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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2005		**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Early Events Leading to the Host Protective Th2 Immune Response to an Intestinal Nematode Parasite	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University of the Health Sciences,F. Edward Hebert School of Medicine,4301 Jones Bridge Road,Bethesda,MD,20814-4799**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT
see report

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**		**119**	

Abstract

Title of Thesis:	Early Events Leading to the Host Protective Th2 Immune Response to an Intestinal Nematode Parasite
Name	John Thomas Pesce
Thesis Directed by:	William C. Gause, Ph.D. Vice Chair, Department of Microbiology and Immunology, and Professor, Microbiology and Immunology, Uniformed Services University of the Health Sciences

Events necessary in the development of Th2 immune responses are poorly understood. A popular model used to study the development of these responses involves intracutaneous inoculation with the intestinal nematode parasite *Nippostrongylus brasiliensis*. Using B7-1/B7-2-/- mice infected with *N. brasiliensis*, we have shown that Th2 effector cells are capable of developing in the absence of B7 signaling interactions, although a substantial decrease in B cell Ag-specific Ab production was observed. To examine the mechanism of T cell activation, OVA-specific DO11.10 T cells were transferred to recipient mice, which were then immunized with a combination of *N. brasiliensis* plus OVA or either alone. Only the combination of *N. brasiliensis* plus OVA triggered T cell differentiation to OVA-specific Th2 cells, suggesting that *N. brasiliensis* acts as an adjuvant to stimulate Ag-specific naive T cells to differentiate to effector Th2 cells.

The adjuvant-like properties of *N. brasiliensis* suggested an innate component of the immune response may be involved in Th2 development. Using

microarray analysis, draining ear lymph nodes from *N. brasiliensis* infected mice exhibited significant increases in CCL2 which is known to be involved in the recruitment of Gr-1^+ neutrophils. Flow cytometric and immunofluorescent analysis of infected lymph nodes resulted in the observation of an increased presence of Gr-1^+ cells. Depletion experiments, using anti-Gr-1 Ab, resulted in disruption of the polarized Th2 *in vivo* immune response, characterized by significantly increased levels of IFN-γ gene expression, IgG2a elevations, and increased worm burden. CCL2-/- deficient mice infected with *N. brasiliensis* were used to determine if CCL2/CCR2 interactions were required for Gr-1 recruitment. CCL2 deficiency resulted in significantly decreased Gr-1bright cell recruitment. Absence of this population had an effect similar to that observed in anti-Gr-1 treatment experiments with increases in IFN-γ and Th1 associated immunoglobulins. Flow cytometric sorting and mRNA analysis of Gr-1bright cells revealed that they consist of a purely neutrophil population which expresses high levels of TNF-α and TGF-β. These studies show the integral role that the innate immune response plays in the development of a highly polarized Th2 immune response.

Overall, these studies have made significant contributions to the understanding of the development of Th2 immune responses. The adaptation of DO11.10 system into a Th2 context provides an essential tool which will allow the determination of specific factors that result in the activation of naïve T cells. As a direct result of developing this tool, we identified a neutrophil population that is essential for the proper polarization of Th2 responses. This finding is

quite significant in that this is the first time that a neutrophil population has been implicated in the development of a Th2 immune response. While this work is still in its infancy, the work detailed in this thesis provides evidence that neutrophils may prove to be a significant target for future drug interventions in the field of allergy and asthma.

Early Events Leading to the Host Protective Th2 Immune Response to an Intestinal Nematode Parasite

By

John Thomas Pesce

Thesis submitted to the Faculty of the Molecular
and Cell Biology Program of the Uniformed Services
University of the Health Sciences in partial fulfillment
of the requirements for the degree of Doctor of Philosophy, 2005

Dedication

While I am responsible for the contents of this thesis, I did not and could not do it on my own. This thesis is a culmination of all the love and support I have received from so many special people over the years. I dedicate this thesis first and foremost to my mom and dad, who sacrificed so much so that I could have every opportunity to succeed. To my Aunt Pat and Uncle Norman, who were closest thing to grandparents I ever had and would not have traded for the real thing. My brothers and sisters, who have proven to be such positive role models and more importantly my friends. Finally, my wife and bestfriend Katie, you are probably the only person who really knows me and I thank you for allowing me to share my life with you. You have provided me with so much love and support that I will never be able to say thank you enough.

My father used to say “who’s got it better than us”, as a child I often thought that people with more money or other material objects had it “better”. In retrospect I finally understand what my father meant and the one thing in life that really matters is family. So I finally have an answer to his question and it truly is “Nobody”.

Acknowledgements

I would like to thank William C. Gause Ph.D. and all the members of the Gause laboratory who have contributed to the development of this thesis and made coming to work a pleasure.

I would also like to thank contributions of my thesis committee:

Clifford Snapper M.D.
William C. Gause Ph.D.
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Ann Jerse Ph.D.
Ajay Verma M.D./Ph.D.

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Introduction

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The adjuvant-like properties of *N. brasiliensis* suggested an innate component of the immune response may be involved in Th2 development. Using microarray analysis, draining ear lymph nodes from *N. brasiliensis* infected mice exhibited significant increases in CCL2 which is known to be involved in the recruitment of Gr-1^+ neutrophils. Flow cytometric and immunofluorescent analysis of infected lymph nodes resulted in the observation of an increased presence of Gr-1^+ cells. Depletion experiments, using anti-Gr-1 Ab, resulted in disruption of the polarized Th2 *in vivo* immune response, characterized by significantly increased levels of IFN-γ gene expression, IgG2a elevations, and

increased worm burden. CCL2-/- deficient mice infected with *N. brasiliensis* were used to determine if CCL2/CCR2 interactions were required for Gr-1 recruitment. CCL2 deficiency resulted in significantly decreased Gr-1bright cell recruitment. Absence of this population had an effect similar to that observed in anti-Gr-1 treatment experiments with increases in IFN-γ and Th1 associated immunoglobulins. Flow cytometric sorting and mRNA analysis of Gr-1bright cells revealed that they consist of a purely neutrophil population which expresses high levels of TNF-α and TGF-β. These studies show the integral role that the innate immune response plays in the development of a highly polarized Th2 immune response.

Why study parasites?

Infection with gastrointestinal nematode parasites results in great morbidity and increased vulnerability to other infectious agents. The World Health Organization estimates that roughly 3.5 billion individuals are infected with either intestinal parasites, including soil-transmitted nematodes, or protozoa. Children infected with these parasites typically suffer from anemia, malnutrition, poor absorption of nutrients, and intestinal bleeding. These factors, taken together, result in growth retardation and decreased cognitive function of exposed children (1-3). While control, through proper sanitation methods, and elimination of these infections is possible in wealthy nations, it is a much larger problem in third world countries where these agents are widespread and funding for healthcare is limited. Currently, our understanding on how immune responses to these agents develop is still at its most basic stages. Therefore, it is important that

research focuses on the basic science behind immune responses to these parasites in order to discover and develop immunological intervention techniques which may be cheaper and more reliable than existing anti-helminthic treatments. Furthermore, research in this field can provide potential treatments against diseases and disorders as evidenced in treatments against inflammatory bowl disease and hyperimmunity (4).

Characterization of immune responses

Host responses to infection with individual pathogens are typically initiated by the innate response. Innate immune responses are fast acting initial defense mechanisms that help rid the host of infectious agents. The skin and mucous membranes act as physical obstacles preventing pathogen entry into the host, while cell populations including macrophages, neutrophils, natural killer cells, and soluble factors including complement and interferons actively aid in the elimination of infection (Fig. 1). In addition to pathogen removal, cell populations involved in the innate response also help activate the adaptive immune response. The adaptive immune responses develop more slowly but provide antigen-specific responses to individual pathogens. This antigen-specific immune response is also characterized by the development of a long lasting memory against the pathogen which provides swift clearance of an infection in subsequent exposures. Adaptive immune responses can be further broken down into T lymphocyte (Cellular) or antibody mediated (Humoral) responses (5) (Fig. 1).

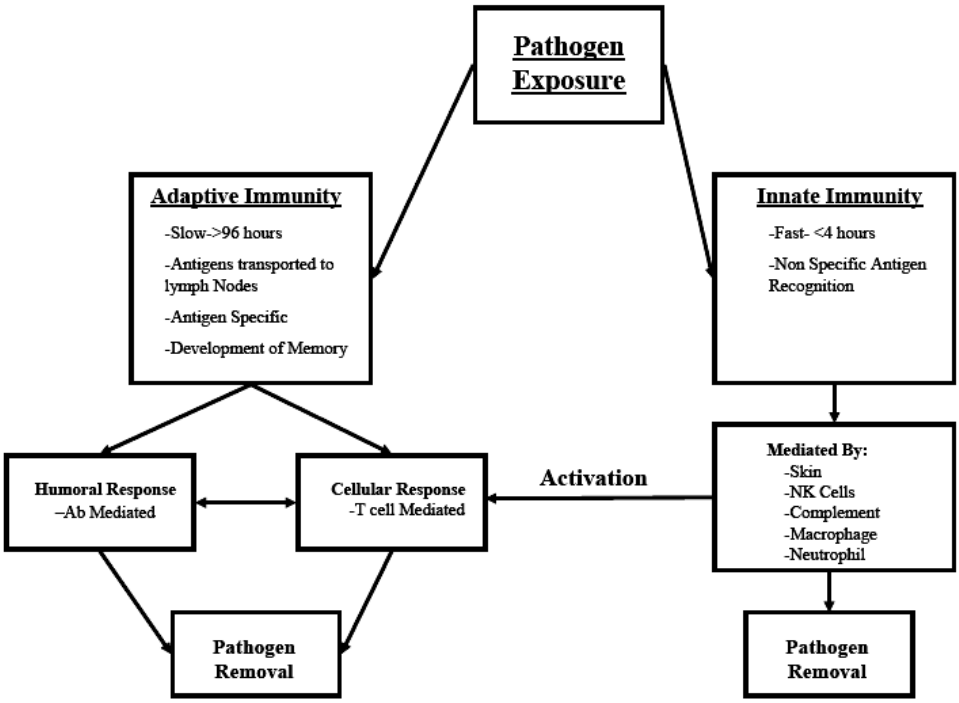
Pathogen Exposure
Adaptive Immunity
-Slow->96 hours
-Antigens transported to lymph Nodes
-Antigen Specific
-Development of Memory
Innate Immunity
-Fast- <4 hours
-Non Specific Antigen Recognition
Humoral Response –Ab Mediated
Cellular Response -T cell Mediated
Activation
Mediated By:
-Skin
-NK Cells
-Complement
-Macrophage
-Neutrophil
Pathogen Removal
Pathogen Removal

Figure 1. Innate and adaptive components of the immune response are involved in the clearance of pathogen infection. The first barrier against infection from pathogens is provided by the innate immune response. Comprised of the skin and mucous membranes, natural killer cells, macrophages, and soluble factors, this arm of the immune response provides an effective first line of defense against infection. The adaptive immune response provides antigen-specific responses and memory responses against pathogens mediated by antigen-specific lymphocytes generated by clonal selection. This response is further subdivided into cellular responses which are mediated by T cells and the humoral response which is mediated by antibodies.

Innate activation of adaptive immune responses

Several components of the innate immune response have been shown to play a role in the activation of adaptive responses including toll-like receptors, found on APCs including macrophages and dendritic cells, natural killer (NK) cells, complement, and neutrophils. This section will focus primarily on toll-like receptors, NK cells, and complement while neutrophils will be discussed in greater detail in a later section.

Viruses and bacteria activate the innate immune response when pathogenic antigens are recognized by dendritic cells; which are known to express pattern recognition receptors (PRRs) that directly bind certain conserved microbial structures referred to as pathogen-associated molecular patterns (PAMPs). Several PAMPs have been described. While the PRR family includes members such as the mannan-binding lectin and the macrophage mannose receptor, the most extensively studied are the Toll-like receptors (TLRs). PAMPs such as LPS or single stranded DNA bind to TLRs and cause the activation of a signaling cascade via the adaptor protein MyD88; which contributes to the activation and the maturation of the dendritic cell. Maturation is characterized by the expression of innate immune response associated genes under the control of the transcription factor NF-κB (TNF-α, IL-6, and IL-12) and the upregulation of B7 molecules on the surface of antigen presenting cells (6) (Fig. 2).

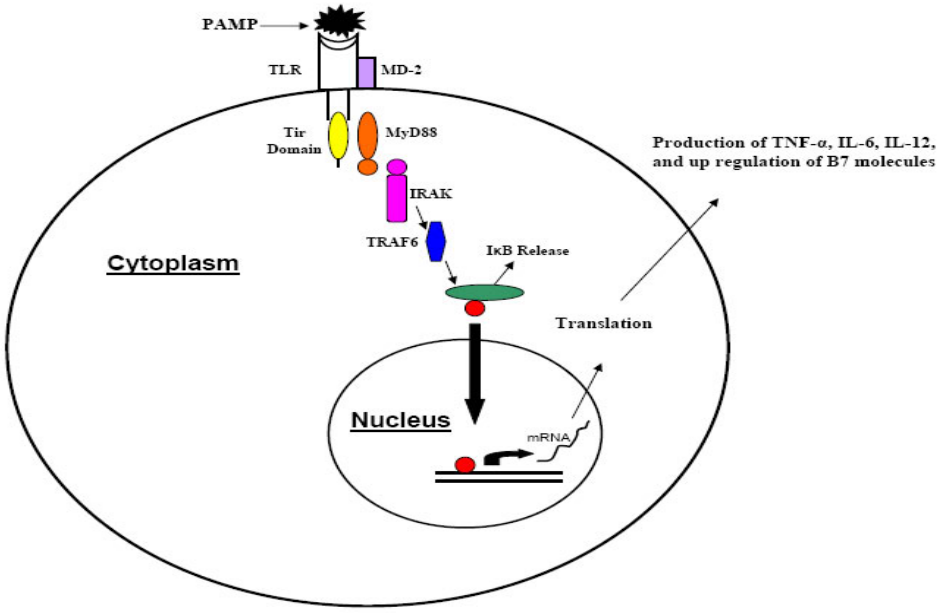

Figure 2. Conserved structures associated with viral and bacterial pathogens activate the immune response via toll receptor signaling. The binding of viral or bacterial elements such as LPS or single stranded DNA to individual toll-like receptors causes the activation of the MyD88 signaling pathway. This results in the transcription and translation of inflammatory genes such as TNF-α, IL-6, IL-12 and B7 upregulation.

NK cells are important in response to pathogen infected cells and in the elimination of tumors (7-9). Innate activation of NK cells occurs through a multiple receptor recognition strategy in which individual NK cells express several receptors which can respond to infection (10;11). Receptor stimulation results in the production of IFN-γ and TNF by NK cells(7). Recent studies have shown that NK cells and dendritic cells can interact initiating a cross-talk between innate and adaptive components. *In vivo* studies have shown that DCs can cause resting NK cells to activate and produce IFN-γ. Conversely, NK cells can interact with immature DCs inducing maturation, and the production of TNF and IL-12 (12-15).

Other studies have implicated complement as a factor that can initiate adaptive responses. Complement is a general name given to over 30 serum proteins and cell surface receptors which are capable of recognizing PAMP's associated with pathogens similarly to toll-like receptors. Components of complement have varying effects including cell lysis, opsonization, and augmentation of T and B cell responses (16;17). The complement factor C3b is described as a mechanism to mark both self and nonself antigens for opsonization by phagocytes or for recognition by CD21 found on B cells. Complexing of, C3b bound toCD21 with CD19, CD81, and B cell receptors results in the production of B cell survival signals and decreased activation thresholds (18;19). Roles for complement have also been described in T cell mediated immune responses. Studies have shown that C3 can assist in the priming of CD4 and CD8 T cells (20). While the mechanism behind this effect is still unclear, it is known that C3 is necessary for the development of T cell responses to influenza. Furthermore, the release of C5aR after pseudomonas infection has been shown to down regulate IL-12 production resulting in an inhibited ability to clear the infection from the lung (21).

Overview of T cell Activation and Costimulation

T cell mediated immune responses are dependent on the activation of T cells via T cell receptor (TCR) complex signaling and its coreceptor, either CD8 or CD4. Upon binding of this complex to peptide bound on the surface of MHC on professional antigen presenting cells (APCs) a series of signals results in the

activation of transcription factors including members of the NFAT, NF-κB, and STAT families (5).

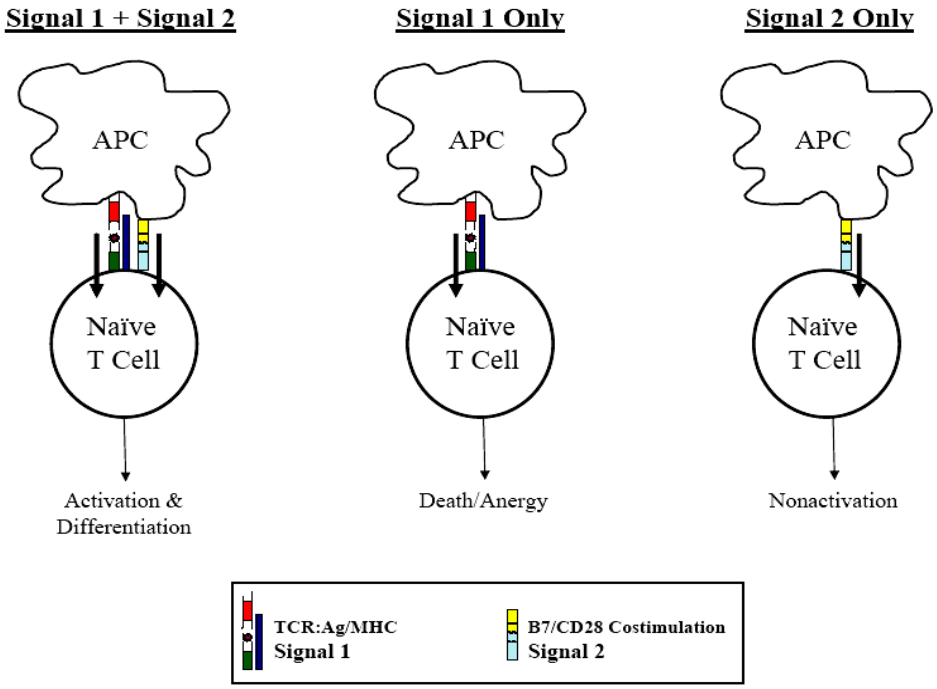

Figure 3. Two signal model for the activation of naïve T cells. In order for naïve T cells to become activated and further differentiate into T cells two signals from antigen presenting cells are required. The first signal is facilitated by the T cell receptor binding the Ag-MHC complex, while the second signal is provided via costimulatory molecule interactions on APCs and T cells. Absence of signal 2 will result in death or anergy of the T cell, while absence of the first signal will result in a nonactivated cell.

In addition, a second signal is required, which is carried out by interactions of costimulatory molecules found on both APCs and T cells. In most cases delivery of both signals will result in the activation of a naïve T lymphocyte to differentiate into a T helper (Th) cell. Absence of costimulation instead results in activation of the T cell leading to anergy or cell death (5;22) (Fig. 3).

While several costimulatory molecules are known to mediate interactions between APCs and T cells, B7/CD28 interactions are of particular importance. B7-1 (CD80) and B7-2 (CD86) are expressed on the surface of dendritic cells, macrophages, B cells, and T cells (23-25). These molecules belong to the

immunoglobulin superfamily and are similar to antibodies in that they are comprised of Ig-like variable and invariable regions in their extracellular domains (26-28). B7-1 and B7-2 have minor amino acid homology to each other and have different expression kinetics. B7-1 is upregulated after activation while B7-2 is constitutively expressed on dendritic cells and upregulated after activation (23;24;29;30). Known ligands for these signaling molecules are CD28 and CTLA-4. As will be discussed in later sections, these interactions have been shown to play an important role in the polarization of Th2 immune responses against intestinal nematode infections.

CD28 is constitutively expressed on the surface of T cells; signaling through this molecule, in conjunction with TCR signaling, results in the production of IL-2 and the expression of CD25 by T cells. In contrast to CD28, CTLA-4 has been shown to be a potent down regulator of T cell activation. While both CD28 and CTLA-4 are capable of bind to B7-1/B7-2, CTLA-4 has the higher binding affinity. Studies involving CTLA-4 deficient mice provided evidence that in the absence of this signal T cell lymphoproliferative disease rapidly develops leading to death at several weeks of age (31).

Differentiation of activated T cells

It was previously thought that activation of naïve T cells resulted in their differentiation into just one of two categories: T-helper 1 (Th1) or T-helper 2 (Th2). This simple delineation has since become more complicated with the addition of unpolarized Th0 to the picture (Fig. 4).

Th1 cells are generally characterized by the production of IFN-γ and TNF (Fig.4). As mentioned above, Th1 immune responses develop due to exposure to structures associated with microbial pathogens, including many virus and bacteria (32). Binding of these agents results in the activation of DCs and subsequent cytokine expression. As dendritic cells undergo maturation they modify their chemokine expression profile to induce chemotaxis from the site of antigen exposure to secondary lymphoid tissue for potential interactions with T cells (33). The cytokine profile expressed by Th1 cells results in the activation of macrophages and NK T cells. Furthermore, Th1 cells migrate towards the B cell zone where they interact with B cells inducing IgG2a isotype switching (34).

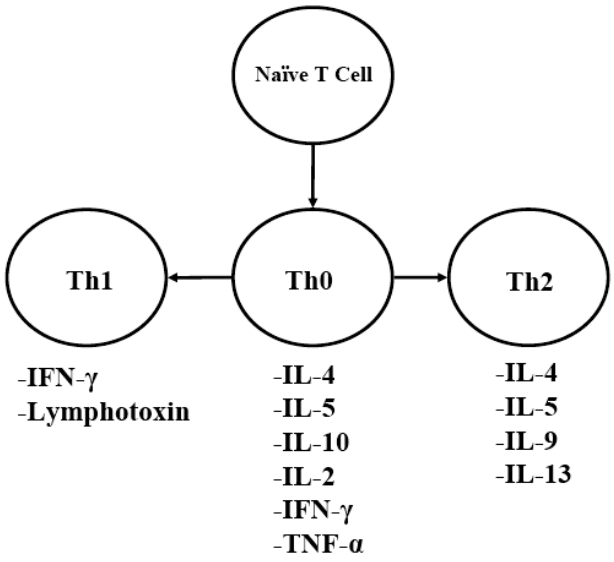

Figure 4. Post activational differentiation of T cells. Upon exposure to antigen, naïve T cells differentiate into Th0 cells expressing a chemokine profile which is a combination of both Th1 Th2, and Th3 cells. Th0 cells can eventually further differentiate to become Th1or Th2,. Th1 cells express IFN-γ and TNF. Th2 cells express IL-4, IL-5, IL-9, and IL-13.

Th2 cells in contrast, are known to express high levels of IL-4, IL-5, IL-9, and IL-13 (35) (Fig. 4). Th2 immune responses result in eosinophilia, mucosal mast cell hyperplasia, and elevations in serum IgG1 and IgE. This immune

response is important for protection from multicellular eukaryotic extracellular pathogens such as gastrointestinal nematodes and for controlling hypersensitivity responses (32;34). While antigen presenting cell/T cell interactions and components of innate immune responses are known to cause the development of Th1 cells, the mechanisms of Th2 cell differentiation remain unclear. It has been show that IL-4 is critical for driving T cells to differentiate into Th2 cells (36), but it is yet to be determined if any portion of innate immunity is involved. IL-4 produced by the T cell is thought to act in an autocrine/paracrine fashion by activating the IL-4 receptor on neighboring T cells (37). Ligation of IL-4 to the IL-4 receptor leads to the activation of the JAK-STAT and IRS2 pathways resulting in the increased expression of IL-4, IL-5, and IL-10 (38). STAT6 and GATA3 are major transcription factors controlling Th2 cytokine production. The dependence of T cells on these two molecules was determined by using conditionally active STAT6 molecules and dominant negative GATA3 mutants. While several studies have shown the importance of $CD4^+$ T cell derived IL-4, other groups have suggested that the source of IL-4 could be from a non-$CD4^+$ cell population like dendritic cells (39), eosinophils (40), mast cells (41), and most recently basophils (42;43). Stimulation of these cell populations may result from PAMPS produced by the parasite which bind hypothetical PRRs thereby inducing IL-4 production (44;45). Recent studies have shown that excretory/secretory products of *N. brasiliensis* (46) and *A. vitae* (47) can induce a Th2 phenotype, while other studies have suggested that a schistosome derived oligosaccharide is capable of inducing Th2 responses (48-50).

B7 Interactions during Th2 Immune Responses

In the context of a Th2 immune response, many models have shown a requirement for B7/CD28 interactions for Th2 cell development. Experiments using antagonistic monoclonal antibodies and fusion proteins against B7 and the CD28 antagonist CTLA4-Ig respectively have shown severe impairment of Th2 development in several model systems. Studies using the nematode parasite *H. polygyrus*, which induces a potent Th2 response, have shown that, in the absence of B7 signaling, MHC II expression is decreased on B cells and IL-4 production by T cells is down regulated. Additionally, decreases were observed in the expression of the Th2 associated cytokines IL-3, IL-5, and IL-9, while also limiting IgE levels in the serum of *H. polygyrus*. Furthermore, in other studies using this parasite, monoclonal antibody blockade of B7-1 and B7-2 resulted in the down regulation of several Th2 associated parameters including serum IgG1, IgE, germinal center formation, $CD4^+$ T cell expansion, eosinophilia, and IL-4 production (51;52). Similar down regulations of Th2 associated cytokines were observed using monoclonal antibodies against B7-1/B7-2 after the whipworm *T. muris* infection. In addition to the down regulation of Th2 associated factors, an increase in Th1 associated factors (53) were observed.

Research on the protozoan parasite, *Leishmania major*, has shown that absence of B7/CD28 signaling, via CTLA-4 fusion protein administration, results in increased susceptibility of BALB/c mice to infection and in some strains of mice a deviation to a Th1 response (54;55). As seen with several of these pathogens, inhibition of the IL-4-associated responses by B7 blockade is

associated with deviation to a Th1 immune response associated with IFN-γ elevations. This suggests that the Th2 response may be more dependent on B7 ligand interactions than the Th1 response (53;54;56). Based on these results, the first manuscript contained in this thesis investigates the development of IL-4 producing T cells in the absence of B7 signaling after *N. brasiliensis* inoculation.

CD28/CTLA-4 signaling during *N. brasiliensis* infection

Previous studies investigating the role of B7 signaling and its role in response to *N. brasiliensis* have focused on the T cell costimulatory signaling molecules, CTLA-4 and CD28. Administration of the fusion protein CTLA4-Ig to *N. brasiliensis*-inoculated mice provided evidence that while the absence of B7 interactions resulted in decreased cytokine levels of both IL-4 and IL-5, a CD4-dependent protective immune response was still able to develop as characterized by worm expulsion (57). In another study, *in vitro* stimulation of mesenteric lymph nodes of mice deficient in CD28 signaling exhibit decreases in both Th1 and Th2 associated cytokine levels (58). While these data suggest that B7 interactions are important for the production of cytokines it cannot explain the development of the protective immunity that develops in their absence. These data thus indicated that Th2 cell could still develop in the absence of B7 signaling and could mediate effective host protection.

Chemokine/Chemokine receptor interactions

Chemokine signaling has proven to be an essential component of the immune response by providing a mechanism for the trafficking of cell populations in and out of lymphoid tissue. Roughly 40 different chemokines have been

identified and are divided into four different groups (CC-, CXC-, C-, and CX3C-) based on the position of cysteine residues near the N-terminus. Further classification is based on the site of chemokine production. This system divides chemokines into groups based on whether they are associated with inflammatory responses or are linked to leukocyte migration. For example, lymphoid chemokines (BLC, SLC, and ELC) are produced by lymphoid tissue, and are used to control cell compartmentalization and direct leukocyte movement (59). Additionally, the inflammatory chemokines (IL-8, RANTES, MIP-1β, CCL2, MIG and IP-10) are used to attract neutrophils and other cell populations associated with innate immunity. These chemokines are produced by a variety of cell types including endothelial cells, epithelial cells, leukocytes, and stromal cells (60).

Chemokines play a major role in the trafficking and eventual activation of lymphocytes. Chemokines accomplish this task by inducing the migration of target cells along a chemical gradient within the host tissue in the direction of increasing chemokine concentration. Cells migrate toward the chemokine source by aggregating chemokine receptors and binding to chemokines attached to proteoglycan molecules within the extracellular matrix and high endothelial venule cell walls (61). Upon reaching the source of chemokine production, chemokine receptor activity is decreased and chemotaxis is halted (62) (Fig. 4). These signaling molecules are known to play an integral role in the migration of dendritic cells from the sites of antigen capture to secondary lympoid tissue based on their expression of inflammatory chemokine receptors such as CXCR1, CCR1,

CCR2, and CCR5. Furthermore, chemokines have been shown to be involved in both Th1 and Th2 mediated immune responses (33).

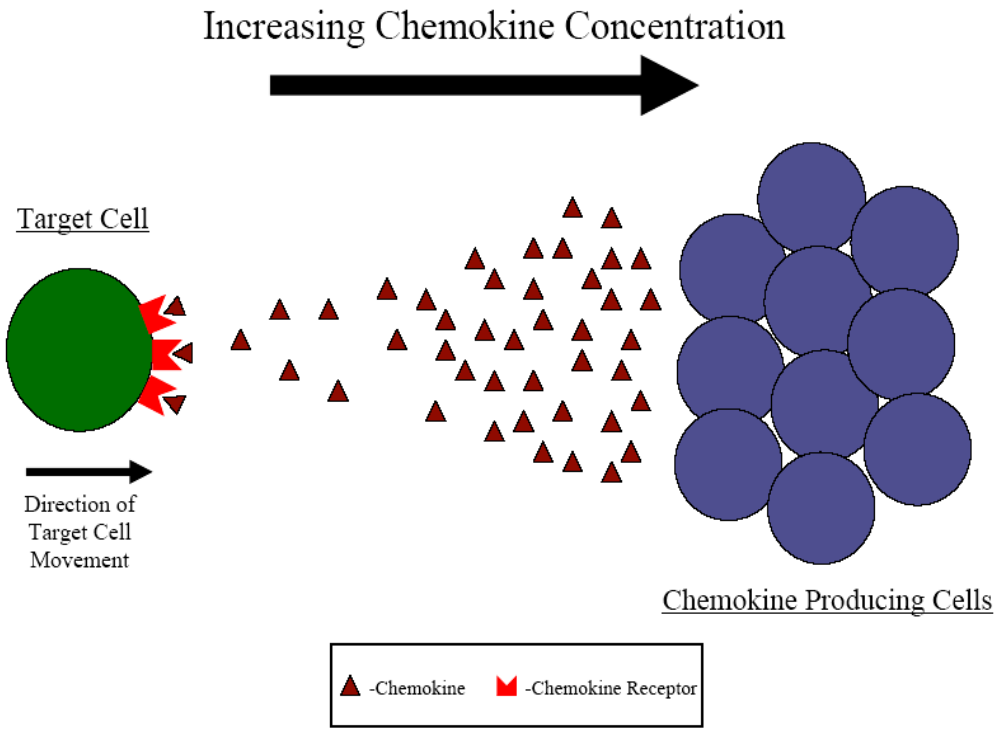

Figure 6. Migration of target cells is induced via chemokines/chemokine receptor signaling interactions. The production of a spatial chemokines gradient from chemokines producing cells causes the attraction of target cells to the source of chemokine production. Target cells bearing specific chemokine receptors migrate along the chemokine gradient from areas of low concentration to areas of high chemokine concentration.

Th1 inducing agents have been shown to up regulate the chemokine receptors CXCR3 and CCR5 on the surface of T cells (33;63;64). Consequently, the ligands associated with these receptors i.e. IP-10, MIG, and RANTES are natural antagonists of Th2 recruiting chemokines (33). Th2 inducing agents have also been shown to induce the up regulation of distinct chemokine profile, including the expression of CCR3 and CCR4. CCR3 is expressed on the surface of eosinophils, basophils, and Th2 cells (65;66). Additionally, CCR4 has been found on basophils (67). These receptors are ligands for eotaxin, TARC, and

MDC. Signaling through these molecules is thought to be necessary for the proper stimulation of function of recruited cells provided by the cytokines IL-4, IL-5, and IL-13 (33). While chemokine receptor profiles can be generalized for both Th1 and Th2 immune responses, it is important to clarify that the expression of these receptors and ligands by these individual cell populations is not exclusive and that Th1 associated receptors have been identified on Th2 cells and vise versa.

Gr-1 involvement in immune responses

Recently, a number of studies have identified cell populations expressing Gr-1 (Ly-6G) that appear to be important in regulating the development of the adaptive immune response. Ly-6 encodes a family of GPI-anchored cell surface proteins that are developmentally regulated (68). Members of the Ly-6 family are typically used as makers for hemopoeitic cells with varying lineages such as lymphocytes (Ly-6C and Ly-6I) (69;70), monocytes (Ly-6C and Ly-6M)(69;71), bone marrow cells (Ly-6M) (71), and neutrophils (Ly-6G) (72).

The study of neutrophil involvement during immune responses has generally revolved around interactions with the pathogen. It is generally accepted that upon reaching the pathogen, neutrophils release antimicrobial agents such as reactive oxygen intermediates and other degradative enzymes. Data has emerged that suggests a role for neutrophils in the modulation of adaptive immune responses. It has been shown that neutrophils possess the ability to store cytokines in preformed pools prior to stimulation by microbial infection. These pools include IL-12, IL-6, MIP-2, TGF-β, and IL-4 (73-77). Excatly how

neutrophils use these pools is still in the process of being determined, but is known that IL-12 plays a critical role in the maturation of neutrophils (74). Furthermore, the de novo synthesis IL-12 and IL-10 by neutrophils has been shown to be important in the development of immune responses to murine candidiasis. Production of these cytokines results in self limiting Th1 response and progressive Th2 response(78). Additionally, it has been shown that neutrophils can function as antigen presenting cells. It has been shown that neutrophils are quite capable of internalizing and processing antigen through either FcR or CR3 internalization. Antigen is processed and expressed on the neutrophil surface complexed to MHC II. Alternatively, it has been found that neutrophils can directly bind antigen on the surface of MHC II (79;80). Neutrophils have also been shown to effectively deliver costimulatory signals to naïve T cells. Surface expression of B7-1 and B7-2 by neutrophils is capable of interacting with CD28 and initiating T cell activation (81).

While the Gr-1 marker is typically used to identify neutrophils, it has been shown more recently to also be associated with several different cell phenotypes including (Tip) dendritic cells, Plasmacytoid Dendritic Cells, and Myeloid Suppressor Cells(82-85). Gr-1^+ cells exhibit considerable heterogeneity with varying functions depending on the specific antigen stimulating the immune response. In response to *L. monocytogenes* infection, a population of $CD11b^+$, Gr-1^+ cells are rapidly recruited to the spleen via CCR2/CCL2 signaling interactions. Upon reaching the spleen, this Gr-1^+ cell population expresses high levels of iNOS and TNFα, which is essential for optimal host resistance (82;86).

Similarly, research involving the Th1 inducing parasite, *Toxoplasma gondii*, has shown that a Gr-1^+/$CD68^+$ cell population is capable of producing IL-12 p40 subunit and generating reactive nitrogen intermediates that aids in the control and inhibition of *T. gondii* replication (87). Furthermore, Gr-1^+ neutrophils have been found to be necessary for early protection against this parasite (88-90). Another dendritic cell population that has been found to express Gr-1 is the plasmacytoid dendritic cell. Murine plasmacytoid dendritic cells are known to express the cell surface markers B220, GR-1, CD11c, and CD8α (83-85). This population has been found to produce IFN-α and IL-12 in response to viral infection. However, several *in vitro* studies have shown that upon maturation via CD40 activation and IL-3 this population can induce the development of IL-4-producing CD4 T cells (84;85).

Gr-1^+ cells have also been described to have not only antigen presenting function but also appear to have T cell regulatory functions. Several studies have shown that a Gr-1^+, $CD11b^+$, $CD31^+$ cell (myeloid suppressor cells) population has suppressor qualities (91). This population has been found to inhibit various aspects of Th1 responses associated in tumor studies. The suppressive properties of this population are known to effect CTL generation, lymphocyte proliferation, and anti-tumor Ab responses. Furthermore, this population is known to accumulate in the spleen and lymph nodes of mice infected with viral vaccines, bacteria, and intracellular parasites (92;93). It has been suggested that TGF-β may be a mechanism through which they mediate suppression (94). Most recently, it has been found that a subset of Gr-1^+ cells, when exposed to alum,

were capable of producing IL-4. Furthermore, this population was found to be required for priming of naïve antigen specific B cells to differentiate into antibody producing cells in vivo (95). These studies raise the possibility that Gr-1^{+} cells may play a role in the development of an adaptive immune response to helminth infection.

***Nippostrongylus brasiliensis* model system**

Nippostrongylus brasiliensis is a gastrointestinal parasite normally infecting rats, which has been adapted for use in mice, and is a well-studied and popular model for Th2 research (96). In this system, third stage larvae are inoculated intracutaneously into the ear of the host. Post inoculation, antigens from the parasite are trafficked to the nearest draining lymph node where a localized Th2 immune response develops. While this immune response is developing, worms migrate to the lungs of the host where they are coughed up and swallowed. Worm maturity is reached in the jejunum of the host 5-6 days after injection. Adult worms are typically expelled from the intestines by day 10 post injection (97;98). Challenge immunization of these mice resulted in reduced worm numbers with little to no egg production (99). For this reason, the *N. brasiliensis* model is useful for studying short-term infections that have gastrointestinal as well as systemic phases (100). Infection with *N. brasiliensis* is associated with increases in serum IgE levels, blood eosinophilia, and mastocytosis (101). Additionally, cytokine expression levels associated with this infection are typical of a Th2 response with large increases in IL-3, IL-4, IL-5, IL-9, and IL-13 (Figure 6) (102).

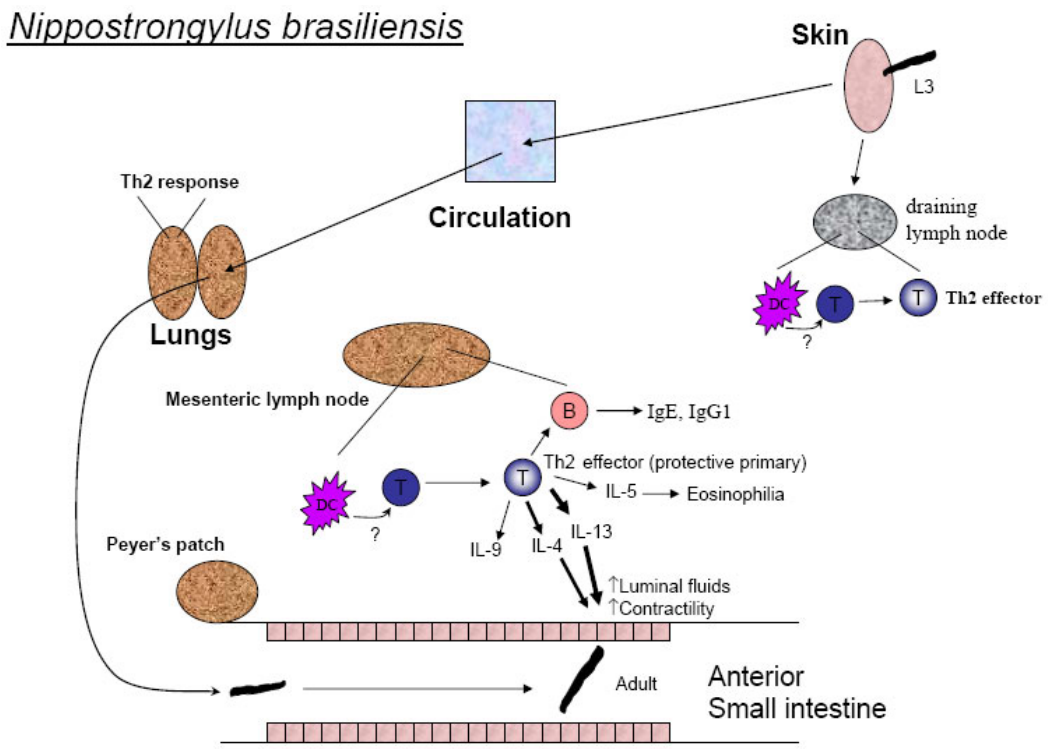

Figure 6. Parasite Host Interactions. Post skin penetration, L3 Nb larvae cause a localized Th2 immune response in the nearest draining lymph node to the site of entry. Additionally, parasites enter into the circulation where they then migrate to the lungs causing a second localized Th2 immune response. Activation of this response causes the host to coughed up and swallow the parasite allowing entry to the gut. Inside the gut, antigens from the parasites interact with APCs which then stimulated naïve T cells to become IL-4 producing effector cells. The gut immune response is characterized by increased levels of IL-4, 13, 5, and 9. Furthermore activated T cells interact with B cells to cause the production of IgE and IgG1. The result of this immune response leads to the clearance of the infection. characterized by high levels of IL-4 and IL-13 with minimal levels of IFN gamma being detected. This model system has proven to be a useful tool to study the development of a Th2 immune response in vivo.

In the studies presented within this thesis, we took advantage of adjuvant-like properties of *N. brasiliensis,* described in Liu et al (97), and used them to develop a model system, which examines nonparasite antigen-specific naïve T cell differentiation to Th2 cells. By adoptively transferring OVA-specific DO11.10 T cells into mice and then later inoculating them with a combination of OVA/Nb we can measure the development of IL-4 producing Ag-specific T cells. As seen in figure 7, DO11.10 T cells which have been fluorescently labeled with CFSE, are injected I.V. through the tail vein and 24 hours later the same mice receive an intracutaneous inoculation of OVA+*N. brasiliensis*. At various timepoints after inoculation, draining cervical lymph nodes near the site of inoculation are removed and analyzed. Using this technique we are able to directly measure IL-4 production from OVA-specific T cells and T cell proliferation.

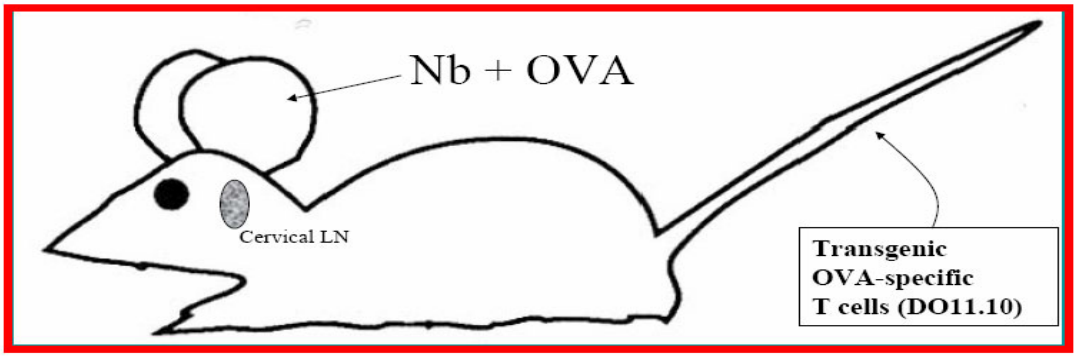

Figure 7. A model for the development of nonparasite antigen-specific Th2 immune responses *in vivo*. Peripheral lymph nodes and spleen are harvested from DO11.10 TCR-transgenic mice and adoptively transferred recipients via i.v. injection. Mice are then inoculated with third-stage Nb and 30 µg OVA peptide intracutaneously in the ear. Draining cervical lymph nodes are removed at specific timepoints for analysis.

Nippostrongylus brasiliensis Can Induce B7-Independent Antigen-Specific Development of IL-4-Producing T Cells from Naive CD4 T Cells In Vivo[1]

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Th2 immune responses to a number of infectious pathogens are dependent on B7-1/B7-2 costimulatory molecule interactions. We have now examined the Th2 immune response to *Nippostrongylus brasiliensis* (Nb) in B7-1/B7-2$^{-/-}$ mice and show that Th2 effector cells develop that can mediate worm expulsion and produce substantial Th2 cytokines comparable with wild-type infected mice; however, in marked contrast, B cell Ag-specific Ab production is abrogated after B7 blockade. To examine the mechanism of T cell activation, OVA-specific DO11.10 T cells were transferred to recipient mice, which were then immunized with a combination of Nb plus OVA or either alone. Only the combination of Nb plus OVA triggered T cell differentiation to OVA-specific Th2 cells, suggesting that Nb acts as an adjuvant to stimulate Ag-specific naive T cells to differentiate to effector Th2 cells. Furthermore, using the DO11.10 TCR-transgenic T cell adoptive transfer model, we show that blocking B7-1/B7-2 interactions does not impair nonparasite Ag-specific DO11.10 Th2 cell differentiation; however, DO11.10 T cell cycle progression and migration to the B cell zone are inhibited. ***The Journal of Immunology,*** **2002, 169: 6959–6968.**

The adaptive immune response is critical for the development of host-protective responses against pathogens. Signaling through the Ag-specific TCR and also through CD28, one of the few costimulatory molecules expressed on naive CD4 T cells, are in many cases required for the development of effector CD4 Th cells that mediate T-dependent responses. In this milieu, there is also evidence that bystander T cells, activated independently of MHC complex-TCR interactions, may also develop that facilitate and amplify the response during infectious disease (1, 2).

Many protective Th2 responses to infectious pathogens require B7-1/B7-2 costimulatory molecule interactions for maturation of naive CD4$^+$ T cells into IL-4-producing Th effector cells. Previous studies have shown that blocking B7-1/B7-2 ligand interactions with the chimeric fusion protein CTLA4Ig or anti-B7 Abs inhibits CD4$^+$ Th2 cell differentiation and the associated cytokine-induced increases in serum IgE and IgG1, after infection of mice with a number of different parasites including the gastrointestinal parasites *Heligmosomoides polygyrus* (3, 4) and *Trichuris muris* (5); the protozoan parasite, *Leishmania major* (6); and the nematode parasite *Schistosoma mansoni* (7). With several of these pathogens, inhibition of the IL-4-associated response by B7 blockade is associated with deviation to a Th1 immune response associated with IFN-γ elevations, suggesting that the Th2 response is more dependent on B7 ligand interactions than the Th1 response (5, 6, 8). The development of the Th1 response during infectious disease is in many cases triggered by signaling through pattern recognition receptors, in particular Toll-like receptors expressed by dendritic cells, which recognize pathogen-associated molecular patterns that function as microbial adjuvants (9). In contrast, during infectious disease, some Th2 responses may develop as "default" responses in the absence of Toll-like receptor signaling (10–14), perhaps partly explaining why Th2 responses are particularly dependent on B7 costimulatory molecule signaling. In addition, a number of studies have found that the strength of signal through the TCR may also influence the generation of Th1 and Th2 effector lymphocytes in vivo (15–17).

Recent studies indicate that an important exception to the requirement of B7 interactions for Th2 responses may occur during the host-protective mucosal immune response to the gastrointestinal nematode parasite, *Nippostrongylus brasiliensis* (Nb).[4] This parasite is a well-defined and widely used model for studying Th2 immune responses. Parasitic larvae migrate into the lungs where they are coughed up and swallowed within 2 days after s.c. inoculation of the infective third-stage larvae (L3). The larvae develop into mature adults that reside in the intestinal lumen and produce eggs that are excreted in the feces. All worms are expelled by ~10 days after infection. This acute host-protective primary Th2 response is associated with increased IgE, and worm expulsion is

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Received for publication June 11, 2002. Accepted for publication October 3, 2002.

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[1] This work was supported by National Institutes of Health Grant AI31678. The opinions or assertions contained within are the private views of the authors and should not be construed as official or necessarily reflecting the views of the Uniformed Services University of the Health Sciences or the Department of Defense or the Department of Agriculture.

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[4] Abbreviations used in this paper: Nb, *Nippostrongylus brasiliensis*; RAG2$^{-/-}$, recombination-activating gene-deficient; WT, wild type; MLN, mesenteric lymph node; PNA, peanut agglutinin; GC, germinal center.

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dependent on $CD4^+$ T cells producing Th2 cytokines (18–20). Surprisingly, CTLA4Ig administration of Nb-infected mice did not impair Th2 cell-mediated worm expulsion; however, Th2 cell cytokine production was impaired after in vitro restimulation (21). In another study, mesenteric lymph node (MLN) cell suspensions from Nb-infected $CD28^{-/-}$ mice showed reduced Th2 and Th1 cytokine production after prolonged in vitro restimulation (22). However, differences have been detected in costimulatory molecule requirements between T cells restimulated in vitro and more physiological measurements of T cell cytokine production and associated host protection (23). The possible B7-independence of the Th2 immune response to Nb suggested that this parasite may function as a potent adjuvant to trigger Ag-specific Th2 cell differentiation, although it was also possible that a previously primed T cell population was cross-reactive with an Nb Ag or, alternatively, that T cell activation occurred through a non-Ag-specific mechanism. B cell activation is often polyclonal in parasite infections (24, 25); however, whether T cell activation is also polyclonal has not been carefully examined.

The development of the adoptive transfer model in which a small number of $CD4^+$, $OVA_{323–339}$-$I\text{-}A^d$ -specific T cells from the DO11.10 TCR-transgenic mouse line are transferred into normal BALB/c recipients, which are then immunized with OVA peptide plus a Th1-inducing adjuvant, such as LPS or CFA, has been widely used to examine Ag-specific naive T cell differentiation and migration in vivo (26, 27). However, this in vivo model has not yet been examined in the context of a Th2 response to determine whether pathogens can also act as adjuvants to trigger naive DO11.10 T cells to differentiate to Th2 cells.

In the studies presented herein, we examined the development of Th2 cells in vivo during Nb infection and the role of B7 interactions in this response. In studies with B7-1/B7-$2^{-/-}$ mice, we found a profound difference between the ability of the differentiated Th2 cells to produce cytokines resulting in worm expulsion and their capacity to provide help for B cell Ab production. Using the aforementioned adoptive transfer model, we determined that Nb can act as an adjuvant which, in the presence of specific Ag, can promote the differentiation of DO11.10 naive nonparasite Ag-specific T cells to Th2 cells even when B7 interactions are blocked.

Materials and Methods

Mice

B7-1/B7-$2^{-/-}$ mice (backcrossed for 10 generations) and the DO11.10 TCR-transgenic mice on an inbred BALB/c background were obtained from Dr. A. Sharpe. The DO11.10 mice contain a large population of CD4 T cells that express a TCR specific for chicken $OVA_{323–339}$-$I\text{-}A^d$ complexes. This TCR is uniquely recognized by the KJ1-26 anti-clonotypic mAb (28). All the mice were maintained in a specific pathogen-free, virus Ab-free facility during the experiments. The studies reported here conformed to the principle for laboratory animal research outlined by the Animal Welfare Act and the Department of Health, Education and Welfare (National Institutes of Health) guidelines for the experimental use of animals.

Adoptive transfers

Peripheral lymph nodes and spleen were harvested from DO11.10 TCR-transgenic mice that were age and sex matched to the adoptive transfer recipients. Single-cell suspensions were prepared by pressing tissue through nylon strainer (BD Biosciences, San Jose, CA). OVA-specific KJ1-26^+ $CD4^+$ T cells (5×10^6) were injected i.v. into recipient mice. The number of KJ1-26^+ cells injected was calculated based on multiplying the percentage of KJ1-26^+ $CD4^+$ cells measured by flow cytometry times the number of live cells obtained. In some experiments, the DO11.10 T cells were incubated with anti-CD4 beads and were further purified by passing through an LS^+ column (Miltenyi Biotec, Auburn, CA). Purified $CD4^+$ T cells (with purity of ~99% as determined by FACS) were resuspended at 5×10^7 cell/ml in PBS containing 0.1% BSA. A final concentration of 10 μM fluorescent dye CFSE (Molecular Probes, Eugene, OR) was added, and the cells incubated for 10 min at 37°C. The labeled cells were washed twice in cell culture medium containing 10% FCS (Life Technologies, Gaithersburg, MD) before transfer. In some cases, parallel experiments were performed with transferred cells from BALB/c DO11.10 recombination-activating gene-deficient (RAG$2^{-/-}$) mice, generously provided by Dr. M. Jenkins (University of Minnesota, Minneapolis, MN).

Parasite infection, CD4 depletion, and OVA immunization

Mice were inoculated s.c. with infective third-stage Nb (L3). Parasite egg numbers and adult worm numbers were evaluated as described previously (29). In several experiments, CD4 T cells were depleted in vivo by i.v. administration of 1 mg of anti-CD4 mAb (clone GK1.5, purified from ascites) on the day of inoculation. This dose has previously been shown to effectively deplete $CD4^+$ T cells in vivo (30). HPLC-purified $OVA_{323–339}$ with the sequence ISQAVHAAHAEINEAGR-COOH was synthesized by Biomedical Instrumentation Center at Uniformed Services University. In some experiments, third-stage Nb and 30 μg OVA peptide were injected intracutaneously in the ear of DO11.10 T cell transfer recipient mice. In some cases, mice immunized with peptide plus Nb were given i.v. 200 μg of murine CTLA4Ig or control fusion protein L6 on days 0 and 1 after immunization.

Quantitation of serum Igs

Total serum IgE, IgG1, and IgG2a levels were quantitated by ELISA. Ag-specific IgG1 level was measured using a modified ELISA. Briefly, individual wells of Immulon IV plates (Thermo Labsystems, Franklin, MA) were coated with diluted (5 μg/ml) Nb excretory/secretory Ag. After 4°C overnight incubation, 1% FBS plus 0.1% sodium azide was used for blocking. Serum samples were added to the plates in 4-fold serial dilutions and incubated for 2 h at room temperature after blocking. Then anti-mouse IgG1-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL; 1/2000 diluted) was added to individual wells, and the plates were incubated for 30 min at room temperature. The substrate (5% 4-methylumbelliferyl phosphate-free acid solution) was then added, and fluorescence of the samples was quantitated using a MicroFLUOR Reader (Dynatech Laboratories, Chantilly, VA).

Immunohistochemical staining

The procedure used for germinal center (GC) staining was as described previously (4). Briefly, 8-μm frozen MLN tissue sections were stained with HRP conjugated to peanut agglutinin (PNA; ICN Biomedicals, Aurora, OH), washed, and then incubated with the peroxidase substrate, 3-amino-9-ethylcarbazole (Sigma-Aldrich, St. Louis, MO). All photographs of the tissue sections were taken at the same magnification (×125).

Cell sorting and cytokine gene expression by RT-PCR

For sorting, MNL cells were labeled with anti-CD4 beads and passed through LS^+ columns (Miltenyi Biotec). The $CD4^+$ and $CD4^-$ populations were collected and assessed for purity using FACS analysis. The $CD4^+$ population was >98% pure, and the $CD4^-$ population was >95% pure in all sorts described. For RT-PCR, total RNA was extracted from purified cell populations with the RNA Isolation Kit (Stratagene, Cedar Creek, TX), specially developed for isolating small RNA quantities, and from tissue as previously described (31). Total RNA was then reverse transcribed as previously described (31). Real-time PCR kits (Applied Biosystems, Foster City, CA), specific for individual cytokines or rRNA, were used to quantitate differences in gene expression, and all data were normalized to constitutive rRNA values. The Applied Biosystems 7700 sequence detector was used for amplification of target mRNA, and quantification of differences between treatment groups was calculated according to the manufacturer's instructions.

Cell cultures and cytokine secretion

Single-cell suspensions were prepared from the MLN, and cells were placed in RPMI 1640 supplemented with 10% FCS that had been heat-inactivated for 30 min at 57°C, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES. The anti-CD3 mAb restimulation assay was based on previously described techniques (32), with some modifications. Briefly, MLN cells (2×10^5 cells/well) from either Nb-infected or uninfected mice were cultured in triplicate wells of 96-well round-bottom plates coated with anti-CD3 mAb (2C11, 10 μg/ml; BD PharMingen, San Diego). After 72 h, the supernatants of each well were collected and stored at −70°C for cytokine production analysis. Cytokine production in the supernatants of cultured cells was measured using commercial ELISA kits (R&D Systems, Minneapolis, MN).

ELISPOT

Two different ELISPOT assays were used. The first was as previously described (3, 33). Briefly, single-cell lymph node suspensions were prepared in RPMI 1640 containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all from Life Technologies). Cells (0.5×10^6) were seeded into each well of an anti-IL-4 (clone BVD4-1D11.2, a gift from Dr. Finkelman)-coated Immulon IV 96-well microtiter plate (Microtiter, Chantilly, VA). After short term culture (5–12 h), the plate was washed several times with PBS followed by washes with PBS-Tween 20. Secondary biotinylated anti-IL-4 Ab was diluted in PBS, 0.05% Tween, 5% FCS; added at 100 μl/well; and incubated overnight at 4°C. Plates were then washed, and a 1/2000 dilution of streptavidin-AKP (Jackson ImmunoResearch, West Grove, PA) was added. Plates were developed, and results were counted as described. The second ELISPOT assay was modified to include the capability to quantitate IL-4-producing cells following in vitro restimulation with OVA peptide. Lymph node cells were cultured with 10 μg/ml OVA peptide for 3 days on anti-IL-4-coated plates, before being washed away with PBS and PBS-Tween. Secondary anti-IL-4 Ab was next added, and subsequent steps were identical with those described for the first ELISPOT assay.

Flow cytometry

Lymph node cells were harvested, and 1×10^6 cells were blocked with Fc Block (BD PharMingen) and then incubated with anti-CD4-Cy-Chrome (BD PharMingen), anti-CD69-PE (BD PharMingen), and KJ1-26-FITC (Caltag Laboratories, Burlingame, CA) or anti-MHCII-PE and anti-B220-FITC (BD PharMingen). After washes, cell were fixed with 1% paraformaldehyde (Fisher, Pittsburgh, PA) and analyzed by flow cytometry using an EPICS XL-MCL (Beckman Coulter, Fullerton, CA). For CFSE-labeled cells, anti-CD4-Cy-Chrome and KJ1-26-PE (Caltag) were used to distinguish the DO11.10 T cells.

Ex vivo intracellular cytokine measurement

For intracellular cytokine detection, the cervical draining lymph nodes of the ear were harvested 2 h after OVA peptide challenge i.v., and 5×10^6 cells were incubated for 5 h with plate-binding anti-CD3 mAb (2C11, 5 μg/ml; BD PharMingen) and Golgi-Stop (BD PharMingen). Lymphocytes were harvested and incubated with Fc Block (2.4G2; BD PharMingen) plus 10% rat serum (Sigma-Aldrich) for 20 min at room temperature. Cell surface markers were stained by anti-CD4-Cy-Chrome (BD PharMingen) and KJ1-26-FITC mAb (Caltag Laboratoaries). Cells were fixed in 4% paraformaldehyde (Fisher) and permeabilized in 0.5% saponin (Sigma-Aldrich) before staining with PE-conjugated rat anti-mouse IL-4 or anti-IFN-γ mAb (BD PharMingen). Over 200,000 lymphocyte-gated events were collected, to obtain $>$2,000 KJ1-26$^+$CD4$^+$-gated events.

Immunofluorescent microscopy

Draining cervical lymph nodes were harvested from sacrificed mice and frozen in liquid nitrogen. Cryostat-cut tissue sections (8 μm) were fixed in acetone and stained as described previously (4, 34) with the following reagents: PE-conjugated KJ1-26 (Caltag Laboratories); biotinylated anti-B220 (BD PharMingen) or biotinylated PNA (Sigma-Aldrich); and streptavidin-Alex 647 (Molecular Probes). Sections were mounted in Fluormount G (Southern Biotechnology Associates) and viewed with a fluorescence microscope (Axiophot; Zeiss, Oberkochen, Germany). Images were acquired on a digital camera and were processed with SlideBook software (Intelligent Imaging, Denver, CO).

Results

Host protection and mucosal cytokine production are not inhibited in Nb-infected B7-1/B7-2$^{-/-}$ mice

Previous studies have suggested that worm expulsion is intact and Th2 cell cytokine production occurs but is significantly impaired in the MLN after CTLA4Ig treatment of Nb-infected mice (21). To examine whether the host protective Th2 response was intact in mice deficient in B7-1 and B7-2, B7-1/B7-2$^{-/-}$ and B7-1/B7-2$^{+/+}$ BALB/c mice were inoculated with 500 Nb L3. Mice (five per treatment group) were killed at days 8 and 14 after parasite inoculation, and adult worm survival and fecundity were determined. As shown in Fig. 1, by day 14 after Nb inoculation, adult worms were not detectable in either B7-1/B7-2$^{-/-}$ or B7-1/B7-2$^{+/+}$ BALB/c mice. Furthermore, worm expulsion was inhibited and

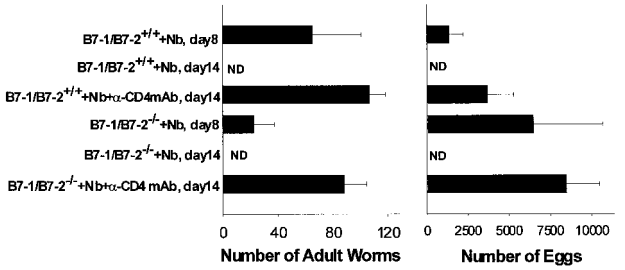

FIGURE 1. CD4-dependent host protection is B7-1/B7-2 independent after Nb inoculation. B7-1/B7-2$^{+/+}$ and B7-1/B7-2$^{-/-}$ BALB/c mice (five per treatment group) were treated with anti-CD4 Abs at day 0 and day 4 after Nb inoculation as described in *Materials and Methods,* and adult worm survival and egg production in the intestine were determined. The mean and SE derived from five individual mice are shown for each treatment group. ND, Not detectable.

worm fecundity was pronounced in Nb-inoculated B7-1/B7-2$^{-/-}$ or B7-1/B7-2$^{+/+}$ mice treated in vivo with anti-CD4 Ab. In a further experiment, B7-1/B7-2$^{-/-}$ mice were immunized with different doses (500, 250, and 50) of Nb L3 to investigate whether B7 interactions may be required at lower Ag concentrations. All worms were expelled at all doses in both B7-1/B7-2$^{-/-}$ and B7-1/B7-2$^{+/+}$ BALB/c controls (data not shown), indicating that the dose of Ag did not influence the B7 dependence of the Th2 immune response.

Real-time quantitative RT-PCR was used to assess changes in IL-4 mRNA gene expression. At days 8 and 14 after Nb inoculation, MLN and Peyer's patches were collected. Pronounced increases in IL-4 mRNA were detected in Nb-inoculated B7-1/B7-2$^{-/-}$ mice that were at least as elevated as and in some cases more elevated than IL-4 mRNA levels in Nb-inoculated wild-type (WT) B7-1/B7-2$^{+/+}$ BALB/c mice. Anti-CD4 Ab treatment blocked elevations in IL-4 in both Nb-inoculated B7-1/B7-2$^{-/-}$ BALB/c mice and Nb-inoculated B7-1/B7-2$^{+/+}$ controls (Fig. 2*a*). CD4$^-$ and CD4$^+$ T cells were also isolated from MLN suspensions of B7-1/B7-2$^{-/-}$ mice at day 8 after Nb inoculation. CD4$^+$ T cells accounted for $>$90% of the IL-4 mRNA detected in two separate experiments (Fig. 2*b*). On day 8 after Nb inoculation, MLN lymphocyte suspensions were collected (five mice per group) and individually analyzed for IL-4 secretion by ELISPOT assay, a method that measures the number of IL-4-secreting cells and requires only a short term in vitro culture without restimulation. Longer in vitro restimulation assays detected markedly reduced levels of IL-4 in MLNs from Nb-inoculated B7-1/B7-2$^{-/-}$ mice compared with Nb-inoculated B7-1/B7-2$^{+/+}$ BALB/c mice (data not shown). As shown in Fig. 2*c*, pronounced and comparable increases in the number of IL-4-secreting CD4 cells were detected in Nb-inoculated B7-1/B7-2$^{-/-}$ mice. These data suggested that mice genetically deficient in B7-1 and B7-2 do not exhibit inhibition in IL-4 gene and protein expression when more physiological measurements of in vivo cytokine expression are used. The pronounced increases in Th2 cytokine gene and protein expression suggested potential expansion, perhaps non-Ag-specific, of CD4 T cells after Nb inoculation. However, increases in total CD4 T cell activation, as measured by CD69 up-regulation, were not observed after Nb inoculation of B7-1/B7-2$^{-/-}$ or B7-1/B7-2$^{+/+}$ mice (Fig. 2*d*), suggesting that a large subpopulation of T cells was not activated. In contrast, the Th2 primary response, including IL-4 elevations, is inhibited in B7-1/B7-2$^{-/-}$ BALB/c mice inoculated with *H. polygyrus* (35) and in CTLA4Ig-treated *H. polygyrus*-inoculated B7-1/B7-2$^{+/+}$ BALB/c mice (3). These latter findings

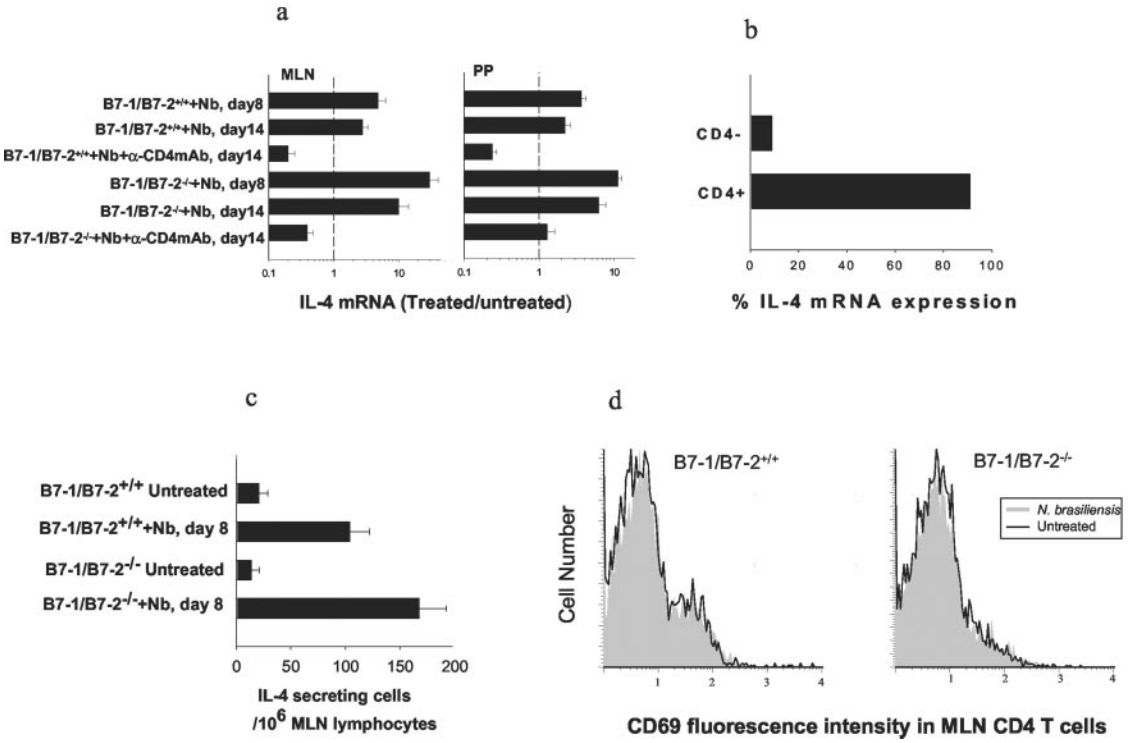

FIGURE 2. CD4-dependent IL-4 expression is up-regulated in B7-1/B7-2$^{-/-}$ mice after Nb inoculation. *a*, IL-4 mRNA expression in the MLN and Peyer's patches (PP) were determined on days 8 and 14 after inoculation. *b*, IL-4 mRNA expression in sorted CD4^{+} and CD4^{-} MLN cell populations (pooled samples from five Nb-inoculated B7-1/B7-2$^{-/-}$ mice) were determined on day 8 after inoculation. *c*, The number of IL-4-secreting cells per 10^6 MLN cells was determined by ELISPOT assay without restimulation. *d*, CD69 expression on MLN CD4^{+} T cells was detected by FACS analysis at day 8 after inoculation.

indicate pronounced differences in B7 dependence in the Th2 response to different parasites and confirm and extend earlier studies suggesting that the Nb immune response is B7-1/B7-2 independent (21).

The humoral immune response is abrogated in Nb-inoculated B7-1/B7-2$^{-/-}$ mice

Our findings that the development of IL-4-producing T cells was unimpaired in Nb-inoculated B7-1/B7-2$^{-/-}$ mice suggested that a humoral immune response should occur. Previous studies have shown that elevations in total serum IgE are decreased but not blocked after CTLA4Ig treatment of Nb-inoculated mice (21). In our studies, elevations in serum IgE levels were completely blocked in Nb-infected B7-1/B7-2$^{-/-}$ mice, whereas in the same experiments CD4-dependent increases in serum IgE levels were detected in Nb-infected WT controls. Nb Ag-specific IgG1 titers were absent in infected B7-1/B7-2$^{-/-}$ mice but were pronounced in B7-1/B7-2$^{+/+}$ mice at day 14 after inoculation (Fig. 3*a*). GC formation is an important CD4 T cell-dependent microenvironment that contributes to Ig class switching and memory B cell development. Immunohistochemical analysis showed an almost complete absence of GC formation in the mesenteric lymph nodes of Nb-inoculated B7-1/B7-2$^{-/-}$ mice in contrast to pronounced increases in GC formation observed in Nb-inoculated WT controls (Fig. 3*b*). Increased B cell MHCII expression is IL-4 dependent during nematode infections and is used as an indicator of B cell IL-4R signaling and activation in vivo (3, 36). MLN cell suspensions from Nb-inoculated B7-1/B7-2$^{+/+}$ and Nb-inoculated B7-1/B7-2$^{-/-}$ mice were stained simultaneously with anti-MHC class II and B cell-specific anti-B220 (6B2) Abs. MHC class II expression was markedly increased in Nb-inoculated B7-1/B7-2$^{+/+}$ mice but was not increased in Nb-inoculated B7-1/B7-2$^{-/-}$ mice (Fig. 3*c*). These findings suggest that although IL-4-producing Th2 cells can develop that mediate worm expulsion in B7-1/B7-2$^{-/-}$ mice, they cannot deliver IL-4 signals to B cells to mediate increases in MHC class II.

Parenteral inoculation with Nb also triggers a B7-1/B7-2-independent Th2 response

A number of studies have suggested that the mucosal immune response favors the development of Th2 cells (37–39). The mucosal microenvironment may thus be permissive for the development of a potent Th2 response that can occur in the absence of B7-1/B7-2 interactions. To test whether Nb could induce a Th2 response in a nonmucosal milieu and, if so, whether the response remained B7 independent, a novel immunization model was developed in which Nb L3 were injected intracutaneously in the ear. Studies of the kinetics of IL-4 and IL-13 mRNA gene expression in the draining cervical lymph node showed that peak levels were attained at day 7 after inoculation with 300 Nb L3 (data not shown). As shown in Fig. 4, pronounced increases in both IL-13 and IL-4, but not IFN-γ mRNA, were detected in the draining ear lymph node at day 7 after inoculation of either B7-1/B7-2$^{-/-}$ or B7-1/B7-2$^{+/+}$ mice. These findings demonstrated that Nb could induce a B7-independent Th2 immune response in a nonmucosal lymphoid environment.

Nb stimulates Ag-specific Th2 cell differentiation

The unusual B7-independent Th2 cell differentiation pathway that occurs after Nb inoculation may involve activation of T cells by Nb through an Ag-specific or an Ag-nonspecific, perhaps bystander or polyclonal, T cell activation mechanism. To distinguish between these alternative mechanisms of T cell differentiation,

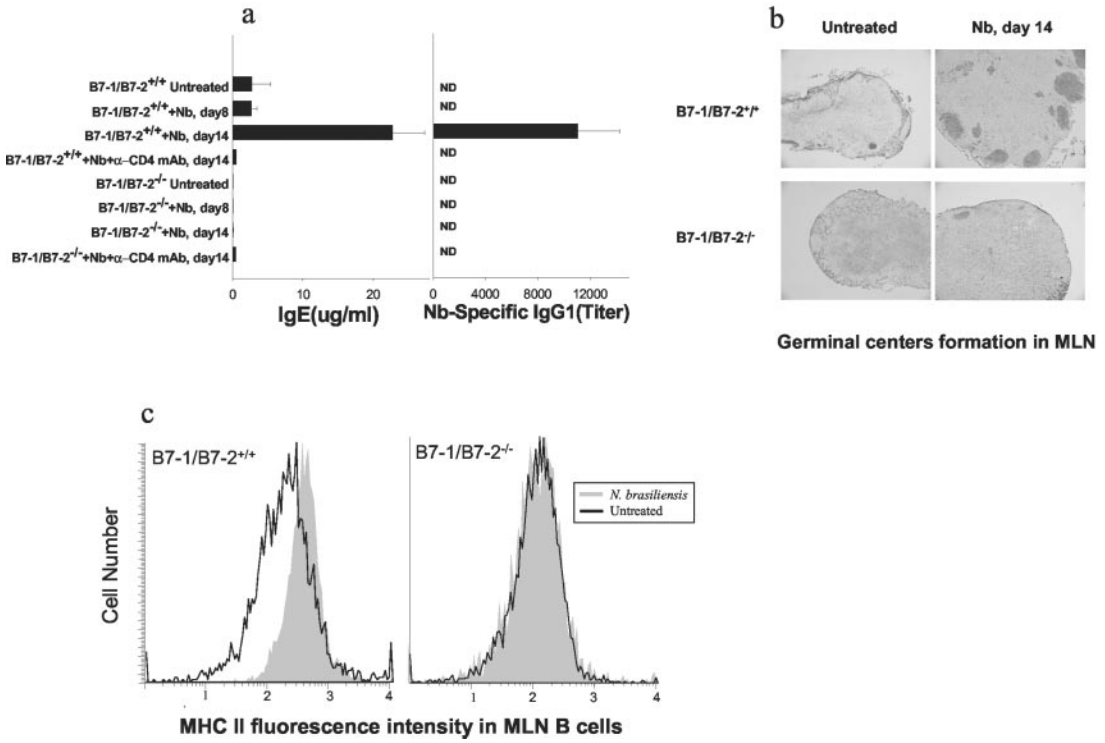

FIGURE 3. Serum Ig elevations, GC formation, and increased MHC class II expression are inhibited in B7-1/B7-2$^{-/-}$ BALB/c mice compared with B7-1/B7-2$^{+/+}$ BALB/c mice after Nb inoculation. *a*, B7-1/B7-2$^{+/+}$ and B7-1/B7-2$^{-/-}$ BALB/c mice were bled on days 8 and 14 after Nb inoculation, and total serum IgE and Nb-specific IgG1 levels were determined by ELISA. The mean and SE derived from five individual mice are shown for each treatment group. *b*, MLNs were collected from B7-1/B7-2$^{+/+}$ and B7-1/B7-2$^{-/-}$ BALB/c mice (five per treatment group) at day 14 postinfection and sectioned and stained with PNA to detect GC formation. *c*, On day 14 after Nb inoculation, MLN B cell MHC class II expression was determined by FACS analysis of pooled samples from five mice per treatment group. ND, Not detectable.

5×10^6 DO11.10 OVA-specific T cells from DO11.10 WT or DO11.10 RAG2$^{-/-}$ mice were transferred to WT recipients (five per treatment group) through i.v. injection. In initial experiments, DO11.10 RAG2$^{-/-}$ transferred cells were also used to confirm that endogenous TCR from the DO11.10 WT mice was not significantly contributing to the activated donor T cell population. Two days after adoptive transfer, recipient WT mice (five per treatment group) were inoculated in the ear with 300 Nb L3 and 30 μg OVA. Seven days later, mice were killed, and cervical ear lymph nodes collected for analysis. The lymph node cell suspensions were dual-stained for CD4 and KJ1-26 (anti-DO11.10 Ab). As shown in Fig. 5*a*, the total number of DO11.10 T cells per cervical lymph node was little increased over untreated mice in treatment groups immunized in the ear with either OVA or Nb alone. However, recipient mice immunized with the combination of OVA plus Nb showed marked expansion of DO11.10 T cells. To detect IL-4-secreting cells, an OVA-specific ELISPOT assay was developed and used as described in *Materials and Methods.* Recipient mice immunized with Nb alone triggered background IL-4 levels in the OVA-stimulated group similar to that observed in cells stimulated with medium alone, whereas cells from recipient mice immunized with Nb plus OVA exhibited marked increases in the number of IL-4-secreting cells (Fig. 5*b*). Taken together, these results indicate that a combined immunization with OVA plus Nb is required to trigger DO11.10 T cell activation and differentiation to Th2 cells. The observation that Nb immunization alone cannot stimulate DO11.10 cells indicates that during this nematode parasite infection Ag-nonspecific mechanisms of T cell activation, such as bystander T cell activation, are not sufficient to activate the naive DO11.10 T cells. Nb can thus act as an adjuvant to promote Ag-specific Th2 cell differentiation in vivo.

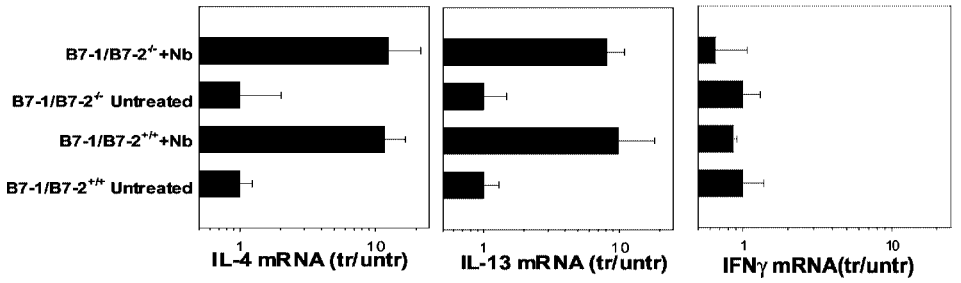

FIGURE 4. Nb can induce a B7-independent Th2 response after parenteral inoculation. Three hundred Nb L3 were injected intracutaneously in the ear of B7-1/2$^{+/+}$ or B7-1/B7-2$^{-/-}$ BALB/c mice (five per treatment group). On day 7 after inoculation, cervical lymph nodes were removed, and RNA was purified. IL-4, IL-13, and IFN-γ gene expression was determined by real-time PCR of cDNA reverse transcribed from total RNA. This experiment was repeated twice with similar results. tr/untr, Treated vs untreated.

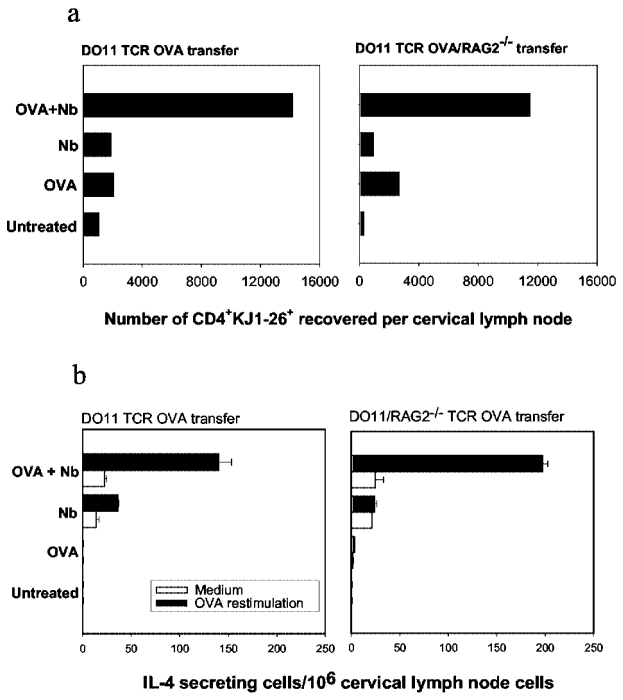

FIGURE 5. Nb acts as an adjuvant to trigger Ag-specific Th2 cell differentiation. Five million DO11.10 or DO11.10 RAG2$^{-/-}$ T cells were adoptively transferred to recipient BALB/c mice, and 2 days later recipient mice were intracutaneously injected in the ear with OVA peptide (30 μg), Nb L3 (300), or the combination of OVA plus Nb L3 (five per treatment group). At day 7 after immunization, cervical lymph nodes were collected and cell suspensions were stained with FITC-anti-CD4 and PE-anti-KJ1-26 to determine the total number of CD4$^+$KJ1-26$^+$ cells recovered per cervical lymph (*a*); cultured for 3 days in vitro with 10 μg/ml OVA peptide, or medium alone, and assessed for IL-4 secretion using a modified ELISPOT assay (*b*). This experiment was repeated twice with similar results.

B7-1/B7-2 interactions are not required for Ag-specific DO11.10 Th2 cell differentiation after Nb plus OVA immunization

The observation that transferred DO11.10 T cells differentiated to Th2 cells in vivo after immunization with OVA plus Nb suggested that this would be a useful model to examine B7-independent Ag-specific Th2 cell differentiation in vivo. In these experiments, rather than using B7-1/B7-2-deficient mice, CTLA4Ig was used to block B7-1/B7-2 interactions in WT B7-1/B7-1$^{+/+}$ BALB/c mice in vivo, so that B7-1/B7-2 expression on transferred DO11.10 T cells could also be inhibited. Five million sorted DO11.10 CD4$^+$ T cells were transferred to BALB/c recipients. At day 2 after adoptive transfer, recipient mice were given 200 μg of murine CTLA4Ig (five mice per treatment group) or control fusion protein L6 (five mice per treatment group) and immunized in both ears with 300 Nb L3 plus 30 μg OVA. At day 3, an additional dose of 200 μg of CTLA4Ig or L6 was administered. This dose has previously been shown to block the Th2 immune response to *H. polygyrus* in BALB/c mice (3). At day 7 after Nb plus OVA immunization, mice were immunized i.v. with OVA and killed 2 h later, and individual cervical ear lymph nodes were collected and prepared for FACS analysis. In vivo restimulation with OVA was necessary because the in vitro OVA-specific restimulation ELISPOT assay did not detect increases in IL-4 in recipient mice treated with CTLA4Ig (data not shown). Instead of ELISPOT, cytoplasmic staining, as described in *Materials and Methods*, was used to detect IL-4 elevations in KJ1-26$^+$ CD4$^+$ T cells. As shown in Fig. 6, pronounced increases in CD69 expression and IL-4 production, but not IFN-γ, were detected in DO11 T cells from the cervical lymph node of BALB/c mice given either CTLA4Ig or L6. These findings suggest that Nb can act as an adjuvant that supports B7-independent nonparasite Ag-specific Th2 cell activation and differentiation.

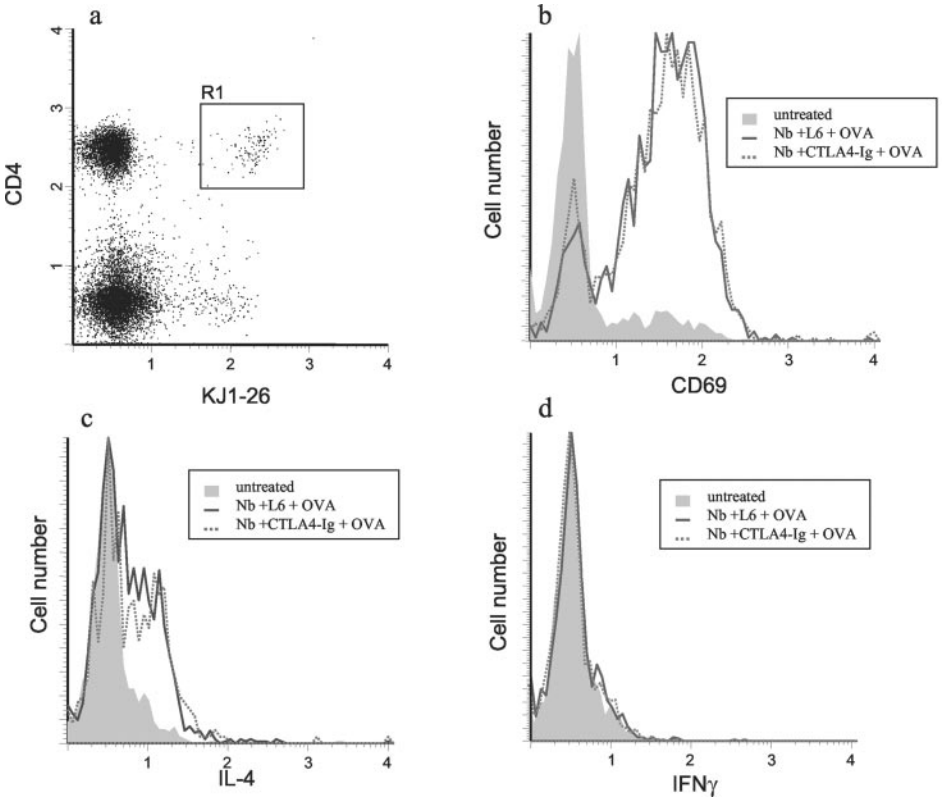

FIGURE 6. Ag-specific T cell activation and IL-4 production are B7 independent in Nb-infected mice. Five million purified DO11.10 CD4$^+$ T cells were transferred to recipient BALB/c mice, and 2 days later recipient mice were immunized intracutaneously in the ear with Nb and OVA peptide and treated with murine CTLA4Ig or L6 on days 0 and 1 after immunization (five animals per treatment group). At day 7 after immunization, OVA peptide (300 μg) was given i.v., and the cervical lymph node cells were collected 2 h later and stained with Cy-Chrome-anti-CD4 and FITC-anti-KJ1-26 (*a*) to discriminate recipient DO11.10 CD4$^+$ KJ1-26$^-$ cells from the transferred CD4$^+$ KJ1-26$^+$ cells (R1). To further examine R1 gated cells, three-color staining was used with anti-CD4, anti-KJ1-26, and either PE-anti-CD69 (*b*), PE-anti-IL-4 (*c*), or PE-anti-IFN-γ (*d*). This experiment was repeated twice with similar results.

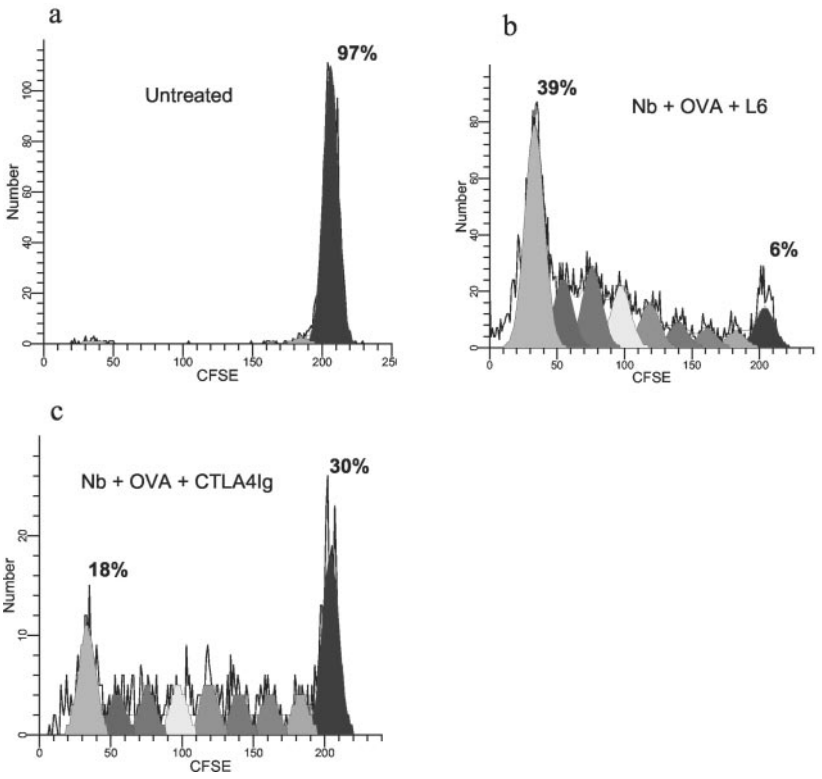

FIGURE 7. DO11.10 T cell cycle progression is partially B7 dependent after Nb plus OVA immunization. The experiment was performed as described in Fig. 5, except that purified DO11.10 $CD4^+$ T cells were labeled with CFSE at the time of transfer. CFSE fluorescence, a measure of cell division, was assessed in the $CD4^+$ $KJ1\text{-}26^+$ T cells from each treatment group by flow cytometry. This experiment was repeated twice with similar results.

DO11.10 T cell cycling is reduced following B7 blockade after Nb plus OVA immunization, and DO11.10 T cell trafficking to the B cell zone is inhibited

The observation that DO11.10 T cells can develop into IL-4 T cells, although B7 interactions are blocked, did not preclude the possibility that the DO11.10 Th2 cells may show impaired cell cycle progression. Five million sorted DO11.10 $CD4^+$ T cells were stained for CFSE, as described in *Materials and Methods*, and transferred to BALB/c recipients. Two days after adoptive transfer, mice were immunized in both ears with Nb plus OVA and treated with CTLA4Ig (five per treatment group) or L6 (five per treatment group) at days 0 and 1 after immunization. At day 7, mice were killed, both draining cervical lymph nodes were collected, and cell suspensions were prepared from one lymph node for FACS analysis whereas the other was imbedded in OCT and frozen in liquid nitrogen for immunofluorescent analysis. Cell populations were stained for KJ1-26 and assessed for CFSE staining. As shown in Fig. 7, cell cycling was detectable up to nine generations in DO11.10 T cells from immunized mice given either L6 (Fig. 7*b*) or CTLAA4Ig (Fig. 7*c*). In both treatment groups, considerable DO11.10 cell cycling was detected compared with untreated controls (Fig. 7*a*). However, the frequency of DO11.10 T cells that cycled nine or more generations was markedly reduced in immunized mice given CTLA4Ig compared with immunized mice given L6. Thus, although Nb can support differentiation to IL-4-producing T cells, subsequent cell cycle progression is reduced.

To examine DO11.10 T cell trafficking in the cervical lymph node of immunized mice, frozen sections were stained from recipient mice injected intracutaneously in the ear with Nb plus OVA or OVA alone. As shown in Fig. 8*a*, the combination of Nb plus OVA triggered markedly increased expansion and migration of T cells to the B cell zone of the draining cervical lymph node compared with mice immunized with OVA alone (Fig. 8*b*). Furthermore, in another experiment, mice given Nb plus OVA plus CTLA4Ig showed a pronounced decrease in DO11.10 T cell migration to the B cell zone compared with the control group given Nb plus OVA plus L6 (Fig. 8, *c* and *d*). These results suggest that the combination of Nb plus OVA is required for DO11.10 T cell expansion and migration to the B cell zone and that B7 blockade inhibits such trafficking. In an additional experiment, DO11.10 T

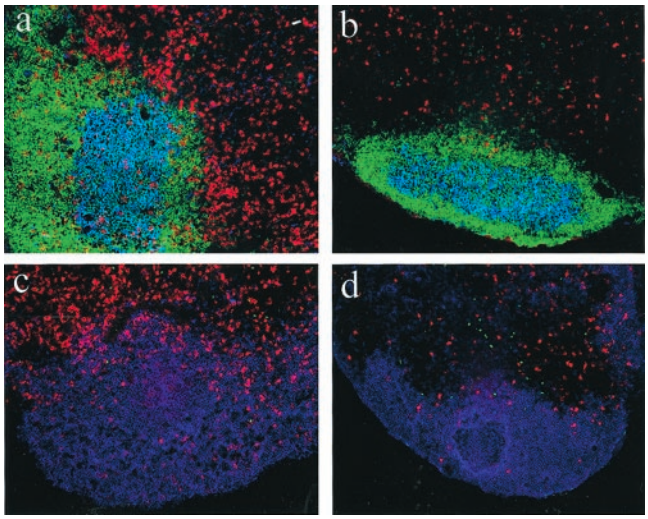

FIGURE 8. Visualization in the lymph node of B7-dependent adjuvant-induced Th2 cell migration to the B cell zone. The experiment was performed as described in Figs. 4 and 6. On day 7 after inoculation of recipient mice, draining cervical lymph nodes were collected, snap frozen, sectioned, and stained. *a*, Recipient mice immunized with Nb plus OVA and stained for DO11.10 T cells (red), B cells (green), and GC (blue); *b*, recipient mice immunized with OVA only and stained as in *a*; *c*, recipient mice immunized with OVA plus Nb plus L6 and stained for OVA-specific T cells (red), B cells (blue), and CFSE (green); *d*, recipient mice immunized with OVA plus Nb plus CTLA4Ig and stained as in *c*. Magnification, ×100. This experiment was repeated twice with similar results.

cells were transferred into B7-1/B7-2$^{-/-}$ recipient BALB/c mice before inoculation in the ear with Nb. CTLA4Ig was also administered using the same protocol already described. The results were very similar to those obtained with Nb-inoculated B7-1/B7-2$^{+/+}$ BALB/c DO11.10 T cell recipient mice treated with CTLA4Ig with respect to both cytokine expression and cell cycling (data not shown).

Discussion

Our findings demonstrate that B7-1/B7-2 blockade during the primary immune response to the nematode parasite, Nb, inhibits humoral immunity but does not inhibit the development of IL-4 producing T cells that can mediate worm expulsion. Furthermore, Nb L3 can act as an adjuvant that induces nonparasite Ag-specific Th2 cell differentiation of naive DO11.10 T cells in vivo and that, if B7 interactions are blocked, naive DO11.10 T cells can still differentiate to Th2 cells.

B7-1/B7-2 costimulation is generally considered a requirement for the development of Th2 cells from naive T cells. The surprising observation that after B7 blockade CD4 T cell effector function was sufficiently intact to mediate host protection during the immune response to Nb suggested that this parasite elicits an alternative pathway for the development of Th2 cells (21). Although these previous studies had suggested that Th2 cell cytokine production was inhibited after in vitro restimulation assays, our findings show that T cell IL-4 production is comparable in B7-1/B7-2$^{-/-}$ and B7-1/B7-2$^{+/+}$ Nb-inoculated mice when assay systems are used that more directly assess cytokine gene and protein expression in vivo. As described in the results, we also found that the B7 independence of the Th2 response was not dose dependent given that low inoculums induced similar Th2 responses in B7-1/B7-2$^{-/-}$ and B7-1/B7-2$^{+/+}$ mice. In contrast, the primary Th2 response to a different nematode parasite, *H. polygyrus*, which is also associated with pronounced T cell IL-4 production, is blocked in B7-1/B7-2$^{-/-}$ mice (35) and in WT mice treated with CTLA4Ig (3). The Th2 response is also blocked after CTLA4Ig administration in *T. muris*-infected BALB/c mice (5).

There are several possible mechanisms that might explain the difference in B7 dependence between the immune response to Nb and both *T. muris* and *H. polygyrus.* Both *T. muris* and *H. polygyrus* are strictly enteric, whereas Nb migrates from the skin to the lungs and finally to the small intestines. Nb may thus encounter and activate cell populations distinct from those activated in the intestine by either *T. muris* or *H. polygyrus*, some of which may mediate B7-independent T cell activation. It is also possible that Nb expresses a particularly effective adjuvant that can support B7-independent T cell activation. Previous studies have shown that CD4 T cell maturation in some Th1 responses can occur without B7 signaling (5, 6, 8). In these cases, microbial adjuvants may trigger the release of cytokines (40–42), which then provide sufficient signaling to circumvent a B7 requirement for Th1 cell activation. It is possible that Nb produces an analogous microbial adjuvant, which when recognized by the immune system can trigger the rapid development of a host-protective B7-independent Th2 response. Recent studies suggest that soluble Nb excretory-secretory proteins can stimulate IL-4 production and polyclonal IgE synthesis, although the response is considerably reduced compared with live parasite infection (43); it is possible that Nb excretory-secretory proteins and/or other structures associated with Nb may be responsible for triggering the B7-independent development of the Th2 response. Th2 adjuvants have also been identified on *Schistosoma mansoni* egg Ags, and recent studies indicate that lacto-*N*-fucopentaose(III), the predominate carbohydrate in *S. mansoni* egg Ags, can stimulate Th2 responses, including Ag-specific IgE production, when conjugated to human serum albumin (44, 45).

The development of an immunization model system to examine whether live Nb can similarly promote a nonmucosal Th2 immune response allows for the direct comparison of the immune response after enteric vs parenteral inoculation with the same live pathogen. Our results demonstrate that a similarly potent and highly polarized nonmucosal Th2 immune response is induced and that this response is B7 independent. In further studies, we examined whether Nb can function as an adjuvant to drive the development of nonparasite Ag-specific Th2 cells by transferring DO11.10-transgenic T cells specific for OVA peptide to recipient mice subsequently inoculated with Nb plus OVA. Previous studies with this adoptive transfer model for studying Ag-specific T helper cell differentiation have used Th1-inducing adjuvants, including LPS and CFA, to trigger DO11.10 Th1 cell differentiation in vivo (26, 27). Our findings show that Nb can analogously act as an adjuvant that instead drives the development of DO11.10 Th2 cells in vivo. Thus, naive T cells with the same specificity and affinity for Ag can rapidly develop in vivo into either Th1 or Th2 cells when sufficiently strong, polarizing microbial adjuvants are available, suggesting that under these circumstances TCR signal strength is not a major factor influencing Th cell cytokine production.

It was possible that during the Nb response, Th2 cells were activated to produce IL-4 through a bystander T cell activation mechanism, as has been observed in other immunization systems (1), particularly given the lack of requirement for B7 costimulatory signals. However, the observation that Nb alone did not significantly activate adoptively transferred DO11.10 T cells even as late as day 7 after inoculation suggests that bystander naive T cells (in this case DO11.10 T cells), which lack specificity for Ags associated with Nb, do not play a major role in this response. Thus, the mechanism of Nb-induced Th2 cell responses is probably restricted to the augmentation of Ag-dependent naive T cell differentiation. Considering that the B cell response is frequently polyclonal in parasitic infections (24, 25), it is possible that the contrasting stringent regulation of Ag-specific T cell activation during this parasitic infection plays an important role in controlling the specificity of the response. There was also the possibility that endogenous TCRs, expressed by the DO11.10 transgenic T cells, may recognize Ags associated with Nb. However, the observation that there was little difference between stimulation of transferred DO11.10 T cells from DO11.10 RAG2$^{+/+}$ mice and DO11.10 RAG2$^{-/-}$ mice in any of the treatment groups showed that endogenous TCRs expressed on T cells from DO11.10 RAG2$^{+/+}$ mice had little effect, indicating that among the transferred cells naive T cells, specific for OVA, were the major activated population.

The finding that, in the context of the Nb in vivo immune response, naive DO11.10 T cells could differentiate to IL-4-producing T cells, although B7 interactions were inhibited, suggests that the in vivo adjuvant properties of this parasite extend to the activation of B7-independent nonparasite Ag-specific Th2 cell differentiation from naive T cells. Previous in vitro studies have suggested that filarial excretory-secretory products may act as adjuvants that promote Th2 cell development; however, T cell differentiation to cytokine production remained B7 dependent, and restimulation with potent mitogens was required to observe the adjuvant effect (46). Other studies have suggested that in vivo a "default" pathway may develop in the absence of microbial adjuvants, which leads to the development of Th2 responses (10–12). Previous studies with adult Nb excretory-secretory products have shown that excretory-secretory products can augment B cell IgE challenge responses to nonparasite Ags (43). Our findings, using transferred DO11.10 T cells, now show that Nb promotes in vivo

Ag-specific naive T cell differentiation during a primary response, even when the TCR is specific for a nonparasite Ag, suggesting that this parasite has structures that can function as a microbial adjuvant to stimulate Ag-specific Th2 cell differentiation in vivo.

In marked contrast to the sustained development of cytokine producing T cells and associated worm expulsion, the humoral response was abrogated in Nb-inoculated B7-1/B7-2$^{-/-}$ mice, consistent with previous studies that elevations in total IgE were substantially inhibited after CTLA4Ig administration of Nb-inoculated mice (21). Our observation that up-regulated B cell surface MHC class II expression, which is IL-4 dependent (3, 36), was also inhibited suggests that although B7-independent IL-4-producing T cells could develop that could mediate worm expulsion, their ability to interact with B cells was severely compromised. Our further finding that, after immunization with Nb plus OVA, adoptively transferred DO11.10 T cells produced IL-4 but showed reduced migration to the B cell zone indicates that the development of IL-4-producing T cells is separable from their differentiation to T helper cells that can migrate to the B cell zone and provide B cell help. These results suggest that IL-4-producing T cells first develop in the T cell zone and then, after additional differentiation stages, migrate to the B cell zone where they contribute to B cell differentiation and GC formation. In vivo cell cycling was reduced in Nb-inoculated mice, when B7 interactions were blocked. Because the majority of the DO11.10 cells that had migrated to the B cell zone in Nb-inoculated mice had undergone multiple cell divisions, as determined by their reduced or undetectable CFSE staining (see Fig. 7), it is possible that T cell migration to the B cell zone requires differentiation stages that are cell cycle dependent and that occur after Th cell differentiation to IL-4 production. These results are consistent with findings suggesting that Th2 cell differentiation (although not necessarily IL-4) may be controlled by the cell cycle (47). It is also possible that B-T interactions, which were inhibited in the absence of B7, promote T cell proliferation; several studies have suggested that B cells are important in Ag presentation during Th2 responses (48, 49).

Taken together, our results demonstrate that after Nb immunization naive T cells can differentiate in the absence of B7-1/B7-2 interactions to Th2 effector cells that can mediate worm expulsion. Furthermore, Nb act as an adjuvant to induce nonparasite Ag-specific Th2 cell differentiation in vivo.

Acknowledgments

We thank Dr. Mark Jenkins (University of Minnesota, Minneapolis, MI) for his helpful discussions and for providing the DO11.10 RAG2$^{-/-}$ T cells. We also thank Dr. Fred Finkelman (University of Cincinnati School of Medicine, Cincinnati, OH), Dr. Mark Jenkins, and Dr. Clifford Snapper (Uniformed Services University of the Health Sciences) for carefully reviewing the manuscript.

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CCL-2-mediated recruitment of neutrophils to the lymph node is required for Th2 polarization

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[1]This work was supported by National Institutes of Health Grants AI31678 and AI55848 and by U.S. Drug Administration CRIS 1265-32000-064-00D.

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[5]Abbreviations used in this paper: WT, wild type; CLN, cervical lymph node; MHCII, MHC class II; APC, antigen presenting cell; DC, dendritic cell; Ab, antibody; Ag, antigen.

Abstract

Components of the innate immune response important in directing the development of the Th2 response are not well understood. To identify early events that contribute to the development of the host protective Th2 response to nematode parasites, microarray analysis was performed on draining lymph nodes shortly after *N. brasiliensis* inoculation. We observed increased expression of several chemokines, including CCL2 and also CXCR3 ligands, which have previously been shown to recruit Gr-1^+ cell populations. Flow cytometric and immunofluorescent analysis showed a transient and marked increase in Gr-1^+ cells in the draining lymph node within 18 hours after *N. brasiliensis* inoculation. Depletion experiments, using anti-Gr-1 Ab, resulted in disruption of the polarized Th2 *in vivo* immune response, characterized by significantly increased levels of IFN-γ gene expression, IgG2a elevations, and increased worm burden. Similar results were obtained in *N. brasiliensis*-inoculated CCL2-/- mice and, at 18 hours after inoculation, recruitment of the Gr-1 bright cell subset was inhibited. Sorting of the Gr-1 bright population demonstrated that these cells are activated neutrophils that express high levels of TGF-β and TNF-α. These studies show for the first time that neutrophils are rapidly recruited to the draining lymph nodes at the initiation of the Th2 immune response, and that this innate immune cell population plays an integral role in the development of the polarized Th2 response.

Introduction:

The immune response that develops following infection with the nematode parasite, *Nippostrongylus brasiliensis*, results in high levels of IL-4 and other Th2 cytokines and low to undetectable levels of IFN-γ. The resulting polarized Th2 response is host protective, leading to rapid worm expulsion following primary immunization (1). The initial events that contribute to the development of this *in vivo* Th2 immune response are not well understood, nor are the mechanisms that contribute to Th2 responses generally.

Nippostrongylus brasiliensis has been shown to function as an adjuvant, driving the in vivo differentiation of Th2 cells from naïve T cells of unrelated Ag-specificity (2). This adjuvant function suggests that an innate response develops *in vivo* that promotes the polarization of Th2 cells. Previous studies have suggested that non-T cell IL-4, produced by eosinophils and basophils, may promote Th2 cell differentiation (3-6); however, recent studies have indicated that non-T cell IL-4 is not required for the development of Th2 cells both *in vitro* and *in vivo* (7;8). It should also be noted that antigen presentation, and presumably Th2 cell commitment, initially occur in the lymph node microenvironment, and that eosinophils and basophils are not present in substantial numbers in this tissue.

In the draining lymph node, naïve $CD4^+$ T cells initially interact with Ag-presenting dendritic cells that express costimulatory molecules, which together provide signals required for T helper effecter cell development and expansion

(9;10). In the case of Th1 responses, dendritic cells producing Th1-inducing cytokines, such as IL-12, and also NK cells have been shown to play an important role in influencing naïve T helper cells to differentiate into effecter T helper cells producing IFN-γ (11). As yet, accessory cell populations that might augment the development of Th2 effecter cells in the lymph node have not been identified. Most studies of the Th2 response to *N. brasiliensis* have involved examination of the mucosal response that develops in either the gut or lung associated lymphoid tissue. We have shown that a potent and highly polarized Th2 response also develops in the draining lymph node at the site of initial inoculation of *N. brasiliensis* in the skin (2;7). This localized response greatly facilitates analysis and manipulation of the Th2 response in vivo (2;7;12;13).

Microarray analysis is a useful method for examining global gene expression to identify specific genes or groups of genes important in an immune response (14;15). In this investigation, we used Affymetrix microarrays to assess elevations in gene expression that occurred at early stages of the Th2 response prior to elevations in IL-4 mRNA. Our studies identified increased levels of specific chemokines known to recruit non-myeloid Gr-1^+ cells to the lymph node (16-18). Analysis of lymph node tissue sections revealed a pronounced influx of Gr-1^+ cells within 18 hours after inoculation. *In vivo* Gr-1^+ cell depletion studies showed a pronounced disruption of the polarized Th2 response resulting in a markedly increased Th1 response. Inoculation of CCL2-deficient mice with *N. brasiliensis* showed a marked decrease in the early influx of a Gr-1 bright subset comprised of activated neutrophils and an associated disruption of the Th2

response similar to that observed with anti-Gr-1 antibodies. These studies indicate a major role for CCL2-recruited Gr-1 bright neutrophils in the development of highly polarized Th2 responses *in vivo.*

Materials and Methods

Mice - All experiments were performed using BALB/c, DO11.10 transgenic BALB/c mice, C57BL/6, or $CCL2^{-/-}$ C57BL/6 mice. Four to five mice were used per treatment group. All strains were obtained from The Jackson Laboratory (Bar Harbor, ME). T cells from DO11.10 transgenic mice express a TCR specific for class II MHC-restricted chicken OVA peptide (323-339)-I-A^d complexes. This TCR is uniquely recognized by the KJ1-26 anticlonotypic monoclonal antibody (19). All mice were maintained in a specific pathogen-free, virus Ab-free facility administered by the Laboratory of Animal Medicine, Uniformed Services University of the Health Sciences. Experiments in this study were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals (Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare (National Institutes of Health) 86-23).

Adoptive Transfers- Peripheral lymph nodes and spleen were harvested from DO11.10 TCR transgenic mice that were age- and sex-matched to the adoptive transfer recipients. Single-cell suspensions were prepared by pressing tissue through a nylon strainer (BD Biosciences, San Jose, CA) and $5X10^6$ DO11.10

OVA-specific $CD4^+$ T cells (5 X 10^6) were injected i.v.. into recipient mice, as previously described (2).

Parasite inoculation, Gr-1^+ cell depletion, and OVA immunization- Mice were intracutaneously inoculated in the ear with infective *N. brasiliensis* L3. Parasite egg numbers and adult worm numbers were evaluated as described previously (20). In several experiments, Gr-1^+ cells were depleted *in vivo* by I.P. administration of 12.5μg of anti-Gr-1 antibody (clone RB6-8C5) 24 hours prior to *N. brasiliensis* inoculation. In some trafficking experiments, *N. brasiliensis* L3 and 0.15mg of Texas Red or FITC labeled OVA (Molecular Probes, Eugene, OR) were simultaneously injected intracutaneously in the ear.

Quantitation of serum immunoglobulins*:* Total serum IgG1, IgG2a, and IgE levels were quantitated by ELISA (21).

Gene Expression by real-time PCR- Total RNA was extracted from cervical draining lymph nodes using RNAzol B (AMS Biotechnology, UK) and total RNA was then reverse transcribed to cDNA using Superscript II (Invitrogen, Carlsbad, CA), as previously described (2). Real-Time PCR Taqman® (Applied Biosystems, Foster City, CA) kits for IL-4, IFN-γ, IL-10, IL-13, TNF-α, and 18s Ribosome or SYBR® green (Applied Biosystems, Foster City, CA) kits for IP-10, MIG, iTAC, MCP-1, TGF-β, and HPRT were used to quantitate differences in gene expression, and all data were normalized to 18s ribosomal or HPRT values. Primer sequences for SYBR green assays were performed using the following primer pairs: IP-10- GACGGTCCGCTGCAACTG (Forward) GCTTCCCTATGGCCCTCATT (Reverse), MIG- TGCACGATGCTCCTGCA

(Forward) AGGTCTTTGAGGGATTTGTAGTGG (Reverse), MCP-1-GCTGGAGCATCCACGTGTT (Forward) ATCTTGCTGGTGAATGAGTAGCA (Reverse), and HPRT-TTGCTCGAGATGTCATGAAGGA (Forward) AGCAGGTCAGCAAAGAACTTATAGC (Reverse), iTAC (22), TGF-β (23). The Applied Biosystems 7500 sequence detector was used for amplification of target mRNA, and quantification of differences between treatment groups was calculated according to the manufacturer's instructions.

Microarray Analysis- RNA was isolated from draining cervical lymph nodes of BALB/c mice (5 mice per group) inoculated with *N. brasiliensis* on days 1.5 or 2.5, or untreated BALB/c mice, and pooled for microarray analysis. Total RNA was prepared using an RNeasy mini kit (Qiagen, Valencia, CA) This RNA was then used to synthesize cRNA probes. cRNA generation, hybridization, and analysis of U74Av2 murine genome arrays were performed under the guidelines of the manufacturer's protocol (Affymetrix, Santa Clara, CA) at the Laboratory of Immunopathogenesis and Bioinformatics, National Cancer Institute, Frederick, MD. Fluorescently labeled cRNA was hybridized to U74Av2 murine genome arrays and measured at 570nm by an Affymetrix Scanner. Affymetrix Microarray Suite 4.0 was used to determine individual transcript fluorescence intensity. Data was compiled and analyzed using Genespring 5.1 software (Silicon Genetics, Redwood City, CA).

Microscopy- Draining cervical lymph nodes were harvested from individual mice and frozen in liquid nitrogen, and 6-µm tissue sections were obtained from near

the center of the cervical lymph node using a HM505E cryostat (Richard-Allan Scientific, Kalamazoo, MI). Tissue sections were allowed to dry at room temperature for 30 min, fixed in cold acetone for 10 min, and stored at -80°C. Cervical lymph node tissue sections were then dual-stained with anti-Gr-1-FITC (Pharmingen) and biotinylated-anti-B220 (RA3-6B2; BD PharMingen) Abs, followed by streptavidin Alexa-350 (Molecular Probes, Eugene, OR), and coverslipped using FlouromountG (Southern Biotechnology Associates, Birmingham, AL). Digital tissue mapping and tiling was performed as previously described (12).

Flow Cytometry- Lymph node cells were harvested and cells suspensions prepared for flow cytometric analysis, as previously described (7;24). Briefly, individual lymph nodes were removed from *N. brasiliensis*-inoculated mice and incubated at 37°C in a cocktail of Liberase Cl (Roche, Indianapolis, IN), DNase (Sigma-Aldrich, USA), and RPMI 1640 containing 10% heat-inactivated FCS, 100U/ml penicillin, 100 µg/ml streptomycin, and 2mM L-glutamine (all from Invitrogen Life Technologies) for a period of 30 minutes, to increase the yield of Gr-1^+ and $CD11c^+$ cells. Cells were passed through a 70µm filter and resuspended. One million cells were incubated with Fc Block (PharMingen, San Diego, CA) and then with anti-Gr-1 PE (BD Pharmingen), anti-B220/CD45 APC Cy7 (BD Pharmingen), anti-CD11c APC (BD PharMingen), anti-CD11b PerCP5.5 (BD Pharmingen), biotinylated-anti-MHC II I-Ad (BD PharMingen), biotinylated-anti-CD86 (BD PharMingen), anti-CD69-PE (BD PharMingen), followed by streptavidin APC Cy7 (BD PharMingen). After washes, cells were

fixed with 2% paraformaldehyde (Fisher) and analyzed by flow cytometry using an LSRII FACS analyzer (BD Biosciences, San Jose, CA).

Cell isolation and Gene expression*:* The Gr-$1^{+\,bright}$ population was sorted using high-speed FACS sorting (BD Vantage with high speed upgrade). More than 95% (purity) of the sorted cells were Gr-$1^{+bright}$. Sorted Gr-$1^{+bright}$ cells were morphologically examined by cytospin centrifugation (Shandon Cytospin 4, Thermo Electron, Corp., UK) and H&E staining. For RT-PCR, total RNA was extracted from purified cell populations with the RNA Isolation Kit (Stratagene, Cedar Creek, TX), specially developed for isolating small RNA quantities, and from tissue as described above. Total RNA was then reverse transcribed and real-time PCR was performed as described above.

Statistical Analysis: Statistical differences (significance level of $p<0.05$) between groups was assessed using ANOVA and Tukey's t test for pairwise comparisons. The software program SigmaStat (Jandel Scientific Software, San Rafael, CA) was used for all statistical analyses.

Results:

Increased expression of CCL2 and CXCR3 ligands is observed prior to IL-4 production after *N. brasiliensis* inoculation.

Little is known concerning the early events that precede and contribute to the development of IL-4 producing T cells *in vivo*. We used Affymetrix microarray technology in an attempt to identify individual genes or groups of genes that may be involved in the initiation of Th2 cell differentiation. Female BALB/c mice were inoculated with 500 *N. brasiliensis* L3 intracutaneously in the

ear. Previous studies have shown that a highly polarized Th2 response develops in the draining cervical lymph node (CLN) by day 7 after *N. brasiliensis* intracutaneous inoculation in the ear (2). We extended these studies to examine earlier time points and found that elevations in IL-4 gene expression were consistently detected as early as day 3 after *N. brasiliensis* inoculation (7).

To identify gene expression changes that precede IL-4 production, we removed draining CLNs at days 1.5 or 2.5 after *N. brasiliensis* inoculation and processed these tissues for microarray analysis. Each time point consisted of a pool of 5 individual mice. Separate microarray analyses were performed on lymph node samples from two independent experiments and highly reproducible results were obtained. Despite screening for *18,000* known genes and transcripts at these two time points, draining CLNs from inoculated mice revealed relatively few significantly changed genes as compared to untreated control mice.

Three chemokines were consistently increased including, CXCL10 (IP-10), CXCL9 (MIG), and CCL2 (MCP-1). CXCL10 and CXCL9 share a common receptor, CXCR3, which is expressed on a variety of cell types including eosinophils, NK cells, and plasmacytoid dendritic cells, and some recent evidence has suggested that CXCL10 is elevated in asthmatic patients and associated with increased eosinophilia and IL-4 production (16;25;26). CCL2 is the ligand for CCR2, which is expressed on populations of granulocytes (17), dendritic cells (27), and also Th1 and Th2 cells (28;29). Recent studies have also suggested that CCL2 may be required for the development of an effective Th2 response in peripheral tissues, although its actual function is unclear (29-32). To confirm

these results with a different assay system, real-time fluorogenic RT-PCR was used to quantitate elevations of these chemokines in the draining lymph node of *N. brasiliensis*-inoculated mice. As shown in Fig. 1, significant elevations in all three chemokines were detected in the draining cervical lymph node at early time points after *N. brasiliensis* inoculation. In addition, CXCL11 (iTAC), another CXCR3 ligand and member of the CXCL10 family, which was not included in the microarray analyses was also assayed and found to be elevated. Taken together, these studies indicate that inoculation with *N. brasiliensis* induces pronounced increases in chemokines known to recruit granulocytes, dendritic cells, and other immune cell populations.

***N. brasiliensis* inoculation leads to an increased presence of a Gr-1$^+$ population within the CLN**.

Our finding that *N. brasiliensis* triggered increased levels of chemokines known to be important in recruitment of granulocytes, neutrophils, and subsets of dendritic cells suggested that a nonresident immune cell population may migrate to the draining lymph node at the initiation of a polarized Th2 response. A common characteristic of many of these different cell populations, including granulocytes (33), neutrophils (34), and dendritic cells (18;35), is their expression of the cell surface marker Ly-6G (Gr-1). Based on the results from the microarray analysis after *N. brasiliensis* intracutaneous inoculation, we next examined whether a Gr-1$^+$ cell population was detected in the draining lymph node at early stages of the type 2 *in vivo* immune response and, if so, whether it was associated with trafficking of Ag. To perform these studies, we utilized a

previously published adoptive transfer model where *N. brasiliensis* is used as an adjuvant to drive transferred DO11.10 T cells to differentiate into Th2 cells (2;7;13). In these experiments, OVA-specific DO11.10 T cells were adoptively transferred to BALB/c mice and two days later, recipient mice were inoculated intracutaneously in the ear with *N. brasiliensis* + OVA. We additionally included fluorescently labeled OVA protein to simultaneously track Ag and DO11.10 T cells in the lymph node during early stages of the immune response, as previously described by others (36).

Remarkably, fluorescence imaging of whole lymph node sections showed for the first time that, by 4 hours after inoculation, Gr-1^+ cells were readily detectable in peripheral regions of the lymph node, and that they increased in number and became distributed throughout the lymph node by 18 hours (Fig. 2A-C). OVA peptide was localized to the same regions as the Gr-1^+ cells at this time point (18 hours). To examine whether the Gr-1^+ cells were interacting with either OVA or the OVA-specific DO11.10 T cells, confocal microscopy (1000X magnification) was used to examine individual Gr-1^+ cells. Distinct clustering was consistently observed between DO11.10 T cells and Gr-1^+ cells with OVA localized at the junction of Ag-specific T cell:Gr-1^+ cell interactions (Fig. 2D). This clustering was primarily observed in the T:B zone and to a lesser extent in the T zone (data not shown).

Using FACS analysis, we similarly identified a pronounced expansion of the Gr-1^+ cell population in the draining cervical lymph node of *N. brasiliensis*-inoculated mice as early as 4 hours after inoculation; by 18 hours this population

had increased from 0.25% to as much as 5.0% of total lymph node cells. The Gr-1^+ cells were greatly reduced in number by two to three days after *N. brasiliensis*-inoculation. Gr-1^+ cells could be resolved into Gr-1 bright and Gr-1 dull subpopulations. Dual detection of Texas Red-labeled OVA and Gr-1^+ cells (FITC anti-Gr-1 Ab) revealed that a subpopulation of both the Gr-1 bright and Gr-1 dull cells had become associated with labeled OVA (Fig. 2 E-G), consistent with a role for these cells in Ag presentation (36), although we cannot rule out that the OVA peptide may be binding the Gr-1^+ cells nonspecifically. It should be noted that lymph nodes not draining the site of inoculation did not exhibit increases in Gr-1^+ cells (data not shown).

Analysis of Gr-1^+ cells reveals two distinct Gr-1^+/CD11b$^+$ cell populations, following inoculation with *N. brasiliensis*.

Our findings indicated that the Gr-1^+ cell populations interact with Ag-specific T cells after *N. brasiliensis* inoculation. To further characterize the Gr-1^+ cell populations in the draining lymph node, we used flow cytometric analysis to assess expression of other cell surface markers associated with Ag presentation. Known populations bearing the Gr-1 marker such as myeloid suppressor cells and plasmacytoid dendritic cells also express high levels of CD11b and CD11c respectively (35;37;38). Immunofluorescent staining of cervical lymph nodes from *N. brasiliensis*-inoculated mice revealed that a majority were positive for CD11b as well as Gr-1 (Fig. 3). Further analysis of gated cell populations revealed distinct phenotypic differences between Gr-$1^{+bright}$ and Gr-1^{-dull} CD11b$^+$ cells. Gr-1 bright cells were CD11c$^-$, but about half were MHCII$^+$, and about a

quarter expressed CD86. Gr-1 dull cells included 42% $CD11c^+$ cells, 79% $MHCII^+$ cells, and 71% $CD86^+$ cells (Fig. 3). The $Gr\text{-}1^+$ cells did not express markers for macrophages (F4/80) (39;40), eosinophils (MBP) (41), and basophils (DX5) (4;42) (data not shown).

Depletion of $Gr\text{-}1^+$ cells results in deregulation of cytokine production after *N. brasiliensis* inoculation.

Our findings that $Gr\text{-}1^+$ cells rapidly increased in draining lymph nodes shortly after inoculation raised the possibility that they might be influencing the lymph node milieu where Th2 cells differentiate. To address this possibility, we performed *in vivo* intervention experiments using the RB6-8C5 anti-Gr-1 Ab., which has previously been shown to specifically and transiently deplete treated animals of $Gr\text{-}1^+$ cells(43;44). BALB/c mice were administered either 12.5μg of anti-Gr-1 Ab or the isotype control intraperitoneally 24 hours prior to *N. brasiliensis* intracutaneous inoculation in the ear. Eighteen hours after *N. brasiliensis* inoculation, draining cervical lymph nodes were removed from sacrificed mice and analyzed for cytokine gene expression by quantitative fluorogenic real-time RT-PCR. Analysis of draining cervical lymph nodes in anti-Gr-1 Ab treated mice at 18.0 hours after inoculation demonstrated markedly higher levels of IFN-γ gene expression, compared with mice receiving *N. brasiliensis* + isotype-matched control antibodies (Fig 4A). IL-4 levels at this time point were not upregulated in any of the treatment groups, consistent with our previous studies of whole lymph node tissue (data not shown). These studies

indicated that Gr-1$^+$ cells downregulated the rapid development of a potent IFN-γ response that would otherwise be induced following *N. brasiliensis* inoculation.

In additional experiments, anti-Gr-1 Ab treated mice were sacrificed at day 7 after *N. brasiliensis* inoculation, to determine whether Gr-1$^+$ cells may influence the development of the Th2 response that mediates worm expulsion. Gene expression analysis of draining cervical lymph nodes showed consistently lowered levels of IL-4 mRNA expression in anti-Gr-1 Ab treated groups compared to isotype control Ab treated groups at day 7 after *N. brasiliensis* inoculation (Fig. 4B). IFN-γ levels returned to baseline levels at this later time point (data not shown). Previous studies have demonstrated that the major source of IL-4 in the cervical lymph node at day 7 after *N. brasiliensis* inoculation is CD4$^+$ T cells (2). Analysis of serum Ig levels at day 7 after inoculation showed a significant increase in IgG2a levels in *N. brasiliensis*-inoculated mice given anti-Gr-1 Ab compared to *N. brasiliensis*-inoculated mice administered isotype control Ab (Fig. 4B), consistent with the early burst of IFN-γ gene expression observed following Gr-1$^+$ cell depletion. It should be noted that IgG1 elevations were not affected, and that at this early timepoint, elevations in serum IgE levels are not detected (data not shown).

An important general indicator of an effective Th2 response in the enteric region is host resistance. Importantly, we observed a significant increase in both adult worm numbers and egg production in *N. brasiliensis*-inoculated mice administered anti-Gr-1 Ab compared to inoculated mice administered the isotype control Ab (Fig. 4B). This finding is consistent with previous studies in which

administration of IFN-γ or induction of endogenous IFN-γ production resulted in impaired development of protective immunity (45;46). Taken together, these studies suggest that the Gr-1^+ cells that rapidly appear in the draining lymph node shortly after *N. brasiliensis* inoculation substantially limit early IFN-γ elevations that would otherwise inhibit the development of an effective Th2 response leading to host protection.

Recruitment of Gr-$1^{+bright}$ cells to the draining CLN after *N. brasiliensis* inoculation is dependent on CCL2 signaling.

Based on our observation of elevated CCL2 mRNA in the microarray analysis and the corroborative RT-PCR assays (see Fig. 1), we directly investigated the role of CCL2 in the recruitment of Gr-1^+ subsets to the draining CLN during the Th2 immune response to *N. brasiliensis*. CCL2 deficient mice or BL/6 WT controls were inoculated with *N. brasiliensis* and, after 18 hrs, when maximal Gr-1^+ cell infiltration is observed, draining CLNs were removed for analysis. It should be noted that we initially determined that pronounced Gr-1^+ cell infiltration of the CLN also occurred in BL/6 mice following *N. brasiliensis* inoculation (data not shown), indicating that Gr-1^+ cell recruitment following *N. brasiliensis* inoculation is not a strain specific phenomenon but instead occurs in diverse genetic backgrounds. In marked contrast to WT controls, CCL2 deficient BL/6 mice inoculated with *N. brasiliensis* showed a marked reduction in the recruitment of the Gr-1 bright subset to the draining CLN. As shown in Fig. 5, up to 22% of Gr-1^+ cells stained Gr-1 bright in *N. brasiliensis*-inoculated BL/6 WT

control mice, while in similarly treated BL/6 CCL2 deficient mice only 1.1% of Gr-1^{+} cells stained Gr-1 bright.

CCL2-deficient mice exhibit pronounced early increases in IFN-γ expression and reduced host protection following *N. brasiliensis* inoculation

The selective inhibition of Gr-1^{bright} cell recruitment to the draining lymph node of CCL2-deficient *N. brasiliensis*-inoculated mice provided the opportunity to assess whether this specific Gr-1^{+} cell subset was important in controlling the early upregulation of IFN-γ. CCL2-deficient or control WT BL/6 mice were inoculated with *N. brasiliensis* and 18 hours later the draining CLN was removed and analyzed for upregulation of IFN-γ mRNA. As shown in Fig. 6A, IFN-γ was markedly upregulated in *N. brasiliensis*-inoculated CCL2-deficient BL/6 mice but not in inoculated WT BL/6 mice by 18.0 hours post inoculation. In contrast, IL-4 mRNA was not elevated in either CCL2-deficient or WT mice at this early timepoint after *N. brasiliensis*-inoculation (Fig. 6A), as also observed in *N. brasiliensis*-inoculated BALB/c mice (see Fig. 4A).

As shown in Fig. 6B, significantly increased levels of IFN-γ-dependent serum IgG2a and also egg burden were detected in *N. brasiliensis*-inoculated CCL2 deficient mice compared to inoculated WT controls, at day 10 post-Nb inoculation. Taken together, these results indicate that CCL2 signaling is required for the recruitment of Gr-1 bright cells to the lymph node after *N. brasiliensis* inoculation and that this Gr-1^{+} cell subset is required to control early elevations in IFN-γ production and associated increased IgG2a levels that would otherwise

occur. Furthermore, CCL2 blockade inhibits the development of the polarized Th2 response required for host protective immunity.

The Gr-1^{bright} cell subset consists of neutrophils that express known regulatory cytokines.

High-speed electronic cell sorting was used to isolate highly purified Gr-1^{bright} cells (>99%) from the lymph nodes at 18 hours after *N. brasiliensis* inoculation. Cells isolated using this technique were adhered to glass slides using a Cytospin centrifuge. Subsequent H&E staining demonstrated that the Gr-1^{bright} subset consisted solely of neutrophils (Fig. 7A). RNA was isolated from the sorted Gr-1^{bright} cells and analyzed for cytokine mRNA expression using quantitative real-time fluorogenic RT-PCR. As a comparison, we isolated CD11c^{+} cells from untreated mice, since Gr-1+ cells were not in sufficient number for isolation. Analysis of these populations show that in comparison to CD11c^{+} cells, Gr-1^{+} cells produced high levels of TGF-β and TNF-α with no detectable increases in IL-4, IL-10, IL-12, or IL-13 (Fig. 7B).

Discussion

This study demonstrates through the use of microarray analysis that CCL2 and CXCR3 ligands are among the few genes exhibiting increased expression in the draining lymph node shortly after inoculation with the Th2 inducing nematode parasite, *N. brasiliensis.* Our findings further show that CCL2 recruits neutrophils to the lymph node, which downregulate IFN-γ expression and are required for the development of the highly polarized Th2 immune response leading to host protection.

Our conclusion that the Gr-1^{bright} cell subset that infiltrates the lymph node is a neutrophil is based on the observation that the cells exhibited the characteristic multi-nucleated phenotype and that they were negative for markers characteristic of basophils (DX5), eosinophils (MBP-1), and macrophages (F4/80). Neutrophils comprise about two-thirds of peripheral blood lymphocytes and are among the first cells to migrate to sites of inflammation, where they perform effector functions including phagocytosis and the secretion of cytotoxic compounds (47-49). Early studies suggested that neutrophils are transcriptionally inactive due to their terminal differentiaton; however, more recent studies have indicated that they are active components of the innate immune response, producing cytokines and chemokines that recruit macrophages and dendritic cells to the peripheral site of inflammation (50). Several studies have also indicated that activated neutrophils express costimulatory molecules, including B7-2, and that they can present Ag complexed with cell surface MHCII (51-53). Our findings now extend the role of neutrophils in the immune response. They show for the first time that neutrophils: 1) infiltrate the lymph node at early stages of the immune response; 2) augment the development of the polarized Th2 response by downregulating early increases in IFN-γ production.

Tissue damage, through injury or infection, can result in the release of chemokines including IL-8, MIP family members, and CCL-2, which cause the rapid recruitment of neutrophils to the site of peripheral inflammation (54-56). Their presence in large numbers in peripheral blood of uninfected animals allows their rapid accumulation shortly after infection, making neutrophils among the

first cells to appear at the site of infection. Our findings indicate that neutrophils are also rapidly recruited to the lymph node at the initiation of the Th2 response. Their appearance in large numbers within 18 hours after inoculation is analogous to their rapid migration to the site of peripheral inflammation. Generally, neutrophils are thought to be short-lived, dying shortly after their recruitment (57). Our kinetics studies in the lymph node showed that the neutrophils passed through in a wave, first being detected in the draining lymph node at 4 hours after *N. brasiliensis* inoculation, peaking at 18 hours, and virtually disappearing from the lymph node by 48 hours. It should be noted that this influx of neutrophils only occurred in lymph nodes draining the site of inoculation and that other peripheral lymph nodes distant from the site of infection did not support the recruitment of neutrophils.

Our studies further indicated that CCL2 is required for the recruitment of neutrophils to the draining lymph nodes. Microarray studies, confirmed with quantitative real-time fluorogenic RT-PCR, demonstrated that CCL2 was one of the first genes elevated in the draining lymph node after *N. brasiliensis*-inoculation and that neutrophil recruitment was blocked in *N.brasiliensis*-inoculated $CCL2^{-/-}$ mice. CCL2 is an important chemokine that can mediate recruitment of a variety of cell types including: dendritic cells, macrophages, and T cells (27;28;40;58). Our findings now extend the role of CCL2 to that of recruitment of neutrophils to the draining lymph node shortly after infection. Recent studies have shown that the host protective Th2 response to the intestinal nematode parasite, *Trichuris muris*, is substantially inhibited in $CCL2^{-/-}$ mice (29).

Our results showed that resistance in *N. brasiliensis*-inoculated mice is similarly inhibited. Taken together, our findings indicate a mechanism for how CCL2 may influence Th2 cell differentiation *in vivo*. It will be interesting in future studies to examine whether the increases detected in CXCR3 ligands in our microarray studies similarly are involved in recruiting neutrophils or other cell populations important in the initial development of the Th2 response.

The nematode parasite, *N. brasiliensis*, induces one of the most potent and highly polarized Th2 responses known to occur *in vivo*. This response is characterized by the rapid development of IL-4 producing T cells, with an absence of IFN-γ elevations, that leads to worm expulsion shortly after primary immunization (1;59). However, few characteristics of the innate immune response have been identified that drive the development of Th2 cells during this response. Most previous studies have concentrated on IL-4 production by non-T cells as providing a necessary cytokine environment for Th2 cell differentiation, but recent *in vitro* and *in vivo* studies have demonstrated that Ag-specific Th2 cells can develop when only autocrine IL-4 is available (4;7;8;60). Our findings indicate that neutrophils play an important role in down-modulating increases in IFN-γ that would otherwise occur at early stages of the Th2 response to this parasite. This is the first study to show that blockade of a specific cell population or cytokine results in a dramatic increase in IFN-γ during the *N. brasiliensis* immune response. Some studies have suggested that dendritic cells can support the development of a Th2 response *in vitro*, when coupled with certain parasite products; however, these cultures have not resulted in the strong and rapid

development of Th2 cells observed under *in vivo* conditions (13;61). Our findings suggest the possibility that an important element lacking in these *in vitro* studies may be the Gr-1 bright neutrophil population that is rapidly recruited to the lymph node during the early stages of the *in vivo* Th2 primary response. The observation that there is an underlying IFN-γ response that can develop when neutrophils are depleted during the *N. brasiliensis* response indicates that these innate regulatory cells actively mediate the development of this polarized Th2 response required for host resistance. Our confocal analyses showed close interactions between Ag-specific T cells and Gr-1 cells at early stages of the response raising the possibility that either Ag-presentation or the expression of cell surface or secreted molecules, or some combination of these may mediate the development of the polarized Th2 response. The finding that the neutrophils that have infiltrated the lymph node express CD86 and MHCII and exhibit increased levels of TGF-β and TNF-α, which have been shown to downregulate Th1 cytokines (62) and enhance the Th2 response (63) respectively, indicate that the neutrophils are in a highly activated state, consistent with their ability to modulate T helper cell differentiation.

Our findings thus indicate that *N. brasiliensis* infection induces the upregulation of CCL2 expression in the draining lymph node, triggering the rapid recruitment of Gr-1 bright neutrophils to the draining lymph node within several hours after inoculation. The Gr-1 bright cell population down-regulates early expression of IFN-γ, thereby facilitating the development of the alternative Th2 response. These results thus provide evidence that an important innate component

of the highly polarized Th2 response that develops following nematode parasite infection includes rapid recruitment of a neutrophil population to the site of Th2 cell differentiation in the draining lymph node.

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Figure Legends

Figure 1. Specific chemokines are upregulated in the lymph node shortly after *N. brasiliensis* inoculation. Pooled draining cervical lymph nodes were removed at days 1.5 and 2.5 after *N. brasiliensis* inoculation and also from untreated control BALB/c mice (5/treatment group). RNA was isolated and cRNA produced as described in the methods, which was then provided to the NCI Affymetrix core facility in Frederick, MD, where cRNA hybridization and Affymetrix oligonucleotide microarray analyses were performed on pooled samples. Fold changes are based on comparisons of treated mice to untreated control mice. This experiment was repeated two times with similar results. Based on the results of microarray analyses four candidate genes were selected for confirmation by real time quantitative fluorogenic RT-PCR. In an additional experiment, BALB/c mice (5/treatment group) were intracutaneously inoculated with *N. brasiliensis*. On days 1, 2, 3, or 6 after inoculation, draining cervical lymph nodes were removed, RNA purified and analyzed for expression of IP-10, MIG, ITAC, and MCP-1. Fold changes are expressed relative to the untreated BALB/c control. This experiment was repeated twice with similar results.

Figure 2. Gr-1$^+$ cells rapidly infiltrate the draining lymph node and interact with Ag-specific T cells shortly after *N. brasiliensis* inoculation. Five million DO11.10 CD4$^+$ T cells were transferred to recipient BALB/c (WT) mice. Two days later recipient mice were inoculated intracutaneously in both ears with *N. brasiliensis* + Texas Red labeled ovalbumin (5 mice per group). Draining cervical lymph nodes (CLNs) were removed at 0.0hrs, 4.0hrs, and 18.0hrs after inoculation. (**A-C**) CLNs were fluorescently labeled for B cells with anti-B220-biotin/Alexa350 (blue) and anti-Gr-1-FITC (green). Individual 400X digital images were tiled together to form a single high resolution composite image of the draining lymph node. **A.** Untreated CLN showed few Gr-1$^+$ cells. **B.** 4.0 hours after inoculation Gr-1$^+$ cells have infiltrated the CLN along with OVA Ag. **C.** At 18.0 hours Gr-1+ cells have reached maximal numbers in the CLN. **D.** 1000X confocal image of OVA-specific D011.10 T cells stained with KJ1-26-Alexa647 (blue), interacting with Gr-1 cells (green) and specific Ag (red). Yellow represents red-green overlap. (**E-F**) In the same experiment, draining cervical lymph nodes were harvested, and 1X10^6 cells were stained with anti-Gr-1-FITC Ab and analyzed by FACS. **E.** Untreated lymph node exhibits limited Gr-1 staining. **F.** At 4.0 hours post inoculation a significant number of Gr-1 cells have entered the lymph node and a subset are positive for Texas-Red labeled OVA staining. **G.** At 18.0 hours post inoculation, a marked increase in Gr-1$^+$ cells is detected with distinct Gr-1 bright and Gr-1 dull populations. This experiment was repeated three times with similar results.

Figure 3. Gr-1^{+} cells from *N. brasiliensis* inoculated mice are comprised of bright and dull subsets. BALB/c mice (5/group) were inoculated with *N. brasiliensis* and 18 hours later cervical lymph nodes (CLNs) were harvested and pooled cell suspensions were prepared for flow cytometric analysis. CD11b^{+}/Gr-1 bright and CD11b^{+}/Gr-1 dull cells were assessed for expression of markers characteristic of dendritic cells by staining with anti-Gr-1-PE, anti-CD11b-PerCP-CY5.5, anti-CD11c-APC, and either anti-MHC II-biotin/Alexa488 or anti-CD86-biotin/Alexa488. This experiment was repeated with similar results.

Figure 4. Gr-1^{+} cell depletion results in a less polarized Th2 immune response. BALB/c mice (5 mice per group) were inoculated I.P. with 12.5μg of anti-Gr-1 Ab 24 hours prior to intracutaneous inoculation of 300L3 *N. brasiliensis*. Draining cervical lymph nodes were removed at 18.0 hours and 7.0 days post inoculation for analysis (mice in day 7.0 group received an additional dose of anti-Gr-1 antibody two days post *N. brasiliensis* inoculation). Additionally, 7.0 days post inoculation serum samples were removed for immunoglobulin measurement. Gut tissue and feces were saved for analysis of worm and egg burden. **A.** 18.0 hour measurement of IFN-γ and IL-4 transcript by real-time quantitative fluorogenic PCR of cDNA reverse transcribed from total RNA. **B.** 7.0 days post inoculation CLN's were removed and analyzed via real-time quantitative fluorogenic PCR for IL-4 mRNA expression. Additionally, total serum IgG2a levels were determined by ELISA. Feces and gut tissue were examined for worm and egg expulsion. The mean and SE derived from five individual mice are shown for each treatment group. Graphs are representative of 3 individual experiments with similar results.

Figure 5. Recruitment of Gr-1^{bright} subset is dependent on CCL2 signaling. CCL2 deficient and WT control BL/6 mice (5/treatment group) were inoculated with 500L3 *N. brasiliensis* and 18.0 hours later, draining cervical lymph node cell suspensions were prepared and stained for anti-Gr-1-PE and anti-CD4-FITC analyzed by FACS. Percentages represent frequency of Gr-1^{+} cells. This experiment was performed three times with similar results.

Figure 6. Gr-1^{bright} subsets are necessary for IFN-γ suppression and polarization of the Th2 immune response against *N. brasiliensis*. BL/6 or CCL2 $^{-/-}$ mice (5 mice per group) were inoculated with 500L3 *N. brasiliensis*. Draining cervical lymph nodes were removed at 18.0 hours post inoculation for analysis. Additionally, 10.0 days post inoculation serum samples were removed for immunoglobulin measurement and feces was saved for analysis of egg burden. **A.** 18.0 hour measurement of IFN-γ and IL-4 transcript by real-time quantitative fluorogenic PCR of cDNA reverse transcribed from total RNA. **B.** 10.0 days post *N. brasiliensis* inoculation, total serum IgG2a levels were determined by ELISA. Feces were examined for egg expulsion. The mean and SE derived from five individual mice are shown for each treatment group. Graphs are representative of 3 individual experiments with similar results.

Figure 7. Isolated Gr-1^{bright} cells are neutrophils which express high levels of TGF-β and TNF-α. BALB/c mice (30/treatment group) were inoculated with *N. brasiliensis* and 18 hours later draining cervical lymph nodes (CLNs) were removed. Pooled cell suspensions were prepared and stained with anti-Gr-1 antibody, as described in the Methods. Gr-1^{bright} cells were isolated using high-speed flow cytometic cell sorting. CD11c^{+} cells were similarly sorted from untreated BALB/c mice and used as controls. **A.** Sorted cells were concentrated onto slides using cytospin centrifugation and then stained with H&E staining to analyze morphology. **B.** RNA was purified from Gr-1^{bright} and CD11c^{+} cell populations, reverse-transcribed to synthesize cDNA, and real-time quantitative fluorogenic RT-PCR was used to assess TGF-β, IL-4, IL-10, IL-12, IL-13, and TNF-α gene expression, as described in the Methods. ND equals not detected. Graph is represented as treated Gr-1^{+} cells/untreated CD11c^{+} cells. Graph is representative of three individual experiments with similar results**.**

Figure 1.

Chemokine/Chemokine Associated Genes	Common Gene Name	Gene Bank Accession Number	Nb Day 1.5 Fold Change	Nb Day 2.5 Fold Change
Small inducible cytokine B subfamily (Cys-X-Cys), member 9	MIG	M34815	**4.0**	**5.0**
Small inducible cytokine A2	Scya2 (MCP-1)	M19681	**4.0**	**3.0**
Small inducible cytokine B subfamily (Cys-X-Cys), member 10	Scyb10 (IP-10)	M33266	**5.0**	**3.0**
Small inducible cytokine B subfamily (Cys-X-Cys), member 11	iTAC	AF167354	**NA**	**NA**

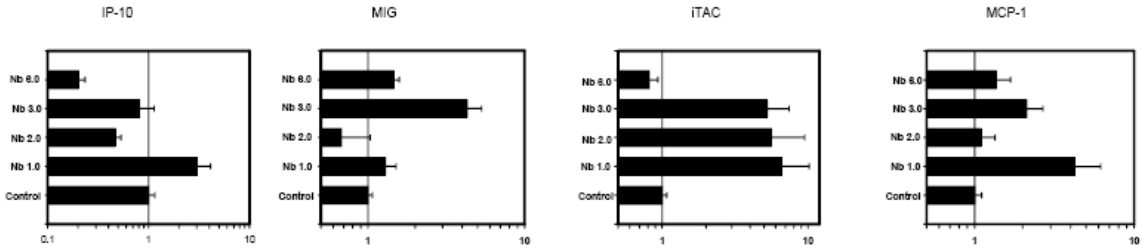


Figure 2.

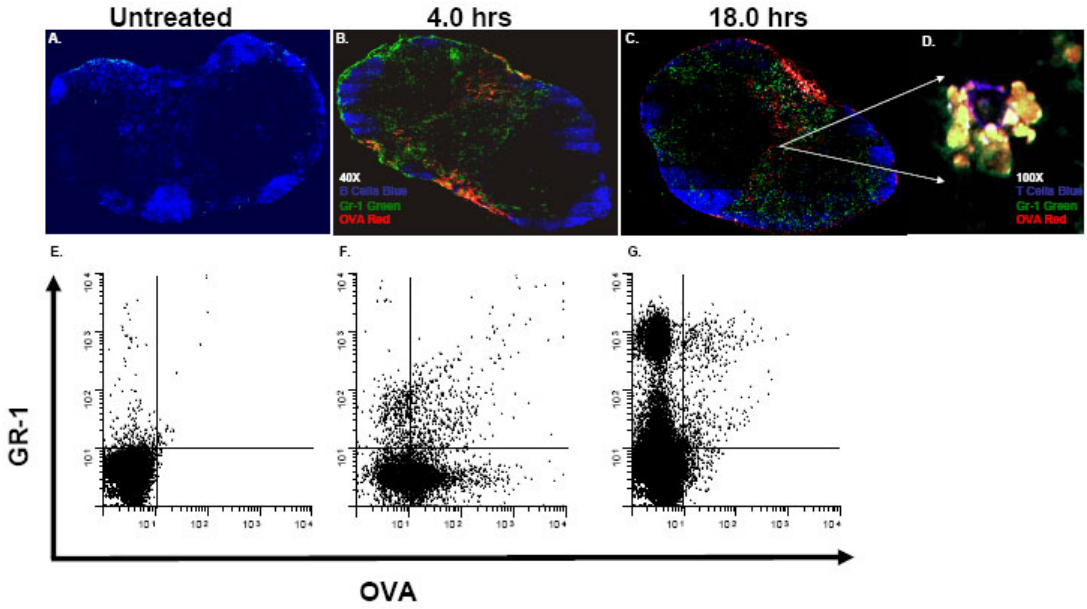

Untreated
4.0 hrs
18.0 hrs
A.
B.
C.
D.
40X
B Cells Blue
Gr-1 Green
OVA Red
100X
T Cells Blue
Gr-1 Green
OVA Red
E.
F.
G.
GR-1
OVA

Figure 3.

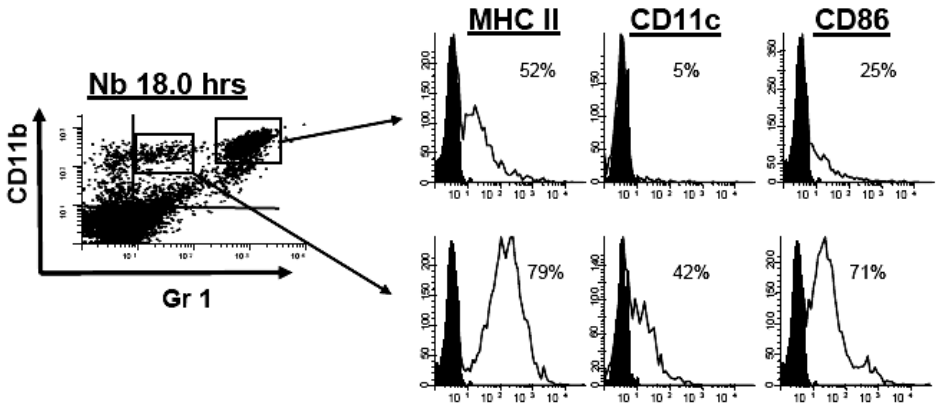


Figure 4A.

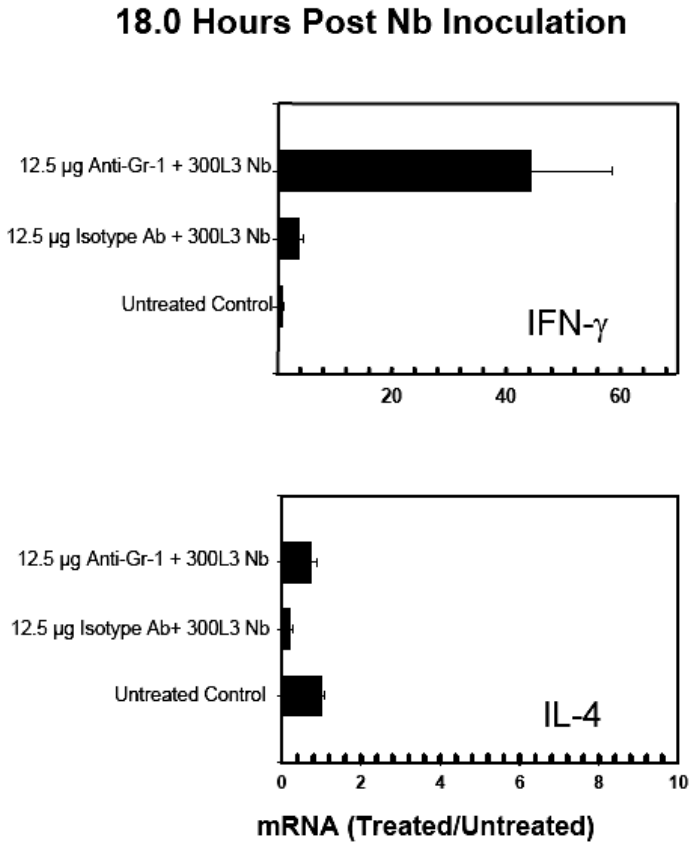

18.0 Hours Post Nb Inoculation
12.5 µg Anti-Gr-1 + 300L3 Nb
12.5 µg Isotype Ab + 300L3 Nb
Untreated Control
IFN-γ
20
40
60
12.5 µg Anti-Gr-1 + 300L3 Nb
12.5 µg Isotype Ab+ 300L3 Nb
Untreated Control
IL-4
0
2
4
6
8
10
mRNA (Treated/Untreated)

Figure 4B.

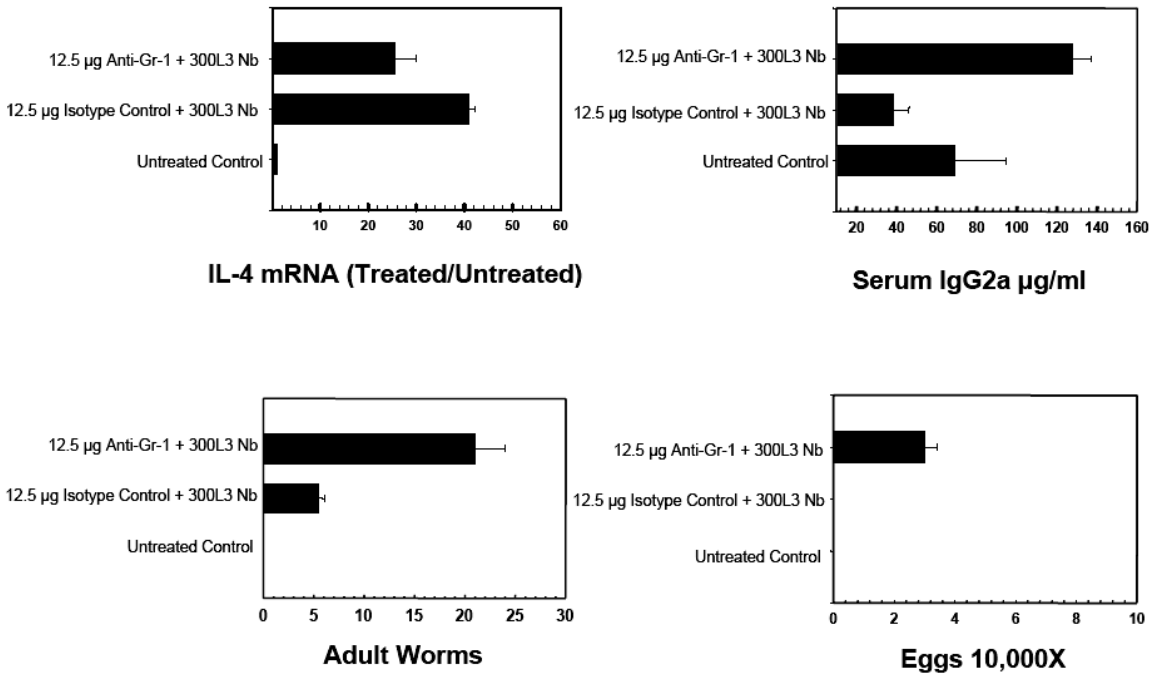

12.5 µg Anti-Gr-1 + 300L3 Nb
12.5 µg Isotype Control + 300L3 Nb
Untreated Control
10 20 30 40 50 60
IL-4 mRNA (Treated/Untreated)
12.5 µg Anti-Gr-1 + 300L3 Nb
12.5 µg Isotype Control + 300L3 Nb
Untreated Control
20 40 60 80 100 120 140 160
Serum IgG2a µg/ml
12.5 µg Anti-Gr-1 + 300L3 Nb
12.5 µg Isotype Control + 300L3 Nb
Untreated Control
0 5 10 15 20 25 30
Adult Worms
12.5 µg Anti-Gr-1 + 300L3 Nb
12.5 µg Isotype Control + 300L3 Nb
Untreated Control
0 2 4 6 8 10
Eggs 10,000X

Figure 5.

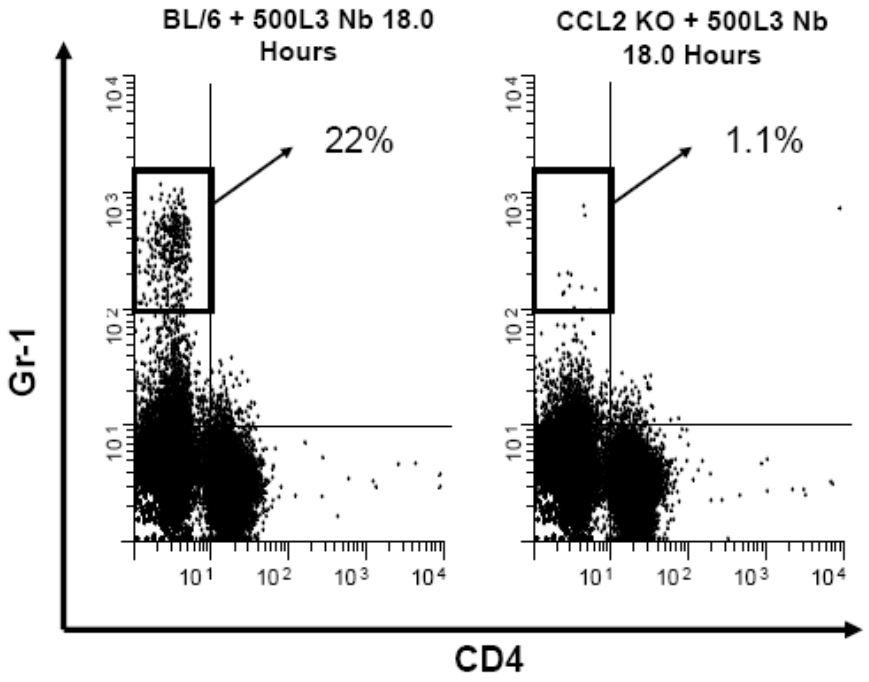

BL/6 + 500L3 Nb 18.0 Hours
CCL2 KO + 500L3 Nb 18.0 Hours
22%
1.1%
Gr-1
CD4
10^1
10^2
10^3
10^4

Figure 6.

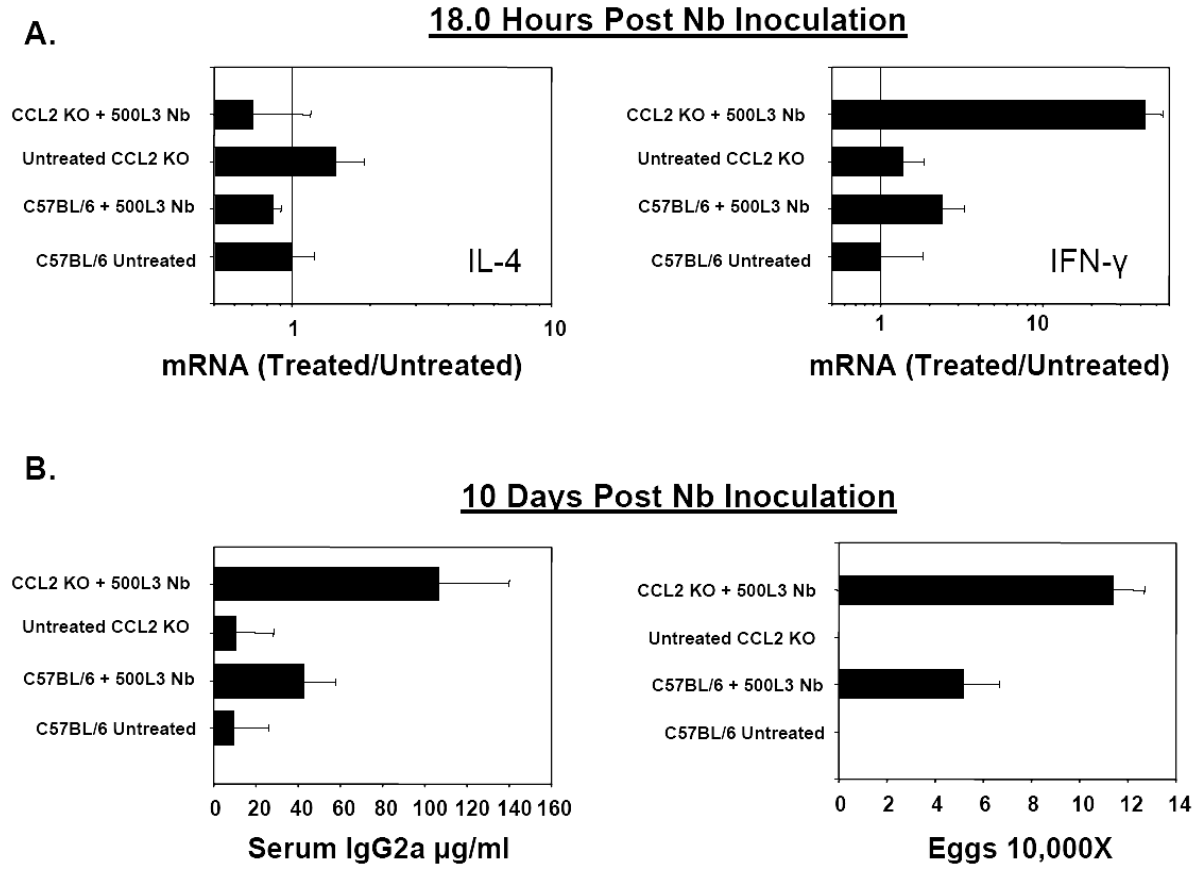
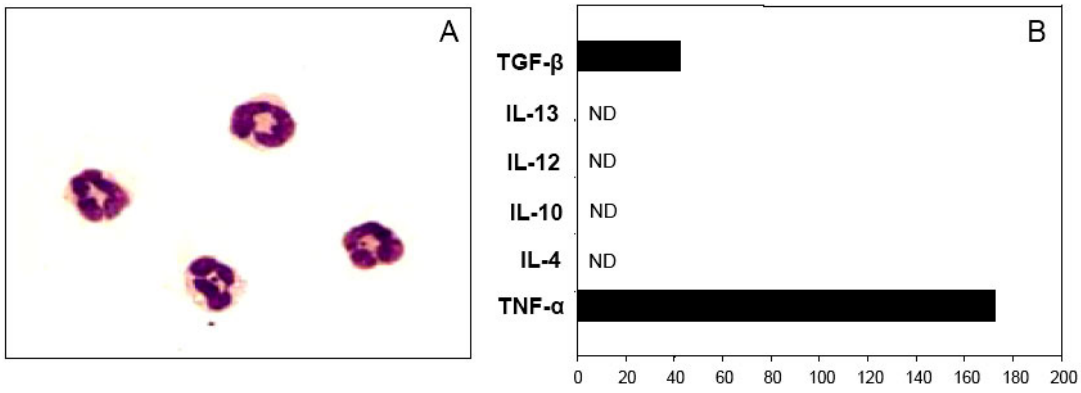

A.
18.0 Hours Post Nb Inoculation
CCL2 KO + 500L3 Nb
Untreated CCL2 KO
C57BL/6 + 500L3 Nb
C57BL/6 Untreated
IL-4
1
10
mRNA (Treated/Untreated)
CCL2 KO + 500L3 Nb
Untreated CCL2 KO
C57BL/6 + 500L3 Nb
C57BL/6 Untreated
IFN-γ
1
10
mRNA (Treated/Untreated)
B.
10 Days Post Nb Inoculation
CCL2 KO + 500L3 Nb
Untreated CCL2 KO
C57BL/6 + 500L3 Nb
C57BL/6 Untreated
0 20 40 60 80 100 120 140 160
Serum IgG2a µg/ml
CCL2 KO + 500L3 Nb
Untreated CCL2 KO
C57BL/6 + 500L3 Nb
C57BL/6 Untreated
0 2 4 6 8 10 12 14
Eggs 10,000X

Figure 7.



H&E staining of sorted Gr-1$^{+bright}$ cells

Sorted Gr-1$^{+ bright}$ Cell cytokine mRNA

Supplementary Figure

Upregulated Genes

Gene Description	Gene Symbol	Gene Bank Accession Number	Nb Day 1.0	Nb Day 2.0
Cytokine/Cytokine Associated Genes				
Transforming growth factor, beta 1	Tgfb1	AJ009862	-2.0	4.0
Interferon activated gene 203	Ifi203	AF022371	7.0	5.0
Interferon activated gene 202A	Ifi202a	M31418	-1.0	5.0
Interferon-inducible GTPase	Iigp-pending	AJ007971	-1.0	4.0
Interferon-stimulated protein (15 kDa)	Isg15	X56602	15.0	5.0
Interferon-inducible GTPase	Iigp-pending	AJ007971	2.0	3.0
Tripartite motif protein 30	Trim30	J03776	3.0	2.0
Cytokine inducible SH2-containing protein 1	Cish1(SOCS1)	U88325	3.0	2.0
Nuclear receptor subfamily 4, group A, member 1	Nr4a1	X16995	3.0	2.0
Cytokine inducible SH2-containing protein 3	Cish3 (SOCS3)	U88328	2.0	3.0
Chemokine/Chemokine Associated Genes				
Small inducible cytokine B subfamily (Cys-X-Cys), member 9	MIG	M34815	4.0	5.0
Small inducible cytokine A2	Scya2 (MCP-1)	M19681	4.0	3.0
Small inducible cytokine B subfamily (Cys-X-Cys), member 10	Scyb10 (IP-10)	M33266	5.0	3.0
Signal Transduction				
Transcription factor Dp 1	Tfdp1	X72310	4.0	4.0
Mitogen-activated protein kinase kinase kinase kinase 4	Map4k4	U88984	4.0	2.0
POU domain, class 2, transcription factor 1	Pou2f1	X68363	8.0	7.0
Cytoplasmic tyrosine kinase, Dscr28C related (Drosophila)	Tec	X55663	3.0	2.0
Mitogen activated protein kinase kinase 5	Map2k5	AB019374	3.0	3.0
Nuclear receptor subfamily 1, group H, member 3	Nr1h3	AF085745	6.0	3.0
MAD homolog 4 (Drosophila)	Madh4	U79748	3.0	1.0
Glycoprotein 49 B	Gp49b	U05265	3.0	1.0
Tnf receptor-associated factor 5	Traf5	D78141	3.0	2.0
GATA binding protein 3	Gata3	X55123	-2.0	3.0
Trans-acting transcription factor 3	Sp3	AF062567	4.0	3.0
Myristoylated alanine rich protein kinase C substrate	Macs	M60474	3.0	2.0
Macrophage/Granulocyte Associated				
Sphingomyelin phosphodiesterase 1, acid lysosomal	Smpd1	Z14132	4.0	4.0
Allograft inflammatory factor 1	Aif1	D86382	4.0	3.0
Guanylate nucleotide binding protein 2	Gbp2	AJ007970	3.0	3.0
Jun-B oncogene	Junb	U20735	5.0	4.0
Paraoxonase 2	Pon2	L48514	3.0	2.0
T/B Cell Development and Activation Associated				
Schlafen 2	Slfn2	AF099973	3.0	2.0
Schlafen 3	Slfn3	AF099974	4.0	2.0
Schlafen 4	Slfn4	AF099977	9.0	4.0
Killer cell lectin-like receptor, subfamily D, member 1	Klrd1	AF030311	3.0	3.0
CD8 antigen, alpha chain	Cd8a	U34881	3.0	2.0
Lymphocyte antigen 6 complex, locus A	Ly6a	X04653	4.0	4.0
Lymphocyte antigen 86	Ly86 (MD-1)	AB007599	3.0	2.0
Haptoglobin	Hp	M96827	3.0	3.0
Growth Factors				
Kallikrein, pseudogene 1	Klk-ps1	V00829	4.0	2.0
Kallikrein 6	Klk6	M13500	4.0	2.0
Kallikrein 9	Klk9	M17962	3.0	2.0
Kallikrein 22	Klk22	M17979	3.0	1.0

Down Regulated Genes

Gene Description	Gene Symbol	Gene Bank Accession Number	Nb Day 1.0	Nb Day 2.0
Cytokine/Cytokine Associated Genes				
Interferon gamma receptor	Ifngr	M28233	-1.1	-3.4
Interferon gamma receptor 2	Ifngr2	U69599	-2.1	-1.5
Transforming growth factor, beta receptor III	Tgfbr3	AF039601	-1.6	-6.3
Interleukin 6 signal transducer	Il6st	X62646	-3.0	-2.0
Transforming growth factor beta 1 induced transcript 4	Tgfb1i4	X62940	-3.0	-3.0
Chemokine/Chemokine Associated Genes				
Small inducible cytokine A21a (serine)	Scya21a (6ckine)	AF035684	-1.1	-2.6
Macrophage/Granulocyte Associated				
Macrophage receptor with collagenous structure	Marco	U18424	-3.0	-5.0
T/B Cell Development and Activation Associated				
Nuclear factor of activated T-cells, cytoplasmic 2	Nfatc2	U36576	-3.0	-3.0
Vascular cell adhesion molecule 1	Vcam1	M84487	-1.3	-3.3
Histocompatibility 2, T region locus 23	H2-T23	Y00629	-3.0	-3.0
Cytotoxic granule-associated RNA binding protein 1	Tia1	U00689	-3.0	-3.0
Pregnancy specific glycoprotein 17	Psg17	M83344	-7.0	-3.0
Calpain 2	Capn2	D38117	-1.0	-3.0
Signal Transduction				
Stress-induced phosphoprotein 1	Stip1	U27830	-5.0	-4.0
Transformation related protein 63	Trp63	AB010152	-3.0	-2.0
Low density lipoprotein receptor-related protein 1	Lrp1	X67469	-3.0	-3.0
RAS-like protein expressed in many tissues	Rit	U71205	-3.0	-3.0
Heat shock protein, 105 kDa	Hsp105	L40406	-4.0	-3.0
Nuclear receptor subfamily 3, group C, member 1	Nr3c1	X04435	-3.0	-4.0

Discussion

The contents of this dissertation have focused on events that are necessary for the development of an *in vivo* Th2 immune response to the intestinal nematode parasite *N. brasiliensis*. The initial studies of this dissertation showed that blockade of B7-1/B7-2 interactions during the primary immune response to *N. brasiliensis* resulted in abrogation of humoral immunity while having no effect on the production of IL-4 from T cells. Blockade of B7-1/B7-2 has been shown to have an impact on IL-4 production in several models (51-54;103). The ability of this parasite to induce a Th2 response in the absence of this signaling suggests that an alternative pathway is being activated. The first pathway to consider is based on the life cycle of individual parasites. Most parasites that are dependent on B7 signaling are strictly enteric in nature. *N. brasiliensis* on the other hand, migrates from the skin and the lungs before finally making it to the intestines. It is possible that during this migration it is activating several populations which may mediate the development of a Th2 immune response circumventing the requirement for B7 interaction.

Adjuvant-like properties of *N. brasiliensis*

Additionally, we were able to show that *N. brasiliensis* does have adjuvant-like properties that can skew nonparasite Ag-specific T cells to become Th2 cells. This finding suggests that an innate component of the immune system, such as toll-like receptors, could provide a potential mechanism for B7 independence. To address this question we were able to adapt a useful tool used in Th1 studies to examine antigen specific Th2 questions, by adoptively

transferring DO11.10 transgenic T cells specific for OVA peptide to recipient mice which were subsequently inoculated with Nb plus OVA.

Previous studies with this model system have used Th1-inducing adjuvants, including LPS and CFA, to trigger DO11.10 Th1 cell differentiation *in vivo* (104;105). Our findings show that *N. brasiliensis* can act as a potent adjuvant which can assist in the development of IL-4 producing T cells. Thus, naive T cells with the same specificity and affinity for Ag can rapidly develop *in vivo* into either Th1 or Th2 cells when sufficiently strong, polarizing microbial adjuvants are available, suggesting that under these circumstances TCR signal strength is not a major factor influencing Th cell cytokine production. There is also the possibility that endogenous TCRs, expressed by the DO11.10 transgenic T cells, may recognize Ags associated with Nb. However, there was little difference between stimulation of transferred DO11.10 T cells from DO11.10 RAG2-/- mice and DO11.10 RAG2-/- mice in any of the treatment groups. This shows that endogenous TCRs expressed on T cells from DO11.10 RAG2-/- mice had little effect and that the OVA-specific transferred cells naive T cells were the major activated population. The finding that, in the context of the Nb *in vivo* immune response, naive DO11.10 T cells could differentiate to IL-4-producing T cells, although B7 interactions were inhibited, suggests that the *in vivo* adjuvant properties of this parasite extend to the activation of B7-independent nonparasite Ag-specific Th2 cell differentiation from naive T cells.

While the induction of immune responses via adjuvants has been well characterized in Th1 model systems, few studies have implicated them in Th2 cell

development (44). Furthermore, studies have shown that Th1 responses can develop in the absence of B7 signaling (53;54;56). Antigens expressed by microbes cause the release of cytokines (106-108), the potency of the signaling associated with these molecules could be sufficient to activate a Th1 response which could bypass the necessity for B7 interactions. It is plausible that a similar instance could be occurring during *N. brasiliensis* infection. Antigens expressed by the parasite could bind to a known toll-like receptor, or a yet to be identified receptor, and cause sufficient signaling to induce Th2 polarization without B7-1/B7-2 being necessary (Figure 8). Studies performed by Holland in 2000 showed that excretory/secrectory products produced by *N. brasiliensis* could induce IL-4 production and polyclonal IgE synthesis (46). Additionally, potential Th2 adjuvants have been described by studying infection with oligosaccharides from *Schistosoma mansoni* egg Ags. When conjugated to human serum albumin, lacto-*N*-fucopentaose(III) has been shown to stimulate the production of an antigen specific IgE response (48;50).

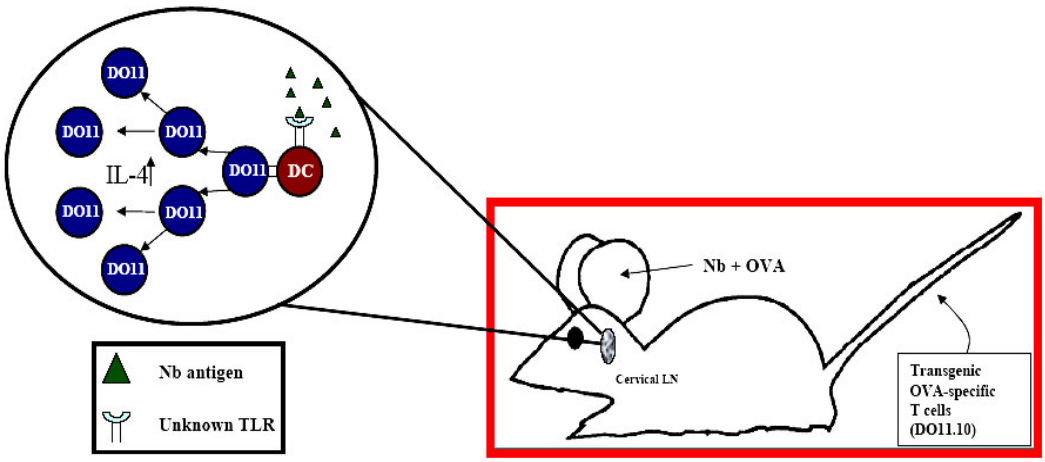


Figure 8. Proposed model for innate activation of T cells by *N. brasiliensis.* Adoptive transfer of DO11.10 OVA-specific T cells, and subsequent inoculation with OVA/Nb, results in significant T cell proliferation and increased IL-4 production. Antigens produced by *N. brasiliensis* are capable of activating naïve antigen presenting cells through a yet to be determined toll-like receptor.

Th2 immune response development against Nb infection is B7 independent

Consistent with previous studies using CTLA-4 Ig, deficiency in B7-1/B7-2 resulted in a marked abrogation of the humoral response, characterized by decreased IgE production, against *N. brasiliensis* infection (57). Our observation that the, IL-4 independent, up-regulation of B cell surface MHC class II expression was also inhibited suggests that B7-independent IL-4-producing T cells could develop that could mediate worm expulsion (52;109). Furthermore, even though antigen specific T cells were able to mature into IL-4 producing cells, migration of these cells into the B cell zone of the draining cervical ear lymph node were reduced. This decreased migration indicates that the development of IL-4-producing T cells is independent from their differentiation to T helper cells that can migrate to the B cell zone and provide B cell help. These data suggest that effecter T cell development occurs initially in the T cell zone, and after further maturation, developing T cells migrate to the B cell zone where they may further mature and provide B cell help. Additionally, we observed a decrease in the level of cell cycling of antigen specific T cells in the absence of B7 interactions. In WT mice a majority of the DO11.10 cells that had migrated to the B cell zone in Nb-inoculated mice had undergone multiple cell divisions, as determined by their decreased CFSE staining. It is possible that T cell migration to the B cell zone requires differentiation stages that are cell cycle dependent and that occur after Th cell differentiation to IL-4 production. This finding is consistent with previously published results suggesting that Th2 cell differentiation is controlled by cell cycling (110). It is also possible that B-T

interactions, which were inhibited in the absence of B7, promote T cell proliferation; several studies have suggested that B cells are important in Ag presentation during Th2 responses (111;112). Taken together, these results demonstrate that after Nb immunization naive T cells can differentiate in the absence of B7-1/B7-2 interactions to Th2 effecter cells that can mediate worm expulsion.

In addition to the findings about B7 involvement in *N. brasiliensis* infection, this work also produced two very useful tools for studying Th2 immune responses *in vivo*. Of particular importance in these studies is the development of the ear model as an effective method for analyzing the immune response to *N. brasiliensis in vivo*. Previous research involving *N. brasiliensis* has focused on mucosal and lung associated responses, but it has been found that a potent Th2 immune response develops within the draining lymph node near the site of inoculation. Upon entry of the parasite, a highly polarized Th2 response develops characterized by high levels of IL-4, IL-13, and low levels of IFN-γ. This has proven to be a useful tool for examining the immune response *in vivo* in a controlled environment that can be easily accessed and analyzed. The second tool developed involved the adaptation of the DO11.10 transgenic OVA system to a Th2 model. This system has been widely used to study Th1 immune responses, but the work described in this manuscript was the first to apply it in a Th2 context. The development of both these tools has proven to be an invaluable asset in subsequent studies. The ability to examine a developing immune response in an easily controlled microenvironment and to also be able to monitor its progress

in an antigen-specific manner was critical for subsequent experiments in the second manuscript.

Analysis of innate components in the development of a Th2 immune response

While the first manuscript provided much insight into the role of B7 signaling during the immune response to *N. brasiliensis*, it also suggested that an innate component of the immune response was involved in Th2 development. To extend this work, the second manuscript used microarray analysis to identify individual genes and cell populations that are critical for the proper polarization of a Th2 immune response *in vivo.*

In these studies, we demonstrated using microarray analysis, that CCL2 and CXCR3 ligands are among the few genes which exhibit increased expression in the draining lymph node shortly after inoculation with the Th2 inducing nematode parasite, *N. brasiliensis.* Our findings further show that CCL2 recruits a population of Gr-1 bright neutrophils that down regulate IFN-γ expression, possibly through a TGF-β dependent mechanism, in the lymph node and are required for the development of the highly polarized Th2 immune response leading to host protection.

Few characteristics of the innate immune response have been identified that drive the development of Th2 cells. Some studies have suggested that dendritic cells can support the development of a Th2 response *in vitro*, when coupled with certain parasite products; however, these cultures have not resulted in the strong and rapid development of Ag-specific Th2 cells observed under *in vivo* conditions (113;114). Other studies have suggested a role for basophils in

the production of IL-4 after infection with *N. brasiliensis*. Using GFP reporter mice two groups have shown that after I.P. or subcutaneous inoculation with Nb that basophils exhibit marked increases in the level of IL-4 production. While $CD4^+$ cells are capable of directing the migration and increasing the levels of IL-4 production by these cells, production of IL-4 by basophils is antigen independent. Our findings suggest that an important element lacking in these *in vitro* studies is an innate population of Gr-1^+ bright neutrophils that is rapidly recruited to the lymph node during the early stages of the *in vivo* Th2 primary response. The observation that there is an underlying IFN-γ response that can develop when Gr-1^+ neutrophils are depleted, during the *N. brasiliensis* response, indicates that these innate regulatory cells actively mediate the development of this polarized Th2 response required for host resistance.

Gr-1 heterogeneity

The cell surface marker Gr-1 has classically been used to identify granulocytes, including neutrophils (115). Recent findings have identified several other populations expressing this marker, with similar adaptive immune response regulatory properties, including plasmacytoid dendritic cells (83;85), TNF-α/INOS producing (Tip) dendritic cells (82), and myeloid cells (91;93;95;116). While these cells share common cell surface markers, such as Gr-1 and CD11b, and have some effect on the development of adaptive immune responses, they are phenotypically and mechanistically different from neutrophils.

Plasmacytoid dendritic cells are known to express B220, CD11c, and Gr-1 and are largely negative for CD11b (85). A subset of the cell population

identified during *N. brasiliensis* inoculation has a phenotype similar to that of plasmacytoid dendritic cells, with the exception of high levels of CD11b expression. However, our *in vitro* studies required the presence of conventional CD11c dendritic cells to augment Th2 cell differentiation, suggesting that they do not potently stimulate Th2 cell polarization from naïve T cells independently.

The phenotype of Tip dendritic cells, identified in the spleen of *Listeria monocytogenes*-infected mice, is similar to that of the Gr-1^+ cells recruited to the draining lymph node during the immune response to *N. brasiliensis*. Both populations express CD11b and Gr-1, low but detectable levels of CD11c, and are F4/80^-, although only Tip dendritic cells express MAC-3 (82). Furthermore, both Gr-1^+ cell populations are dependent on CCL2 signaling for their recruitment to draining peripheral lymphoid tissue (86). However, Tip dendritic cells have been shown to present Ag and express high levels of TNF-α and iNOS. Sorted Gr-1^+ cells were examined for cytokine gene expression and while detecting significant levels of TNF-α elevations they were negative for iNOS.

Myeloid suppressor cells express high levels of both Gr-1 and CD11b and can down regulate Th1 responses associated with tumor rejection (93;116;117). However, unlike the Gr-1^+ cells identified in the Th2 response to *N. brasiliensis*, myeloid suppressor cells express high levels of surface F4/80, a myeloid cell marker. Another Gr-1^+, F4/80^+ population, identified in the spleen of alum-inoculated mice has recently been described as producing IL-4, which can prime B cells for expansion during an *in vivo* immune response (95). It should be note that Gr-1^+, F4/80^+ cells were not detected in the draining cervical lymph node

following *N. brasiliensis*-inoculation in any of the studies presented in the second manuscript. Recent studies have also implicated Gr-1^+ cells in the development of Th2 immune responses in nonlymphoid peripheral tissues. In response to schistosome oligosaccharide inoculation, a Gr-1^+ cell population expressing CD11b and F4/80, and expressing both IL-10 and TGF-β has been found to rapidly expand within the peritoneum and there augment the peripheral Th2 response (50).

After *N. brasiliensis* inoculation, sorted Gr-1^+ neutrophils express high levels of TGF-β and TNF-α while being negative for IL-4, IL-10, IL-12, IL-13, and IFN-γ production. Furthermore, our studies are quite different from previously published literature in that they demonstrate that a neutrophil population recruited to the lymph node shortly after *N. brasiliensis* inoculation influences the development of Th2 cells in the lymph node at the initiation of the primary immune response.

A population similar to that described in this text is a Gr-1 bright neutrophil population known to be important during the early phases of the immune response to *Toxoplasma gondii*. Absence of this population resulted in early susceptibility to infection while having no effect on later stages of the response *(88)*. This scenario is similar to observations made in *N. brasiliensis* infected, neutrophil depleted mice, where inoculation results in significant increases in Th1 associated factors and increased susceptibility to infection, . Additionally, *T. gondii* associated neutrophils are known to migrate to the site of infection via CCL2(118), while *N. brasiliensis* associated neutrophils use CCL2

signaling to migrate to secondary lymphoid tissue. Furthermore, *T. gondii* associated neutrophils help maintain a Th1 response by releasing reactive oxygen intermediates and proinflammatory cytokines at the site of infection (89). In contrast, *N. brasiliensis* associated neutrophils are negative for the pro-nitric oxide precursor iNOS and produce high amounts of the anti-inflammatory cytokine TGF-β. It is possible however, that the *N. brasiliensis* associated neutrophil population recruited to the lymph node during the Th2 response is of the same lineage as the populations discussed above, and that their functional differences result from differentiation along distinct pathways depending on the particular pathogen infecting the host.

The observation of different differentiation states of Gr-1 cells is not unprecedented. Heterogeneity in cell differentiation has been well documented in macrophages and dendritic cells. Dendritic cells are all bone marrow-derived cells which constitutively express MHCII (119;120). These cells have proven to be one of the most heterogeneous populations within the body containing six distinct subsets. The three major subsets, "myeloid" $CD11b^+$ DCs (121), "lymphoid" $CD8\alpha^+$ and $CD205^+$ DCs (121), and "plasmacytoid" $CD11c^+$, $B220^+$, and Gr-1^+ DCs (83;85), are found within secondary lymphoid tissue. The labels myeloid and lymphoid have fallen under scrutiny due to the fact that while they express lineage specific markers they may not actually belong to those lineages (122). Myeloid DCs have been shown to rapidly migrate to the lymph node and present soluble OVA to antigen-specific CD4 T cells within 18 hours of inoculation (123). Lymphoid DCs on the other hand, are poor presenters of

soluble antigen and are primarily involved in the presentation of particulate antigens through MHC II complexes (124;125). Plasmacytoid DCs exhibit increased MHCII expression after TLR ligand binding resulting in the production of high levels of IFN-α (126).

Classically and alternatively activated macrophages are derived from the same progenitor cell and express similar cell surface markers. Depending on the cytokine milieu that they are exposed to, unactivated macrophages will differentiate into one of two pathways. Exposure to IFN-γ, TNF-α, or IL-1 will result in classical activation, while exposure to IL-4, IL-10, IL-13, or GM-CSF will result in alternative activation. Classical activation leads to the production of iNOS which catabolized L-arginine to synthesize NO. Alternatively activated macrophages on the other hand, express high levels of arginase which competes with iNOS for the substrate L-arginine to produce L-ornathine and cause collagen formation (127).

CCL2 dependent recruitment of Gr-1 neutrophils

Our results from the microarray analyses indicated that chemokines associated with leukocyte recruitment, including CCL2, were rapidly elevated shortly after *N. brasiliensis* inoculation. Blocking CCL2 interactions inhibited the migration of Gr-1 bright neutrophils into the draining lymph node of *N. brasiliensis*-inoculated mice and further resulted in the in situ rapid up regulation of IFN-γ. These findings implicate the CCL2-dependent Gr-1 bright population as being the principal Gr-1^{+} subset that down-regulates IFN-γ during the initiation of the Th2 response. Recent studies have shown that the host protective Th2 response to the

intestinal nematode parasite, *Trichuris muris*, is substantially inhibited in CCL2$^{-/-}$ mice (128). Our results showed that resistance in *N. brasiliensis*-inoculated mice is similarly inhibited. Taken together, our findings indicate a mechanism for how CCL2 may influence Th2 cell differentiation *in vivo*.

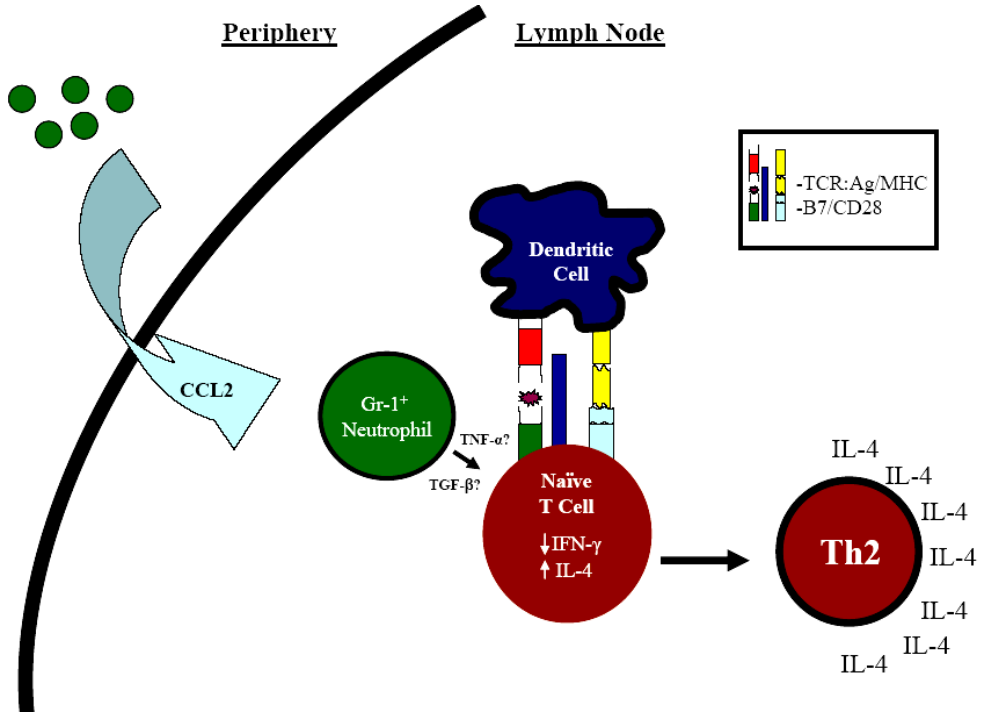


Figure 9. Proposed model for Gr-1^{+} neutrophil involvement in Th2 polarization. After inoculation with Nb, Gr-1+ neutrophils are recruited to the T cell zone of the lymph node in a CCL2 dependent mechanism. Through the expression of cytokines or direct cell:cell contact, Gr-1 cells cause the down regulation of IFN-γ production in newly activated T cells allowing Th2 polarization to occur.

In this model, *N. brasiliensis* infection induces the upregulation of CCL2 expression in the draining lymph node, triggering the rapid recruitment of Gr-1 bright neutrophils to the draining lymph node within several hours after inoculation. The Gr-1 bright cell population, perhaps through expression of TGF-β, TNF-α, or through direct cell:cell contact, down-regulates early expression of

IFN-γ thereby facilitating the development of the alternative Th2 response. These results thus provide evidence that an important component of the highly polarized Th2 response that develops following nematode parasite infection includes rapid recruitment of a neutrophil population to the site of Th2 cell differentiation in the draining lymph node (Fig. 9).

Future Studies

In future studies it will be necessary to investigate the role of CXCR3 ligands in the recruitment of Gr-1$^+$ subsets. Based on current data, it is unlikely that CXCR3 ligands play a role in Gr-1 bright cell recruitment, but it is possible that they are involved in the recruitment of Gr-1 dull cells. Furthermore, it is important to identify the source of CCL2 production from within the lymph node. To look at this, laser capture microdissection (LCM) will be employed to isolate known regions of infected lymph nodes such as B and T cell zones. These areas would then have mRNA isolated and analyzed for CCL2 gene expression. Once a region of interest is identified, individual cell populations will be stained immunofluorescently and dissected out. mRNA from these homogeneous populations will be analyzed for CCL2 expression. An alternative method for accomplishing these studies could involve intracellular staining of Nb treated lymph node cells and then adding known cell lineage markers such as B220 or CD4 to identify double positive populations. Based on immunofluorescent image data of Nb treated whole lymph node sections, I hypothesize that the source of CCL2 will be a non-B cell antigen presenting cell population. This hypothesis is supported by the migration data of Gr-1 neutrophils into the lymph node which

has corresponded with localization within the T cell rich region of the lymph node, and notable absence from B cell follicles. In the event that a dendritic cell population is responsible for the production of CCL2, it would be interesting to examine the individual cell population for the production of the notch ligands Delta and Jagged-1. These two ligands have been shown to signal the differentiation of naïve T cells into Th1 and Th2 effecter respectively. While the mechanism for how Delta induces Th1 differentiation, it is known that Jagged-1 acts directly on GATA3 to induce IL-4 expression and antagonize IFN-γ production (129). Furthermore, while it is known that DCs express IL-12 which leads to the development of Th1 cells, an analogous cytokine produced by DCs has not been identified for Th2 development. Although some data has suggested that IL-4 production by DCs maybe responsible for Th2 polarization (130).

An area of particular interest is the biology of Gr-1 neutrophils themselves. As we have observed, these cells are specifically recruited to the lymph nodes after *N. brasiliensis* inoculation and this leads to questions about the origins of these cells within the host. Are these cells from the site of inoculation, or are they nonspecifically recruited from the periphery of the host? To answer this, untreated Gr-1 cells will be isolated from Nb treated mice and intracellularly stained with a fluorescent marker such as CFSE or SNARF. The labeled cells would then be adoptively transferred into WT mice which are then inoculated with Nb. At varying times post inoculation, the site of inoculation will be removed by ear punch. The removed sections will then be sectioned and analyzed via immunofluorescent microscopy to determine the presence/ absence of Gr-1

cells. Based on imaging and FACS data I hypothesize that the Gr-1 cells we have observed are from the site of inoculation due to the demonstration of fluorescent OVA antigen on their surface. These experiments can then potentially be extended to examine the role of Gr-1 bright neutrophils in the development of an immune response. Using a similar isolation and labeling protocol as described above, WT Gr-1 bright cells will be isolated and transferred into CCR2 deficient mice, which presumably will be unable to recruit Gr-1 cells to the lymph node after Nb inoculation. Reconstitution of the a WT CCR2 signaling pathway into CCR2 deficient mice should provide definitive data as to the role of Gr-1 bright neutrophils in the regulation of IFN-γ production and in the development of protective immunity.

Conclusions

The contents of this dissertation have focused on events that are necessary for the development of an *in vivo* Th2 immune response to the intestinal nematode parasite *N. brasiliensis*. This body of work has made several significant contributions to the overall understanding of the Th2 immune response associated with *N. brasiliensis* infection. Of particular importance, we were able to develop an ear inoculation model system that has proven to be a successful and useful tool for examining Th2 immune responses *in vivo* before and after the production of IL-4. Furthermore, we have successfully adapted the DO11.10 system to function in a Th2 environment which has allowed us to examine antigen-specific questions and further elucidate the kinetics behind the development of a Th2 immune response. Finally, we were able to show that a Gr-1^+ neutrophil subset is critical

for the successful polarization of a Th2 immune response and development of protective immunity, and that recruitment of this population to the lymph node is dependent on CCL2/CCR2 signaling.

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Appendix A

Abbreviations Used

Ag- Antigen

APC- Antigen Presenting Cell

CTL- Cytotoxic T Lymphocyte

DCs- Dendritic Cells

IFN - Interferon

MHC- Major Histocompatibility Complex

Nb- Nippostrongylus brasiliensis

NK Cells- Natural Killer Cells

OVA- Ovalbumin

PAMPs- Pathogen Associated Molecular Patterns

PRR- Pattern Recognition Receptor

TCR- T Cell Receptor

TGF- Transforming Growth Factor

Th1- T helper 1

Th2- T helper 2

TLR- Toll Like Receptor

TNF- Tumor Necrosis Factor

Appendix B

Statement of Authors Contribution:

The author was personally involved in the development of immunofluorescence techniques, and made significant contributions to the analysis and writing of sections of the manuscript entitled “Nippostrongylus brasiliensis can induce B7-independent antigen-specific development of IL-4 producing T cells from naïve CD4 T cells in vivo”. The author was personally involved in the planning and execution of all experiments described in the second manuscript. Additionally the author was directly responsible for writing the second manuscript presented here, as partial fulfillment of the dissertation requirements.