Distinct CD4:CD8 T Cell Ratio in

Adult and Neonatal Mice

Correlates with Either Th1 or Th2 CD4 Immunity,

Respectively, Specific for Transplantation Antigens

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ABSTRACT

Previous studies employing the generation of MHC-incompatible embryonic chicken chimaeras by injecting MHC-incompatible stem cells resulted in an unexpected finding. Chimaeras made late in gestation developed as adults a severe autoimmune syndrome resembling the human syndrome of Systemic Lupus Erythematosus.

Work in our laboratory aims to understand the role of CD8 T cells in immunity and/or autoimmunity. We have tested a three-cell model of CD4 T cell activation and differentiation during the development of the immune response specific for MHC transplantation antigens in one way mixed lymphocyte reactions. Our model proposes that whether Th1 or Th2 immunity is generated depends on both the ratio of CD4:CD8 T cells specific for antigen at the initiation of the immune response and on the ability of antigens to coordinately induce both CD4 and CD8 T cells.

Previous studies employing parent into F1 models of graft-versus-host disease in mice have shown that the injection of parental cells results in two distinct outcomes. Parental cells which do not have a sufficient number of CD8 T cells present produce an autoimmune syndrome characteristic of systemic lupus erythematosus and a chronic graft-versus-host disease mediated by a Th2 response. Conversely, the presence of an adequate number of CD8 T cells results in a Th1 immune response and acute graft-versus-host disease resulting in the death of the F1 host.

Our findings indicate that the ratio of the number of CD4 T cells to the number of CD8 T cells present in the spleen is crucial in whether naive CD4 T cells differentiate into Th1 or Th2 cells. We refer to this ratio as the CD4:CD8 T cell ratio or CD4:CD8 ratio. Thus, the differentiation of naive CD4+ T cells towards a differentiated Th1 phenotype is critically dependent on the concomitant induction of CD8 T cells by the same antigen, driven by a low CD4:CD8 ratio. In contrast, inefficient induction of CD8 T cells during the initial priming of

lymphocytes greatly facilitates the differentiation of CD4 T cells towards the Th2-type lineage, and occurs when the CD4:CD8 ratio is high.

Given our findings on the significance of the ratio of CD4:CD8 T cells in the decision making process of CD4 differentiation stimulated by antigen, we hypothesized that different CD4: CD8 ratios at different stages of development might contribute to the immune response generated at these stages.

We tested this hypothesis in mice by comparing the CD4:CD8 ratio in adults and neonates and the Th1/Th2 responses generated *in vitro*. This CD4:CD8 T cell ratio is significantly higher in neonates than adults resulting in predominant Th1 responses by adult spleen cells and Th1/Th2 responses by neonatal spleen cells as demonstrated by the ELISPOT assay.

We have compared the CD4:CD8 T cell ratio of a large number of adult and neonatal spleens in several mouse strains and have studied it systematically in BALB/c and C57BL/6 mice by flow-cytometry. We have consistently found a 3-5 fold higher CD4:CD8 T cell ratio in neonates as compared to adults in the strains tested. Furthermore, we found that neonatal spleen cells generate a predominant Th2 response whereas adult spleen cells generate CD4 and CD8 Th1 immunity when activated under the same conditions.

We have further studied the role of CD8 T cells in CD4 T cell differentiation by reconstructing the adult CD4:CD8 T cell ratio in neonatal spleen cells with age-matched, isolated CD8 T cells. We found that in these "CD4:CD8 ratio-reconstructed cultures", the Th2/IL-4 immunity is suppressed with concomitant generation of Th1/IFN-γ immunity upon activation by allo-antigen. Additionally, we have characterized the phenotype of the T cell mediating

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Th1/IFNγ immunity in the "CD4:CD8 ratio reconstructed cultures" and we found that while CD8 T cells produce exclusively IFN-γ, CD4 T cells now produce IFN-γ rather than IL-4.

We suggest that physiologically distinct CD4:CD8 ratios at different stages of life should be considered in designing protocols of neonatal vaccination against pathogens that are contained by Th1-type immunity upon infection as adults. Moreover, as elaborated in the discussion, our studies might be pertinent in understanding by which mechanism autoimmunity arises in some cases.

DEDICATION

I would like to dedicate my thesis to my family and especially to my grandmother Dora Warner, who passed away in 2013 from cancer while I was in the first year of graduate studies. She helped raise me and was the nicest and most generous person I have ever known. She has been one of the most important and influential people in my life and she is the reason I am who I am today.

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ABBREVIATIONS

APC ....Antigen-Presenting Cell

CTL ....Cytotoxic T Lympocyte

DC ....Dendritic Cell

ELISPOT... Enzyme-linked Immunospot Assay

FBS ....Fetal Bovine Serum

FITC ....Fluorescein Isothiocyanate

GVHD..Graft Versus Host Disease

IFN-γ .... Interferon-gamma

IL ....Interleukin

MACS .... Magnetic Activated Cell Sorting

MHC .... Major Histocompatibility Complex

MLR ....Mixed Lymphocyte Reaction

PBS ....Phosphate-Buffered Saline

PBS-T ....Phosphate-Buffered Saline with Tween 20

PE ....Phycoerythrin

RPMI .... Roswell Park Memorial Institute

Th ....T helper i.e. T helper 1/T helper 2 (Th1/Th2)

CHAPTER 1

Introduction

1. INTRODUCTION

The cells and molecules responsible for protection of vertebrates against disease, most notably infectious disease, are collectively referred to as the immune system. From a historical perspective, the first mention of immunity was made by the Greeks, specifically Thucydides, who noted that those who had survived infection with a specific "plague" could tend to the sick without becoming sick themselves. The ancient Chinese also may have recognized a similar phenomenon with their custom of making children inhale powder from smallpox lesions to make them resistant to infection (Abbas at al. 2011). Modern immunology started to take shape in the 1700's when Jenner was the first person to successfully manipulate the immune system to protect against smallpox by injecting the material from pustules of cowpox-infected milkmaids into a young boy. This boy did not contract the disease when intentionally inoculated with the smallpox virus (Abbas et al. 2011). Hundreds of years of research have culminated in what is today's immunology; an experimental field that studies the many aspects of immunological events.

The immune system has two distinct means of protecting an individual from foreign invaders with the ability to cause disease; the innate immune system and the adaptive immune system. The innate immune system is an early line of defense for the host against invading pathogens. Innate immunity is relatively non-specific and the cells and molecules of the innate system are resident in tissues before infection takes place and mount a rapid response. The adaptive immune system carries the hallmark features of memory and specificity. The adaptive immune system's memory refers to its ability to mount a stronger, more rapid response upon a second encounter with the same pathogen. Its specificity refers to the ability to respond in a discriminating manner to a very large number of different microbes and molecules, while also not reacting against the host's own tissues. This memory and specificity are what distinguishes the innate and adaptive immune systems in the classical sense. There is recent evidence that the innate arm of the immune system also maintains some immune "memory" after encounter with pathogens (Netea et al. 2011). This immunological memory has been coined "trained immunity", and refers to the innate immune system's ability to provide protection against

pathogens that have previously been encountered independently of T and B cell responses (Netea et al. 2011). While this new evidence shows that the enhanced immune response upon secondary exposure to a pathogen cannot be completely explained by T and B cell responses and while both innate and adaptive immunity play an important role in host defense, it is generally the adaptive immune response that is responsible for the clearance of pathogens and producing the effector functions of the immune system. There are two different types of responses that make up adaptive immunity; humoral responses and cell-mediated responses. The type of response made can be critical for clearing certain infections and determining host survival.

1.1 PHYSIOLOGICAL FUNCTIONS OF CD8 T CELLS

There is a vast literature on studies involving CD8 T cells leading to a characterization of the T cell receptor (TCR) as well as the MHC restricted nature of their recognition of antigen. I will not discuss the long history of the discovery of MHC restriction and TCR interaction with the MHC in the detail it deserves, but will briefly touch on what is important in the context of this thesis. Pertinent to this thesis, the discovery of T cells requiring compatibility at H-2 to perform effector functions led to the important revelation of MHC restriction (Zinkernagel and Doherty 1974; Zinkernagel and Doherty 1975). It was also shown that peptides could be produced from antigen inside cells and that some of these are expressed on the cell surface, bound to grooves of the MHC. The receptors of T cells recognize the peptide/MHC complex (Masopust et al. 2007). Additionally, in the 1980's, the structure of the TCR was being examined by several groups. It was discovered that the TCR complex consisted of a heterodimer, with both constant and variable regions (Masopust et al. 2007). The question remained of how T cells, which possessed this TCR, while also being dependent upon interaction with MHC on target cells, recognized antigen. Studies on the crystal structure of the class I MHC molecule helped pave the way for the proposition that the TCR engages the peptide binding groove of the MHC molecule with bound antigenic peptides (Bjorkman et al. 1987). For the purposes of this thesis, the discovery that CD8 T cells were MHC class I-restricted, and that CD8 is a co-receptor for class I provided a clear picture of how T cells recognize both the antigenic peptide and the grooves of the class I MHC molecule.

Both CD4 and CD8 T cells play a role in the development of the immune response. What is now known about the different subsets of T lymphocytes, namely CD4 and CD8 T cells, has not always been so clear. T lymphocytes were shown by Medawar and others to play a role in rejection of transplants, but it was not known until years later that this population of cells, capable of transferring immunity to naïve recipients was a heterogeneous group of cells. The experiments of Miller showed, through studies of the thymus, that there are two different types of lymphocytes, thymus-derived helper cells and bone marrow-derived antibody-producing cells (Masopust et al. 2007). As seen, in *in vitro* studies in neonatal mixed lymphocyte cultures, lymphocytes capable of destroying allogeneic cells can be generated in said cultures. This cytotoxicity was shown to be due to T cells through experiments by using anti-thy-1 antibody and complement to abolish the cytotoxic activity of spleen cells against allogeneic cells (Cerottini et al. 1970). The separation of functional subclasses of T cells was established by Cantor in the mid 1970's. Cantor showed that in response to allogeneic cells, T cell populations which had been treated with anti-Ly1 antibody and complement lost all helper function, while populations treated with either anti-Ly2 or anti-Ly3 antibody and complement retained helper function, but lost the ability to lyse target cells (Cantor and Boyse 1975a). The modern terminology of CD4 and CD8 cellular markers would come along years later, but this work showed that there are distinct populations of T cells with different functional capacities. Cantor also showed that the generation of Ly23 with cytotoxic ability was facilitated by the presence of Ly1 cells, giving an early indication that CD4 T cells could help CD8 T cells produce a cytotoxic response as well as help B cells produce antibody (Cantor and Boyse 1975b).

1.1.1 CD8 T Cells as Cytotoxic T Lymphocytes

Following the invaluable studies in which CD8 T cells were shown to mediate cellular cytotoxicity, and recognize antigen in the context of MHC class I, it was realized that these cells play an important role in cell-mediated immunity, especially in response to intracellular pathogens such as bacteria or viruses. The classification of CD8 T cells as cytotoxic T lymphocytes (CTLs) highlights their important role in eliminating pathogens through the lysis of infected cells. The role of CD8 T cells as cytotoxic effector cells is not the main area of investigation pertaining to this work, however as this role is well-established I will discuss the important aspects of how CTL are generated and their role in the immune response. Naïve CD8

T cells are activated through the interaction of their TCR with class I MHC and with co-stimulatory molecules expressed on cells presenting antigenic peptides and are important for the clearance of many viral and bacterial infections. The activation phase for CTLs involves this interaction of the T cell with antigen-presenting cells causing the T cell to undergo expansion, acquire effector function, and traffic to sites of infection to mediate pathogen clearance by elimination of infected cells (Williams and Bevan 2007). After clearance of infection, the population of effector CTLs drops dramatically, leaving a small number of memory CTLs behind for protection against subsequent infection.

There are two distinct mechanisms by which effector CTLs mediate killing of infected cells: exocytosis of granules containing molecules capable of mediating cell killing and through a Fas-FasL cell-cell surface interaction (Russell and Ley 2002). In the former mechanism, upon the activation of CD8 T cells into effector cells, transcription of certain genes encoding cytolytic molecules and the formation of granules containing these molecules ensues. Thus the cells become "armed" with the ability, upon the next engagement of their TCR with class I MHC, to release the contents of their cytotoxic granules. The main molecules contained within cytolytic granules that can lead to the death of infected cells are perforin and granzyme proteins (Russell and Ley 2002; Podack et al. 1991). Perforin, when released from the CTL through granule fusion with the cell membrane, polymerizes in the presence of calcium ions, and creates pores in the membrane of the target cell. The pores created by perforin in the target cell membrane allow for the entrance of granzyme molecules, with granzyme B playing a major role (Russell and Ley 2002). Granzyme B is a serine protease that can induce cell death upon cellular entry via trafficking to the nucleus and cleaving substrates that are important for DNA repair (Russell and Ley 2002). During the Fas-FasL pathway of cell death, activation of T cells induces the expression of Fas ligand which interacts with Fas expressed on the surface of target cells. Fas is a death receptor that leads to programmed cell death upon interaction with its ligand FasL (Russell and Ley 2002). The granzyme/perforin pathway seems to be a more important effector function in class I-dependent cellular killing, but these mechanisms are responsible for the cytotoxic function of CD8 T cells.

1.1.2 CD8 T Cells as Suppressors of the Antibody Response

Aside from the role of CD8 T cells as cytotoxic effector cells, their function as suppressor cells came to prominence during the years when Cantor was performing his experiments on Ly subsets of T cells. In experiments involving the induction of tolerance in mice against sheep red blood cells (SRBC), Kondo was one of the first to show that T cells can suppress the antibody response (Gershon and Kondo 1970; Gershon and Kondo 1971). In this work, mice that had been thymectomized, lethally irradiated, and bone marrow-reconstituted were exposed to high doses of SRBC. Mice were unable to respond to further challenges with SRBC, but were able to make antibody after the injection of thymocytes days after the termination of treatment. However, mice that had received thymocytes at the time of challenge were tolerant of SRBC and their response could not be restored with the injection of thymocytes. This population of thymus-derived cells also suppressed the response made against horse red blood cells given days later. Furthermore, the receipt of T cells from tolerant animals could abrogate the antibody response made by normal mice (Jiang and Chess 2000). This work first suggested that a population of T cells is responsible for the suppression of the antibody response.

Following this work, many people showed that tolerance to a certain antigen can be mediated via antigen-specific T cells. Basten expanded upon previous experiments showing that the antibody response, in most cases, required B and T cells. He also showed that T cells could have a suppressive effect on B cells (Basten et al. 1974). He demonstrated that the suppressive activity of T cells was transferrable to irradiated hosts challenged with antigen, and that this could be abolished with anti-Thy1 antibody treatment. In addition to this and many other experiments showing that T cells could inhibit antibody responses, was the demonstration that supernatants from T cells activated with the mitogen concavalin A (Con A) are able to inhibit the antibody response through some secreted factor (Rich and Pierce 1974; Dutton 1975). In these examples, the suppression mediated by T cells is directed against the antibody response. However, there was also evidence that certain populations of T cells had a suppressive effect on other T cell populations. Given what we now know regarding differential phenoytpes of T cells and their immune responses, this is not surprising. Jadinski *et al* were able to show that cell surface markers could distinguish cells which helped the antibody response from those which were suppressive, similar to those done by Cantor and Boyse mentioned above (Okumura et al. 1976). This work showed that T cells which expressed Ly1 were primarily helper T cells and did

not generate "killer responses", while Ly2 and Ly3 T cells became suppressor or killer cells upon stimulation with Con A (Okumura et al. 1976).

In the subsequent years, work that was done attempted to characterize the means by which suppressor T cells were able to inhibit immune responses. In two papers published in 1976 by Murphy and colleagues, they showed that T helper and T suppressor populations could be distinguished not only by Ly surface markers but by specific I-region expression (Okumura et al. 1976; Murphy et al. 1976). They showed that the subpopulations of T cells expressed different I-region markers and that these markers were distinct from the B cell I-region marker Ia. At the time of this work, they named this new immune response region, which they believe to be encoded in the I-region of the H-2 complex, the I-J region. The soluble suppressive factors expressed by suppressor T cells were also shown to be encoded in the I-J region, these being separate from factors which enhanced the production of antibody (Pierres et al. 1978; Eardley et al. 1980). Anti-I-J antibodies were shown to be able to restore the production of antibody, thus further strengthening the thought that this I-J region, found within the MHC, encoded for both cellular receptors and soluble factors that inhibited the humoral response. These studies were undermined by detailed studies on the nature of the MHC complex. Experiments by Steinmetz and colleagues revealed that I-J, thought previously to be encoded between I-A and I-E, was in fact not encoded within the I-region (Steinmetz et al. 1982; Kronenberg et al. 1983). This work demonstrated that the region found between I-A and I-E was only two kilobases, and that much of this stretch of DNA was in fact part of the E_β region. Their conclusions were that the I-J region must map elsewhere in the genome (Kronenberg et al. 1983).

Indeed, it became clear through the use of monoclonal antibodies that the newly discovered surface markers CD4 and CD8 of T cells were analogous to Ly1 and Ly2 phenotypes shown on helper and suppressor T cells by Cantor years earlier (Jiang and Chess 2000). This was followed by the demonstration from multiple groups that there was an interaction between CD4 and CD8 T cells that was needed for suppression of the antibody response, and this required CD4 helper T cells to activate "suppressor" CD8 T cells (Jiang and Chess 2000; Shoenberger et al. 1998; Jiang and Chess 2004). It would eventually become clear that cell-cell interactions as well as cytokines produced by activated T cells are responsible for the suppression or production of antibodies.

With the skepticism regarding observations on I-J molecules, the potential role of CD8 T cells as regulators was largely forgotten. However, there are still many instances where CD8 T cells play an important regulatory role in developing immune responses (Jiang and Chess 2004). CD8 T cells have been shown to play a role in regulating autoimmune diseases in experimental autoimmune encephalitis and in staphylococcal enterotoxin B models (Jiang and Chess 2004; Jiang and Chess 2006; Shevach 2006; Najafian et al 2003). In the 1970's it was proposed that there was exclusiveness between humoral and cell-mediated immunity, that an antigen, in most cases, would induce one type of response, but not the other (Bretscher 1974). The fact that this reciprocal control of different types of immune responses was regulated by the CD8 T cell was largely disregarded, but it was later shown that the cells which were responsible for inhibition of humoral responses were indeed CD8 T cells (Ramshaw et al. 1977). More recently, there has been work done on the CD8 T cell-dependent suppression of antibody responses in various systems. The presence of CD8 T cells has been shown to play a regulatory role in the inhibition of allergic responses and IgE production (McMenamin and Holt 1993; Kemeny et al. 1995) as well as in productive Th1 responses to viral proteins which normally results in a Th2 response and eosinophilia (Srikiatkhachorn and Braciale 1997).

1.1.3 CD8 T Cells in Transplantation

The importance of the regulatory role of CD8 T cells in the development of immune responses is critical for disease outcome in many situations. A lack of CD8 T cells during various situations can result in incomplete clearance of pathogens and the manifestation of atopic disease due to insufficient Th1 immunity and resultant Th2 predominance. The role of CD8 T cells in response to alloantigens is included here due to the particular importance these cells play in the occurrence of disease in transplantation systems. A murine model of autoimmunity that resembles systemic lupus erythematosus (SLE), a disease that is characterized by autoantibodies against a diversity of self-antigens, particularly against the kidneys of the host causing glomerulonephritis, is achieved by the injection of parental lymphocytes into F1 recipients (Pickel and Hoffmann 1977; Via et al. 1987). The disease is mediated by a graft-versus-host (GVH) reaction in the recipient that is mediated by donor T cells. There have been extensive studies using this model, which I will delay here, but the function of CD8 T cells in this model has been shown to be regulatory as well as having effector function. Early studies on GVH

reactions implicated CD8 T cells as suppressors of the antibody response. They inhibited the antibody responses *in vitro* to sheep red blood cells by F1 cells after inoculation in F1 mice (Pickel and Hoffmann 1977). *In vivo,* a lack of CD8 T cells in the inoculum injected into recipient mice resulted in characteristic lymphoid hyperplasia, autoantibody formation, and glomerulonephritis, all manifestations of SLE in humans, indicative of the model used (Via et al. 1987). The occurrence of SLE-like symptoms in this model is mediated by a Th2 type immune response against the host. The presence of CD8 T cells in the same experiments resulted in immune deviation toward a Th1 type response, implicating CD8 T cells as the main regulators in this instance, and that this phenomenon is critically dependent upon CD8 T cells being engrafted (Via et al. 1987; Rus et al. 1995). In more recent experimental work examining target organ injuries in GVHD, it was shown that cytotoxic CD8 T cells were present only in acute GVHD, while there absence was indicative of chronic GVHD (Kataoka et al 2001). Furthermore, studies in rats showed that in an *in vivo* GVHD model, differential subsets of CD8 T cells are induced and their induction influences the differentiation of host-reactive CD4 T cells (Xystrakiz et al 2004). Additionally, a functional impairment of CD8 T cells has been linked to the incidence of SLE in humans (Filaci et al. 2001). Functional differences in CD8 T cells as suppressor cells were found in patients with SLE who were in remission compared with those who have active disease. I will discuss further the impact that the presence of CD8 T cells has on disease outcome in this transplantation model in section 1.3, but I think that it is clear that in this GVH situation as well as in situations involving pathogen clearance and atopic diseases, as well as autoimmunity, CD8 T cells have a regulatory role.

1.2 PHYSIOLOGICAL FUNCTIONS OF CD4 T CELLS: THE CD4, Th1/Th2 PARADIGM

CD4 T cells are important mediators of the immune response. They are responsible for helping in the production of antibody, for helping in the induction of the cytotoxic T cell response, and for macrophage activation; they also mediate immunological memory, and have regulatory functions for the control of autoimmunity (Zhu et al. 2010). CD4 T cells become activated through the recognition of their cognate antigen presented by APC in an MHC

restricted manner, as well as through the interaction of co-stimulatory molecules on the surface of the T cell and APC. Naive CD4 T cells can be activated to differentiate into different subsets, depending on many factors during the onset of the activation of the T cells. The first evidence of distinct populations of CD4 T cells came from observations by Mossmann and Coffman in the 1980s, who demonstrated the existence of distinct subsets of helper T cell clones called T helper 1 (Th1) and T helper 2 (Th2), respectively (Zhu et al. 2010; Mossmann et al. 1986). Th1 clones produce IL-2, IFN-γ, GM-CSF, and IL-3 upon interaction with APC, while Th2 clones produce IL-3, BSF1, a mast cell growth factor distinct from IL-3, and IL-4, a T cell growth factor distinct from IL-2. Th1 and Th2 cells could be distinguished by the profile of cytokines they produced. It is now known that the signature cytokine produced by Th1 cells is IFN-γ as well as some IL-2, while Th2 cells produce mainly IL-4, IL-5 and IL-13. Th1 cells play a role in the development of cytotoxic T lymphocytes, and are involved in cell-mediated responses. Th2 responses mediated by CD4 T cells promote the production of antibody and the development of antigen-specific B cells into memory B cells and plasma cells. The differentiation into Th1 or Th2 cell types is reciprocally regulated, as Th2 cells inhibit the production of cytokines by Th1 type cells (Fiorentino et al. 1989). There are multiple other T helper subsets that have since been recognized, those being Th17, Th9, Th22, T follicular helper cells, and regulatory T cells. Though these cell types are important in many immunological situations and have interesting roles of their own, I will only mention these subsets of helper T cells here, in that my project and thesis was concerned strictly with the development of Th1 and Th2 responses.

1.2.1 Factors that Influence the Th1/Th2 Nature of the Immune Response

There is a vast literature on the many factors that play a role in determining whether a Th1 or Th2 response is made. In the thirty years since the introduction of different T helper subsets there have been many reports as to what determines the nature of the immune response. There is substantial evidence in the literature for many different factors, but I will mention some that I think are important, and are not involved with my project briefly, before discussing the important factors involved with my work. Also, in the following section I will highlight the important transcription factors involved in Th1/Th2 differentiation.

An important factor in determining whether a Th1 or Th2 response is made is the antigen dose. Early studies in guinea pigs showed that the administration of diphtheria toxoid results in delayed type hypersensitivity followed by production of antibody (Salvin 1958). The period of delayed type sensitivity was decreased with the administration of a higher dose of antigen, while this period could be extended by giving a lower dose. This can be readily explained in today's terms of Th1 and Th2 immunity, with Th1 responses typically occurring first, before a Th2 response predominates. More recent experiments using *Leishmania major* and *Mycobacteria* (Bretscher et al. 1992; Power et al. 1998) showed that the dose of the parasite or bacteria determines the nature of the immune response regardless of the route of immunization.

The number of antigen-specific CD4 T cells present at the initiation of the immune response has been shown to play a role in the Th1/Th2 decision. Generally, if the number of responding CD4 T cells is low, below a certain threshold, a Th1 response will be made while a Th2 response will be made if the number of CD4 T cells is above that critical number, if the dose of antigen is held constant (Ismail and Bretscher 1999; Bretscher 2014). This "Threshold Hypothesis" seems to hold true in many instances, for instance in *in vitro* experiments where only CD4 T cells are the responders. The response to allo-antigens, for which there are likely many antigen specific CD4 T cells, probably relies on CD8 T cells to mount the proper type of immune response (Bretscher 2014). Nevertheless, the number of T cells specific for antigen likely plays an important role in determining a cell-mediated vs. antibody response.

The nature of the APC presenting cognate antigen to the T cell has been thought to play a role in differentiating T cells. The standard antigen-presenting cells are macrophages, dendritic cells, and B cells. Due to their important role as APCs, dendritic cells (DCs) have been called "professional APCs", and are responsible for the uptake of foreign molecules and pathogens for their processing and presentation to T cells. There have been many different subsets of DCs characterized in terms of cytokine production and cell surface marker expression, and it is thought by some that the development of a Th1 or Th2 response depends on the maturation status of the DC (Palucka and Banchereau 2012). The co-stimulatory molecule expression as well as MHC class II expression may play a role in the DC's ability to activate T cells, and this is important in direct recognition pathways of the response against allo-antigen to be described later (Game and Lechler 2002). The phenotype of DCs presenting antigen to T cells has been

shown to play a role in the type of immune response generated (Moser and Murphy 2000). There is a vast literature on what transcription factors and cytokines in APCs play a role in differentiating T cell subsets as well as the surface marker expression of these cells, and while the phenotype of DCs has been shown to have an effect on the type of immune response made and I think that this phenomenon probably has some merit, I did not study the effects of APC maturation and presentation in my work, so it is only briefly mentioned here.

There are other factors that have been discussed in the literature that may impact the type of immune response, such as the foreignness of antigen, the type of antigen, co-stimulatory molecule expression, and affinity of the antigen for the TCR and the MHC. However, for many of these factors there is insufficient literature describing them to evaluate their role decisively. Moreover, they do not appear to be directly pertinent to this thesis, so are only mentioned here.

1.2.2 The Cytokine Environment's Role in Determining Th1/Th2 Immune Responses

The phenotype of effector T cells is determined by the transcription factors they express and is assessed by characterizing that cell population. These subsets of CD4 T cells are typically generated by antigen *in vitro* in the presence of distinct cytokines. The first experiments showing that cytokines are important in determining the fate of helper T cells were done by multiple groups which revealed that *in vitro* IL-4 was needed to generate IL-4 producing Th2 cells (Le Gros et al. 1990; Swain et al. 1990). The fact that the cytokine being produced by this type of T helper subset was also required for its induction was surprising. However this was also found to be the case later for Th1 cells and the production of IFN-γ, which is enhanced in the presence of IFN-γ itself through the activation of the transcription factor T-bet (Lighvani et al. 2001). The first cytokine to be implicated in Th1 differentiation was IL-12. It was demonstrated that IL-12 produced by macrophages in the presence of heat-killed *Listeria monocytogenes* induced Th1 development *in vitro* (Hsieh et al. 1993). The characteristic cytokines of each T cell subset play a role in differentiating T cells by inducing specific transcription factors as well as inhibiting the expression of transcription factors important for other subsets (Zhu et al. 2010). Indeed, the presence of cytokines *in vitro* will inhibit the formation of certain T helper lineages. The presence of IFN-γ inhibits the proliferation of Th2 clones, but not Th1 cells (Gajewski and Fitch 1988) and reciprocally, IL-4 inhibits the production of Th1 cells (Fiorentino 1989).

The reciprocal regulation seen in the presence of different cytokines is due to the interaction of these cytokines with their target receptor on the surface of lymphocytes and subsequent induction of transcription factors. Each cytokine has a specific receptor, which upon interaction with that cytokine will promote downstream effects leading to the expression of certain transcription factors that will induce the production of subset-specific cytokines as well as inhibit transcription of a different subset's genes. The interaction of lymphocytes with a particular cytokine will lead to an autocrine loop, whereby the cell will produce the cytokine that mediated its induction. This is what is responsible for the observation, as noted above, that IL-4 and IFN-γ induce the differentiation into effector cells which produce that cytokine.

The major transcription factors for Th1 and Th2 helper cells are T-bet and GATA-3, respectively. These major regulators are downstream factors that are induced by the transcription factor family of STAT proteins, which consists of multiple factors, each with different regulatory functions that I will briefly discuss. GATA-3 was the first master transcriptional regulator to be identified; it is essential for Th2 cell generation, but is also expressed in naïve CD4 T cells (Zhu et al. 2010). GATA-3 is essential for Th2 production downstream of both STAT5 and STAT6, with the latter being the most important regulator of Th2 immune responses (Zhu et al. 2010). GATA-3 also functions by inhibiting the production of Th1 cells. T-bet is the major transcription factor involving in Th1 differentiation, by inducing the production of IFN-γ and down-regulating Th2 cell development through inhibition of GATA-3, which may be a more important role for T-bet, as suggested in (Usui et al. 2006). The STAT proteins upstream of T-bet during Th1 differentiation are STAT1 and STAT4. STAT1, upon activation via IFN-γ induces T-bet expression resulting in Th1 cell differentiation, while STAT4 is induced by IL-12 and directly promotes IFN-γ expression (Zhu et al. 2010). Additionally, the expression and induction of transcription factors important for differentiation into one T helper lineage down-regulate the transcription factors important for other lineages. Thus a cross-regulation is seen among transcription factors induced by cytokines analogous to that seen in the presence of cytokines *in vitro*.

There is a substantial literature on the role the cytokine environment plays at the initiation of the immune response in determining Th1 or Th2 immunity. The importance of this role in terms of my work is critical, due to the fact that activated CD8 T cells produce large amounts of

IFN-γ and the presence of this cytokine functions in favor of the induction of Th1-type immunity.

1.2.3 The Presence of CD8 T Cells

I described above how the number of CD4 T cells present at the initiation of the immune response could impact what type of response was made. Experiments examining the effect of CD4 T cell density typically involve only CD4 T cells present in culture, without CD8 T cells present. The presence of CD8 T cells both *in vivo* and *in vitro* will have an effect on the response mounted. Aside from the cytotoxic role of CD8 T cells, it has been shown that these cells can have a regulatory effect (Jiang and Chess 2004; Jiang and Chess 2006), and that their presence has a dramatic outcome on disease stability or progression, as briefly mentioned above. Studies by Via using murine acute and chronic GVHD as a model showed that the presence of CD8 T cells in a parent into F1 lymphocyte allograft will result in acute GVHD and a cellular response, while in the absence of CD8 cells antibody is produced with chronic GVHD (Rus et al. 1995). CD8 T cells regulating the response made by CD4 T cells in a GVH model has also been shown in rats, as mentioned previously (Xystrakis et al 2004). The presence of CD8 T cells in these experiments show a regulatory role for these cells and the transition into a cell-mediated response was dependent upon elimination and down-regulation of the B cell response and production of IFN-γ by CD4 T cells.

1.3 TRANSPLANTATION IMMUNOLOGY

One of the important situations where an immune response is generated is when a host is transplanted with a foreign graft. Transplantation is the process of taking cells, tissues, or organs, called the graft, from one individual and placing them into another, the host (Abbas at al. 2011). A graft from one individual into another genetically identical individual will typically be successfully transplanted without any issues. Grafts from one individual into a genetically non-identical individual (individuals which are MHC incompatible) will cause the host to mount an immune response against the graft. Allografts are grafts from a member of one species into a genetically different member of the same species. Allografts will generally elicit an immune

response by the host. Early work by Medawar showed that skin grafts between MHC incompatible strains of mice caused their rejection within two weeks (Billingham et al. 1954; Medawar 1946; Billingham, Brent at al. 1954). He found out that skin transplanted between members of inbred strains of mice would act normally, as a graft from the members of the same inbred strain would, but then "in due course it is invariably destroyed" (Medawar 1946). The importance of these experiments was the fact that Medawar showed that the rejection of skin grafts was mediated by the immune system. The rejection of skin grafts between allogeneic mice exhibited the hallmarks of adaptive immunity, namely memory, shown by the fact that skin grafts transplanted onto a genetically different mouse will typically reject the graft in about 11 days, while a second graft from the same donor strain will be rejected much faster in this mouse, 6 days or less (Billingham, Brent et al. 1954). The response was also specific, shown in experiments whereby mice transplanted with a skin graft from one donor, say A, resulted in a secondary response and rejection in about 6 days, while grafts from a separate donor, B, elicited a primary response and rejection in about 11 days. The fact that a second set rejection was not elicited by B grafts shows the specificity of the immune reaction against the transplant. Another striking observation showing that transplants are rejected by the immune system was the fact that immunity to grafts could be transferred to naïve hosts via the transfer of lymph node and spleen cells of actively immunized mice to the host. Medawar as well as Mitchison showed that mice given lymph node or spleen cells of a syngeneic mouse that has been previously immunized with an allogeneic graft will reject a genetically identical transplant of the same donor origin in a second set fashion despite not having encountered the graft previously (Billingham, Brent et al. 1954; Mitchison 1954). The transfer of these cells from the secondary immune organs conferred immune memory on the host. These experiments showed that the rejection of skin grafts is mediated by an immune response against the graft. Medawar called this phenomenon "actively acquired immunity" to the skin graft (Billingham, Brent et al. 1954). Further experiments done by Mitchison helped to determine which arm of immunity was in fact responsible for mediating graft rejection. Previous experiments had shown that immunity could be transferred to naïve mice via the transfer of lymph node or spleen cells of immunized mice. Mitchison had attempted to transfer immunity via serum from immunized mice, but was unsuccessful (Billingham and Brent 1956). Therefore it was shown that cell-mediated immune responses were needed for

successful graft rejection and that antibodies were not capable of causing complete rejection of allogeneic grafts.

1.3.1 MHC Antigens are the Main Transplantation Antigens

We have seen that transplantation of grafts between genetically non-identical individuals results in rejection of the graft and subsequent immune memory against grafts of donor type (Billingham et al. 1954; Medawar 1946; Billingham, Brent at al. 1954). Therefore the immune system must be recognizing something on the allograft which is different from self, to which it mounts an immune response. The major histocompatibility complex (MHC) was discovered via studies using tumor and tissue transplantation. Early work by Little and Tyzzer showed that tumors taken from Japanese waltzing mice were able to grow in mice of that strain, but not in mice of other strains (Little and Tyzzer 1916). In the 1940's, Snell and colleagues were, through many different experiments to determine the genetics of the transplantation response, able to identify alleles at the histocompatibility complex of mice involved in the rejection of transplants. The work of Snell was subsequent to earlier experiments done by Gorer. Gorer showed that the laws governing tumor and tissue transplantation were similar in that inoculation of either into genetically different individuals will be unsuccessful, whereas transplants between inbred mice that have undergone brother-sister breeding, behaved similar to autographs (Gorer 1942). He also noted that the genes responsible for this transplantation rejection were dominant genes. After the establishment of a set of genes responsible for the rejection of tumor and tissue transplants, Snell suggested these genes be called histocompatibility genes (Snell 1948). He theorized that the same set of genes responsible for the rejection of tumors between strains was also responsible for the rejection of tissue transplants. Studies done by Snell and his colleagues identified a histocompatibility antigen identical to the antigen II found on erythrocytes, linked to a gene for a "fused" tail, and responsible for tumor rejection (Gorer et al. 1948). The antigen, so named H-2, was thought to be responsible for stimulating an immune response that mediated the rejection of tumors and transplants. Of the histocompatibility antigens in the mouse, genetic differences in H-2 result in graft rejection in less than two weeks, as noted by Medawar, while differences in H-1, H-3, etc. produce much slower rejection (Bretscher 2010). This was also shown to be the case by Snell who determined that differences in H-2 antigens elicit much stronger reactions than differences in H-1 or H-3 upon grafting (Counce et al. 1956). Further

experiments were performed to determine specific H-2 alleles present that mediate rejection. Multiple studies followed, using multiple mouse strains and tumor lines, which set out to determine different H-2 alleles (Snell 1953; Snell et al. 1953; Amos et al. 1955). The establishment of rejection of transplants between mice differing in H-2 led to experiments showing that F1 crosses between different inbred strains will grow a tumor of parental origin of either strain (Snell 1953). After the discovery of the H-2 "locus" and its alleles and their role in transplant rejection, it was shown that recombination events can occur between strains within the H-2 "locus", leading to the recognition of several linked genes encoded within H-2. This led to the renaming of H-2 as the major histocompatibility complex (Abbas et al. 2011). The major physiological roles of the MHC antigens, nor the fact that these antigens induced T cell responses were known at the time of these experiments. They were indeed significant in defining the role of MHC antigens in transplantation immunology. The role of MHC antigens in antigen processing and presentation were discovered later.

1.3.2 Direct and Indirect Allo-recognition

The discovery of MHC antigens in the context of transplantation led to the realization that differences in the MHC were the cause of rapid graft rejections. The recognition of MHC antigens is the initial event leading to rejection. When MHC incompatible tissues were grafted onto mice, or when leukocytes from MHC disparate individuals were cultured together, it was realized that the strength of the immune response generated was exceptional. It was demonstrated that the immune response is responsible for graft rejection through the transfer of primed lymph node and spleen cells and the ability of these cells to cause second set rejection. In the 1970s it was shown that T cells are the main population of cells responsible for graft rejection (Gelfand and Steinberg 1973). The strength of the T cell response against MHC incompatible allografts was found to be substantially higher than T cell responses against foreign antigens as measured by antigen-dependent proliferation of T cells (Sherman and Chattopadhyay 1993; Rogers and Lechler 2001; Hornick 2006). Over the years there have been multiple hypotheses developed as to why there is such a vigorous response made against MHC antigens; however, these hypotheses are not pertinent to this thesis so I will not describe them here. I will simply state that the frequency of T cells specific for a given, foreign MHC antigen is roughly 10-100 times higher than the frequency of T cells specific for other antigens (Hornick 2006).

The activation of T cells specific for MHC allo-antigens can be accomplished via two distinct pathways: direct or indirect (Rogers and Lechler 2001). The direct pathway involves recipient T cells recognizing intact MHC molecules on the surface of donor cells, usually APC, resulting in their activation. The indirect pathway requires recipient APC to process and present donor MHC molecules to recipient T cells through traditional antigen presentation, i.e. in a self-MHC restricted fashion (Rogers and Lechler 2001). It is generally thought that the direct pathway is involved in the acute rejection of transplants, whereas the indirect pathway is needed for chronic rejection (Game and Lechler 2002). The direct pathway requires the presence of donor APC and is therefore limited by the presence/absence of these cells. Once the graft is devoid of APC capable of stimulating allo-specific T cells, there must be another mechanism whereby the immune system can cause rejection, namely the indirect pathway. Due to the involvement of the direct pathway and its correlation to the mixed lymphocyte reaction (MLR), discussed below, in acute rejection, I will focus on it rather than the indirect pathway, as the latter likely has little impact on the rapid responses made in the *in vitro* experiments described below. Experiments in support of direct allo-recognition were carried out by Lafferty, who cultured mouse thyroid gland for 12 days before transplanting it into an allogeneic recipient (Lafferty et al. 1975). A prolonged survival of the graft was presumably due to the loss of donor leukocytes which are capable of stimulating allo-specific T cells. Further experiments by Lechler involved the "parking" of rat kidney allografts in intermediate recipients, before their transplantation resulting in permanent survival of the grafts (Rogers and Lechler 2001; Lechler and Batchelor 1982). This is due to a presumed loss of "passenger" cells during time spent in the intermediate recipient, presumably APC which would stimulate allo-reactive T cells under normal conditions. The infusion of allogeneic dendritic cells back into the renal grafts induced a rapid rejection response, showing that it is the donor's APC that are responsible for an acute rejection response (Lechler and Batchelor 1982). A good method for studying the mechanisms behind transplantation and graft rejection is the *in vitro* correlate to direct allo-recognition, the mixed lymphocyte reaction, which I will discuss briefly.

1.3.3 The Mixed Lymphocyte Reaction

The first mention of the mixed lymphocyte reaction was by Bain, Vas, and Lowenstein in the 1960's, who, when looking at slides of blood smears from human patients, found a number of

cells that were "large with large clear nuclei and prominent nucleoli" (Bain et al. 1964). At the time it was known that when peripheral blood leukocytes were cultured in the presence of phytohemagglutinin, the cells could transform and undergo mitosis. What they discovered was that a similar phenomenon could occur in the absence of phytohemagglutinin. It turned out that the culture with the enlarged cells was a mixture of blood from two patients. They set out to determine whether a mixture of blood leukocytes from two patients would consistently produce the same results. What they found out was that mixtures of blood leukocytes from different patients reliably produced the same type of large cells undergoing mitosis as in their original discovery. Such cultures are said to give rise to a mixed-lymphocyte reaction. They showed that cultures with blood cells from two patients also significantly increased the uptake of radiolabelled thymidine as compared to cultures of one patient's blood alone (Bain et al. 1964). This multiplication of leukocytes from a donor required the presence of stimulator leukocytes rather than plasma or platelets from the donor of the stimulator leukocytes. Moreover, culture of blood leukocytes from monozygotic twins did not show any reaction, thus the effect was likely due to some genetic differences between individuals from whom the leukocytes were obtained. This study led to the hope that a lack of response in the MLR may be a valuable indicator that individuals from whom lymphocytes were obtained are MHC compatible.

These observations led to many studies on the nature of the immune response made during MLRs. Wilson showed in rats that a sizable immune response is made only when the leukocytes cultured together in an MLR come from individuals differing at the MHC, and that the induction of mitosis as determined by the uptake of thymidine indicates an immune response at the cellular level (Wilson 1967). Thus, it was assumed that the reaction against the MHC during the MLR *in vitro* was analogous to the "sensitizing" phase seen during immune rejection of an MHC-incompatible allograft (Hayry and Defendi 1970). Hayry and Difendi set out to demonstrate the production of effector cells in the MLR. Such a finding made it plausible that what occurs in an MLR corresponds to what happens when an MHC-incompatible graft is rejected *in vivo* (Hayry and Defendi 1970). They showed that incubation of murine leukocytes from DBA/2 and C3H mice cultured with leukocytes from their F1 offspring underwent mitosis and uptake of thymidine, as determined previously in rat and human studies, and that lymphocytes that were sensitized in the MLR were capable of lysing chromium labelled target cells *in vitro.* The MLR also showed the essential features of specificity and memory,

characteristic of an immune response. Therefore it was reasonable to consider the MLR as a suitable *in vitro* analog for the rejection of MHC-incompatible grafts. Due to the swift nature of the proliferative and cytotoxic response made by lymphocytes during an MLR, the reaction corresponds well with acute rejection (Wilson et al. 1968). As described above, the cells responsible for acute rejection of grafts in vivo are likely generated due to direct recognition of allogeneic MHC antigens on APC, and thus it is likely that during MLRs there is activation of T cells through the direct pathway, as evidenced by the rapid nature of the response.

1.4 MOUSE STRAINS AND THEIR RESPONSES TO TRANSPLANTATION ANTIGENS

1.4.1 Differences in Class I and II MHC and the H-2^b Haplotype

Experiments described above involving the presence of CD8 T cells during an immune response expanded upon previous work on immune responses against allo-antigens when lymphocytes from parental mice were grafted into F1 recipients. A large influence for this thesis comes from these models and their implication that CD8 T cells play a role in promoting cell-mediated responses, leading to acute GVH reactions. A series of reports published in the 1980's by Gleichmann and colleagues demonstrated that when parental T cells are injected into F1 hosts, there were two distinct kinds of responses that can occur. These responses were made by parental lymphocytes against allo-antigens of the F1 host. What was found was that either the host mice would succumb to a lethal graft-versus-host reaction, characterized by aplastic anemia, severe immunodeficiency, hypogammaglobulinemia, and sepsis, or the mice would suffer from a longer term chronic reaction resulting in lymphoid hyperplasia, hypergammaglobulinemia, autoantibody formation, and symptoms similar to that of human SLE (Rolink et al. 1982). The mechanism behind the two different disease outcomes, named acute and chronic graft-versus-host disease (GVHD) respectively, was not well understood until this series of experiments. We have seen already that the rise of suppressor T cells was in full swing, and it was thought that the different pathological outcomes seen in these allogeneic responses was due to differing subpopulations of T cells of the donor. It was found that T cells of C57B10 mice, activated for five or six days against recipient MHC antigens could, when injected into F1 recipients, cause

severe lethal, acute GVHD if activated for five days, while the six day activated cells caused chronic GVHD and SLE like symptoms (Rolink et al. 1982). What was critical in this experiment was that the five-day and six-day lymphocyte populations were different. The five-day activated cells contained many more activated CD8 T suppressor cells, while six-day cells contained more T helper cells. The five-day activated cells suppressed the antibody response made by F1 hosts, as well as by syngeneic lymphocytes *in vitro*. This work showed that different subpopulations of T cells could impact the type of disease in this GVH model.

It was known that the presence of T suppressor cells in the population of responding lymphocytes resulted in acute GVHD leading to death of the mice. The graft-versus-host reaction caused by responding T cells against the incompatible H-2 regions of the host was assessed by Gleichmann to determine a genetic basis for disease outcome in the same series of experiments. Knowing that differences in I-A and I-E of the MHC provided strong stimulation in mixed lymphocyte culture, they used mice which differed across I-E, and found that B10.A(4R) mice, which were able to recognize the I-E antigens of the recipient, respond dramatically against the F1 host and cause a chronic GVHD (Van Rappard-van der Veen et al. 1982). Mice which did not recognize I-E molecules on the surface of recipient B cells and APC did not respond similarly to B10.A(4R) mice despite a different haplotype across H-2. Therefore, it was shown that this difference across the I-E region was enough to stimulate the activation of responder T helper cells characteristic of chronic GVHD.

The genetic basis for the different pathological outcomes was further investigated in a series of experiments with mice having mutations at the class I or class II MHC and determining the phenotype of the responding cell population. It was shown similarly to the above experiment that differences in class II across the responder and the host caused chronic SLE-like symptoms, but a difference in only class I did not result in disease (Rolink et al. 1983). To cause acute GVHD, a difference in class I and class II MHC was needed. What was found was that when there was a difference in both classes I and II MHC, T helper cells responding against the class II MHC of the host were activated and proliferated for approximately one week, before alloreactive T suppressor cells became abundant (Rolink et al. 1983; Pals et al. 1984a; Gleichmann and Gleichmann 1985). Thus, it was shown that T suppressor cells reacting against class I MHC of the host needed T helper cells reacting against class II MHC of the host to become activated and

in sufficient numbers before allowing for the development of a suppressive response and acute disease. The Ly phenotypes of these allosupressor and allohelper cells were shown to be Ly2 and Ly1 respectively, consistent with the idea that "CD8" suppressor cells were responsible for acute GVHD and inhibition of the antibody response (Rolink and Gleichmann 1983). The subpopulation of responding T cells was critical, as the injection of only T helper cells even in hosts incompatible at both class I and class II MHC could not cause acute disease, reflecting a requirement for "CD8" T suppressor cells. Therefore, the induction of T helper cells alone caused chronic SLE-like disease, while the induction of T helper cells, followed by T suppressor cells, in the presence of incompatibility across class I MHC, caused acute or lethal GVHD.

Differences across the MHC of the responder and host as well as the subsets of lymphocytes responding were known to be critical for the development of disease upon injection of parental lymphocytes. An interesting observation by Gleichmann (Pals et al. 1984b) was that the administration of T cells from different donors resulted in different disease outcomes despite the injection of the same number of unseparated spleen cells. In experiments using either C57BL/10 or DBA/2 mice as donors into C57BL/10 x DBA/2 F1 recipients, the activation of F1 specific T suppressor cells was only found in those mice which developed acute GVHD, which turned out to be only mice injected with C57BL/10 spleen cells (Pals et al. 1984b; Via et al. 1987). F1 recipients of DBA/2 spleen cells did not activate the donor cells to generate F1-specific "CD8" T suppressor cells, following the activation of T helper cells, and did not develop acute GVHD, but instead developed chronic, SLE-like disease. The failure to develop a lethal reaction in DBA/2 mice was thought to be due to a lack of T suppressor cells, and later experiments showed that the levels of CD4 T cells was comparable in F1 mice injected with lymphocytes of both strains, and that treatment with anti-Ly2 antibody and complement in mice injected with C57BL/10 spleen cells could alter the effect on disease outcome to a chronic rather than acute state (Via et al. 1987). Thus, there was some genetic reason, unknown at the time, for the lack of lethal disease development in DBA/2 mice compared with C57BL/10 mice. This is consistent with contemporary knowledge of murine haplotypes. Mice which are of the H-2^b haplotype do not express class II I-E molecule on the surface of their B cells and APC (Flavell et al. 1985), due to a lack of the expression of the E$_\alpha$ chain. The consequences of this is that there is no positive thymic selection of CD4 T cells which are I-E restricted during development, and therefore fewer CD4 T cells present in these mice. It was discussed above that the number of

responding T cells of a given subset could impact the type of immune response developed and thus the CD4:CD8 T cell ratio would impact disease development. In the case of C57B10 mice used in these experiments, the relative numbers of CD4 and CD8 T cells and thus the ratio of these cells differ from that of DBA/2 mice, affecting the type of disease progression. The presence of more CD8 T cells relative to CD4 T cells in H-2^b mice compared to other haplotypes results in a more cell-mediated bias and this is responsible for the acute GVHD seen upon injection of C57BL/10 spleen cells. This is confirmed by the selective depletion of these cells resulting in chronic disease similar to that seen upon DBA/2 spleen cell injection. The genetic difference between haplotypes is important in the resistance or susceptibility to disease.

1.4.2 Parabiosis of Mice to F1 Offspring

At the same time that these studies were carried out, similar studies were reported involving the parabiosis of mouse strains that differed at H-2. Parabiosis is the process by which a common blood supply can be established between two indiviudals. The blood vessels of the two individuals are sutured together, and this allows for blood flow between each party (Drell and Wegman 1979). This parabiosis of a mouse belonging to one strain, A, with an H-2 incompatible A x B F1 mouse results in a graft-versus-host reaction akin to that seen in the experiments done by Gleichmann discussed previously. The GVH reaction that occurs is called parabiosis intoxication disease and typically leads to the death of both animals in two or three weeks (Drell and Wegman 1979; Eichwald et al. 1959). An exception to the fatal outcome of parabiosis was found in an experiment by Rubin who showed that DBA/2 mice parabiosed to F1 (DBA/2 X C57BL) mice could survive up to 60 days and beyond in almost half the cases (Rubin 1959). These pairs of mice would also accept F1 skin grafts. Years later it was confirmed that this was indeed the case, and it was found that the parental mouse, in this case DBA/2 performed a takeover of the F1's red blood cells and lymphoid system. After long-term survival of DBA/2 mice parabiosed to (DBA/2 x C3H) F1 mice, the circulating red blood cells of the F1 are nearly of 100 percent DBA/2 origin (Drell et al. 1975). It was shown that the takeover of the F1 mouse also included the lymphoid system, when spleen cells taken from F1 mice were able to react against C3H lymphocytes in a mixed lymphocyte reaction (Shaw et al. 1974). Therefore it was concluded that the cells of the homozygous mouse strain replaced the red blood cells and lymphoid system of its parabiosed, F1 partner.

As seen earlier, genetic differences between DBA/2 and C57BL/10 mice were responsible for differential disease outcome after injection of parental cells in F1 mice. In experiments using parabiosed mice, differences could be found in the survival frequency of pairs of mice when different H-2 haplotypes were used. DBA/2 mice were parabiosed to mice with the different heterozygous haplotypes H-2$^{d/k}$, H-2$^{d/p}$, or H-2$^{d/b}$ (H-2^{b} being the same as C57BL/10 mice in Gleichmann's experiments) and survival rates were assessed. When DBA/2 mice were parabiosed with H-2$^{d/p}$ mice, the survival of the mice increased to 69% as compared to the standard survival of around 50% for the H-2$^{d/k}$ haplotype mice, while H-2$^{d/b}$ mice had the lowest survival rate (Drell and Wegman 1976). That this genetic background was least susceptible to takeover by DBA/2 lymphocytes is not surprising when compared to the aggressive reaction of H-2^{b} haplotype cells in the experiments by Gleichmann and the ability of these cells to react strongly in mixed lymphocyte reactions.

The difference in the immune response made by mice differing in H-2 haplotypes has implications for disease outcome, as we have seen, and these result from differences in the T cell subsets inherently found in these strains of mice. The importance of these differences is a large part of the work presented in this thesis.

1.5 DEVELOPMENT AND ONTOGENY OF THE IMMUNE SYSTEM

1.5.1 Early Neonatal Immunology

The earliest experiments involving the immunity of newborn mice were done in the late 19th century. In 1890, Behring and Kitasato discovered the immunity to diphtheria and tetanus toxins could be transferred to non-immune recipients and that immunity to the toxin is due to some substance in the serum of individuals, which they called antibodies (Silverstein 2000). After the discovery of antibody, it was found that newborns possessed some antibody in their serum. It was known that mothers who are infected with certain diseases could pass their affliction onto the newborn child, so it was thought that perhaps the presence of antibodies in the serum of the newborn was passed on from their mothers. Ehrlich performed studies whereby females, immune to certain toxins, mated with males that were non-immune, and vice-versa to

determine whether this passive immunity was transferred from the mother or the father (Silverstein 2000; Bona 2005). He found out that antibody could only be passed on to the newborn through immune mothers, as these mice were resistant to a normally lethal challenge of the toxin, whereas mice born to immune fathers were susceptible. The presence of antibody in the serum of newborn mice indicated that maternal antibody could be transferred to the fetus *in utero,* but that the presence of the antibody in the newborn's serum lasts longer than is typically seen in adult mice. This longer term presence of antibody was provided by the mother's milk. Another experiment by Ehrlich showed that neonates born to non-immune mothers, but transferred into cages where they would receive milk from immune "foster mothers" became progressively resistant to challenges with the toxin (Silverstein 2000; Bona 2005). Therefore, he concluded that maternal antibody is important for early protection of the newborn, and that this could be passed on to the offspring both *in utero* and through suckling. These early experiments by Ehrlich were likely the first in neonatal immunology, and the discovery of the transfer of maternal antibodies it still important today.

1.5.2 Models of Tolerance During Ontogeny

In the decades since Ehrlich's experiments there has been substantial progress in the understanding of neonatal immunity. Half a century later, during the formative years of Medawar's work on transplantation Billingham, Brent, and Medawar showed that if given allogeneic cells sufficiently early in life, a mouse will acquire tolerance to the foreign cells, whereby a second challenge with these cells in adult life produces no immune response, or at least a diminished one (Billingham et al. 1953). Medawar called this phenomenon "actively acquired tolerance" as opposed to "actively acquired immunity" which is seen when adult mice are challenged similarly with foreign cells. The mice that had been challenged with allogeneic cells *in utero* were unable to mount an immune response, and a response could be restored upon administration of lymphoid cells from syngeneic mice previously challenged, showing that there is some deficiency in the ability of the fetal immune cells to respond to foreign cells. In another experiment, it was shown that dizygotic cattle twins were tolerant of skin grafts from each other, but not of grafts from separately born siblings or of their parents' skin. This was due to a presumed sharing of circulating cells through fetal blood vessels, which expanded upon work by Owen showing that dizygotic twins were often red blood cell chimeras with circulating red cells

of both twins (Billingham et al. 1955; Owen 1945). The presence of the cells of both twins during the prenatal life of the fetuses renders them tolerant. These experiments were some of many that showed that the administration of a number of antigens, not only foreign cells, during development could render the individual tolerant to that antigen. This was shown in many species including mice, rats, rabbits, chickens, turkeys, and cattle as reviewed by Billingham, Brent and Medawar in 1956 (Billingham et al. 1956).

The idea that tolerance could be induced before birth was a proposal that has been well studied in models other than those of mice and rats. As noted above, experiments were carried out in many species, including in chickens, in which the parabiosis of chorioallantoic membranes of the embryos of different strains could lead to tolerance, providing further evidence of the immaturity of the developing immune system. Chicks of one strain, SC, which had been given stem cells of another histoincompatible strain FP on days 12 or 15 of embryonic development, were unable to respond to challenges with FP cells at both the humoral and cell-mediated levels (Havele et al. 1982; Havele 1981). The success of achieving an unresponsive state that was generated by making chimeras between two strains was dependent on the time of embryonic development at which chimeric formation was initiated, as chimeras that were generated at day 17 of development and beyond were capable of mounting an immune response against the foreign alloantigens. What was interesting was that chimeras generated at day 17 of embryonic development differed from those generated at day 21, right before hatching. Day 17 chimeras were not unresponsive to donor alloantigens, as seen in day 12 and 15 generated chimeras. These chicks were able to produce alloantibodies that were detectable at seven weeks of age, and by ten weeks, all chimeras generated at day 17 of development had some alloantibodies against the donor strain, but could not mount an effective cell-mediated response (Havele et al. 1982). Chimeras created at day 21, however, were able to respond similarly to older chicks upon challenge with allogeneic cells. These chicks were able to mount competent cell-mediated graft-versus-host responses against the foreign cells (Havele et al. 1982; Havele 1981). The fact that day 17 chimeras were able to produce antibody, but not a cell-mediated response, while chimeras generated later in development could mount effective responses akin to those of adult individuals gave clues to the maturation of the immune response and the ability to mount effective responses at different stages of the individual's development.

Studies attempting to examine the nature of self-tolerance moved from employing fetal to newborn animals. It was demonstrated by Billingham that newborn mice and rats could also be made tolerant against allogeneic skin grafts by the injection of allogeneic cells early in life (Billingham and Brent 1959; Billingham et al. 1962; Schwarz 1968). Early studies, to determine whether mixed lymphocyte reactions could be used as tests of tolerance, took advantage of what was known about neonatal tolerance at the time. Newborn rats could be made tolerant, by injecting cells from histoincompatible donors, as was custom at the time, and the lymphocytes from the tolerant rats were tested in mixed lymphocyte reactions for their responsiveness to cells of the donor strain (Schwarz 1968). This demonstrated their tolerance. These experiments were done to expand upon the knowledge that was being accumulated regarding mixed lymphocyte cultures at the time, but took advantage of what was known regarding the development of the immune system and the establishment of tolerance, namely that newborn animals did not mount effective immune responses. This was confirmed *in vitro*. The experiments outlined here were important in gaining knowledge of the developing immune system. The models of tolerance in neonates turned out not to be completely correct; it was found that these mice generated an antibody response against donor antigens and were unresponsive for the induction of a cell-mediated response. They were in a state of humoral immune deviation, rather than tolerance, with respect to donor antigens.

1.5.3 The CD4, Th2 Nature of the Neonatal Immune Response

Following the early studies on neonatal immune responses described above, more advanced studies began to show that the individual animals, exposed as neonates to donor antigens, were not tolerant in the classical immunological sense. Experiments in the 1990's were able to show that newborn mice, when immunized with a peptide in incomplete Freund's adjuvant, and then challenged later, a typical method for the induction of tolerance, were impaired in their lymph node responses (Forsthuber et al. 1996). This impaired response was used as the hallmark of tolerance in early studies. What was found was that the lymphocytes of the neonate had an impaired ability to home to the lymph nodes, resulting in their accumulation in the spleen. Lymphocytes, mainly CD4 T cells found in the spleen had down-regulated the expression of L-selectin, thought to be needed for migration into lymph nodes (Forsthuber et al 1996). The CD4 T cells found in the spleens of neonatal mice were capable of responding

vigorously against the peptide they were challenged with. What was found was that priming with peptide enabled the splenic CD4 T cells to mount a response upon secondary challenge *in vitro*, as well as support antibody responses *in vivo*. The IgG antibody made by neonatal mice on secondary challenge was predominantly of the IgG1 isotype, characteristic of Th2-type responses (Forsthuber et al. 1996). This Th2-dominated immune response was confirmed by ELISPOT assay with primed splenic neonatal T cells and their production of IL-5, but not of IFN-γ. Separately, at the same time, Matzinger showed that neonatal female mice could mount a mature response against male H-Y antigens when challenged with donor dendritic cells (Ridge et al. 1996), and another group led by Sarzotti showed that CTL responses could be made in newborns challenged with virus at low doses, in contrast to the strong Th2-type response seen upon challenge with a normal dose (Sarzotti et al. 1996). Thus, it was determined that in studies in which neonates had been exposed to antigen, they were not tolerant as adults to antigen in the classical sense, which had been thought to be the case for years, but in fact were immunocompetent and this competence typically resulted in a strong Th2 bias of the immune response.

1.5.4 Factors Influencing the CD4, Th2 Bias of the Neonatal Immune Response

The recognition that neonatal rodents, upon exposure to antigen, can produce antibody to the antigen rather than become tolerant naturally led to an interest in why the production of antibody was favoured. The Th2 nature of immune responses made by neonates makes them particularly susceptible to certain types of infection, particularly those that require a cell-mediated response for effective containment. In the years after the studies that showed neonates could mount strong antibody responses, the focus of much research shifted toward why there was a predominant antibody response. Though many hypotheses exist in the literature, the underlying mechanism has still not been resolved.

1.5.4.1 Innate Immunity of Neonates

There is substantial literature on the innate immune system of newborns, and how it differs from that of adults. A large portion of work involving neonatal innate immunity is centered on toll-like receptor (TLR) function in newborn APCs. In newborn mice, the expression of TLRs is usually around the levels of adult mice (Kollmann et al. 2012; Kollmann

et al. 2009; Cuenca et al. 2013), but APCs of newborns have a poorer capacity to support the generation of Th1-type cells. Macrophages and dendritic cells of newborns, upon TLR stimulation that typically results in production of Th1 cytokines, produce significantly less IL-12p70, Type I IFNs, TNF-α, and IFN-γ, with increased production of the tolerogenic cytokine IL-10 (Kollmann et al. 2012; Kollmann et al. 2009). The lack of production of certain cytokines by newborn leukocytes has been linked to altered expression of transcription factors downstream of TLRs, such as interferon response factors, which have been shown to be reduced in neonates (Kollmann et al. 2012). The low production of these cytokines leads to altered activation of T helper cells correlating with susceptibility to certain pathogens encountered in early life. The role of innate immunity is not directly pertinent to my project, but the interaction between the innate and adaptive immune response is important and critical for the generation of effective responses. The role of this differential cytokine profile can be critical for the generation of particular T helper subsets, which will be discussed later.

1.5.4.2 Deficiencies in Neonatal Antigen-Presenting Cells

The previous subsection on innate immunity touched on neonatal APCs, stating that their expression levels of TLRs is typically similar to that of APCs from adults. However, it has been shown that APCs in newborns can differ from adult APCs in different ways. Studies have shown that delayed maturation of specific subsets of DCs in newborns leads to a Th2 bias of immune responses generated in neonates. DC subsets which are CD8-positive are not present at high levels at birth, but gradually increase, and this subset is important for significant production of IL-12 that can lead to Th1 responses by helper T cells (Zaghouani et al. 2009; Lee et al. 2008). The delayed maturation of this subset of DC could explain a delay in the development of cell-mediated responses.

In addition to the differing subtypes of dendritic cells present in newborns, the absolute number of APCs present in neonates is also substantially reduced (Verhasselt 2010). Studies of lung DCs in neonatal and adult mice showed that distinct populations of DCs and their capacity to provide proper co-stimulation play a role in directing the immune response. A recent study showed that CD103-positive DCs are present in the neonatal lung and that this type of DC predominated while CD11b DCs, which can direct Th1 type responses in adults, were severely

diminished in neonates (Ruckwardt et al. 2014). Additionally, CD103 DCs from adult lungs stimulated T cell responses differently than CD103 DCs found in neonatal lungs. Blocking of CD28 co-stimulation upon interaction of CD103 DCs with CD8 T cells in adults provided a similar level of co-stimulation to that provided by neonatal CD103 DCs, suggesting that impairment in co-stimulation, in addition to altered numbers of certain subsets of DCs, could be responsible for altered Th1 responses (Ruckwardt et al. 2014). Again, the role of APC deficiencies in neonatal immunity is not directly pertinent to my studies, but I feel it is appropriate to describe the findings as they provide alternative hypotheses for the Th2 bias of neonatal immune responses. The role of DCs, however, is important in the development of neonatal responses in an allogeneic system, where the presence of DCs in the donor inoculum is critical for direct recognition of MHC antigens by T cells as explained above. It was determined experimentally by Matzinger that DCs were important in stimulating neonatal T cells (Ridge et al. 1996). The basis for the experiments done by Matzinger was to show that the critical players in tolerance induction were APCs, as suggested by her "danger" model of T cell activation. According to this model, T cells encounter APCs which have been activated by some danger signal resulting in the up-regulation of co-stimulatory molecules required for T cell activation. This showed that donor APCs were critical for the activation of T cells in an allogeneic system, in which neonatal T cells could be activated (Ridge et al. 1996).

1.5.4.3 Antigen Dose

The first group to show that antigen dose could have a major impact on the immune response of newborns was Sarzotti's group in 1996. It was known that infection of neonatal mice with 100 PFU of murine leukemia virus led to severe disease, without the generation of protective CD8 T cells and IFN-γ, but in mice that were a few weeks old, the same viral dose elicited a strong Th1 protective response. They performed experiments showing that protective CTL responses in newborns are correlated inversely with viral dose (Sarzotti et al. 1996). Infection with 0.3 or 1 PFU of virus was capable of eliciting a protective response with strong CTL activity. These early studies on antigen dose in the neonate showed that, at least experimentally, neonates could produce a cell-mediated response. This has important implications for vaccination, and it was subsequently shown that various antigens, such as that produced by DNA immunization, protein antigens, and oligonucleotide sequences with CpG

motifs, when given at an appropriate dose could stimulate protective Th1 immunity (Adkins et al. 2004; Siegrist 2000; Adkins 2000). Despite being potentially important in vaccine development, the dose of antigen being used to produce protective Th1 responses may not be physiologically relevant when encountering most pathogens naturally. Thus it is still important to develop an understanding of why neonates are biased toward Th2 responses.

1.5.4.4 T Cell Immunity in Neonates

The important studies from the mid 90's discussed above were critical in showing that newborn lymphocytes were capable of mounting immune responses and that this generally resulted in Th2-type immunity. Investigations into the role of T cell immunity in neonates has taken different paths. The work by Sarzotti on antigen dose originated from work which showed that the total number of T cells in neonatal spleens was up to 2 logs lower than that found in adult mice (Sarzotti et al. 1996; Garcia et al. 2000). The T cells of the newborn were shown to be capable of mounting effective responses under appropriate conditions, thus were functionally mature, and also able to express a full Vβ repertoire in the first week of life (Garcia et al. 2000). Therefore, the neonatal Th2 bias, due to differences between adult and newborn T cells, is likely due to differences in numbers of T cells and not in their ability to respond to antigen.

Other studies by Zaghouani focused on the neonatal development of T cells. His group showed that Th1 and Th2 cells are initially present in similar numbers in neonatal spleens upon exposure to antigen, but that each type of cell expresses the IL-13Rα1 chain of the IL-13 receptor (Li et al. 2004). The secretion of IL-4 during the immune response causes apoptosis of potential Th1 cells expressing this receptor, resulting in a Th2 bias. Blocking of IL-4 or the IL-13Rα1 receptor restored Th1 immunity under the same conditions. Thus the kinetic development of lymphocytes could play some role in Th1/Th2 differentiation early in life. Additionally, it has been shown that *in vitro,* human neonatal CD4 T cells produce less IFN-γ than naïve adult CD4 T cells, and are methylated at CpG and non-CpG sites within the IFN-γ promoter, which limits IFN-γ production and Th1 responses (Marchant and Goldman 2005; White et al 2002).

Another experiment centered on the neonatal role of regulatory T cells (Tregs) during immune responses. Tregs are responsible for negatively regulating immune reponses and maintaining self-tolerance, and are identified by the surface molecule CD25 along with

expression of the transcription factor Foxp3 and the production of IL-10. The induction of Tregs is dependent upon IL-2 signalling through STAT5 and TGF-β production (Wang et al. 2010). A 2010 study was published which showed that under similar stimulatory conditions, a larger population of neonatal T cells develop into Tregs compared to adult populations of T cells (Wang et al. 2010). The Tregs produced in neonates were able to suppress the immune function of other T cells *in vitro,* and the generation of Tregs under these conditions declined until two weeks of age, when they fell to the levels seen in adult mice (Wang et al. 2010).

Neonatal T cells are capable of responding to antigenic challenge and can produce the correct type of immune response needed if activated under appropriate conditions. However, the relative number of neonatal lymphocytes of different types, the development of these neonatal lymphocytes, and their capacity to differentiate into separate subsets makes neonatal and adult lymphocyte populations different. It is certainly possible that some of these differences contribute to the Th2 bias of newborn individuals.

1.5.4.5 Cytokine Environment

The antigen dependent generation of distinct activated helper T cells differs in neonatal and adult mice. An important contributor to the type of immune cells generated by an antigen is the presence of cytokines during the primary response, as discussed in detail in section 1.2.2. The cytokine milieu during activation has been shown to affect the nature of the immune response made by neonatal as well as adult lymphocytes. A shift in the cytokines present during immune activation, or being produced by cells present during activation, can alter the response from a Th2 to a Th1 mode

. A series of studies in the 90's, using allogeneic spleen cells injected into neonatal mice, showed that the blocking of IL-4 with a monoclonal antibody prevents the acquisition of tolerance that is seen when the cells are injected in the absence of anti-IL-4 (Schurmans et al. 1990). The presence of anti-IL-4 antibody also resulted in the rejection of allogeneic skin grafts onto neonatal mice and allowed for the emergence of T cells secreting IL-2 and IFNγ leading to graft rejection in contrast to the predominant Th2 response seen in the absence of neutralizing anti-IL-4 antibody (Donckier et al. 1995; Gao et al. 1996). Another recent paper showed that immature erythroid cells present in the spleens of neonatal mice produce IL-6 that can facilitate

the generation of IL-4 producing T helper cells (Rincon et al. 2012). A depletion of these cells, and thus a loss of IL-6 production, diminishes the Th2 CD4 T cell response. Additionally, IL-6 knock-out mice have a reduced ability to produce splenic Th2 cells compared to normal mice (Rincon et al. 2012).

In addition to these studies on the role of IL-4 and IL-6 in affecting the generation of Th1 cells, cytokines made by Th1 cells can facilitate Th1 responses. In experiments done *in vitro,* neonatal T cells are capable of producing large amounts of IFNγ in the presence of exogenous IL-12 (Adkins 2000; Tregoning et al. 2013). The addition of exogenous IFNγ has also been shown to facilitate Th1 responses. As previously described, injection of allogeneic spleen cells into neonatal mice typically results in a humoral response against the donor spleen cells resulting in an SLE-like disease. The administration of IFNγ at the time of donor cell inoculation results in graft rejection by the host and a marked reduction in antibodies secreted by both host and donor B cells (Donckier et al. 1994; Chen et al. 1996). Additionally, in a respiratory syncytial virus model in neonates, the presence of NK cells as well as CD8 T cells is responsible for the attenuation of the antibody response against the virus, and this is mediated through IFNγ secretion by these cells. These experiments show that the presence of cytokines can have a dramatic impact on the type of response made, and that neonates as well as adult mice are susceptible to the effects of cytokines on immune modulation.

1.5.4.6 Other Hypotheses for the Th2 Nature of Neonatal Immune Responses

Besides the nature of their T cells and APCs, there are other alternative reasons as to why neonates have an inherent Th2 bias that have been studied. Recent studies investigating the presence of erythroid cells present in the spleens and gut of newborn mice have led to the hypothesis that the immunosuppressive function of these cells is to allow for the colonization of the gut soon after birth (Elahi et al. 2013; Bordon 2014). CD71 positive erythroid cells identified in these studies were capable of suppressing the function of adult lymphocytes upon adoptive transfer, and culture of the cells together with mature lymphocytes renders the mature lymphocytes incapable of producing a protective Th1 response against *Listeria* (Elahi et al. 2013). The authors suggest that the presence of these cells, in addition to other mechanisms, is

responsible for attenuating a strong inflammatory response during colonization of the intestine with commensal bacteria.

Maternal immune responses during pregnancy are modulated from inflammatory to regulatory (Prendergast et al. 2012).   This modulation of the mother's immune response allows the fetus to survive.   The maternal-fetal environment of the uterus is also thought to help establish tolerance of the fetus during development and a less inflammatory environment through the development of regulatory T cells (Prendergast et al. 2012). There are many hypotheses as to how the mother's immune response is regulated so as to not reject the fetus throughout pregnancy.   The maternal environment shapes the neonatal immune system, leading to unresponsiveness or immune deviation in the neonate after birth.

1.5.5 Implications for Vaccination

The Th2 bias of the neonatal immune response has important implications for the development of vaccines. The protection provided by vaccination is typically due to a robust antibody response upon administration of the vaccine resulting in antibodies that can neutralize certain pathogens like bacteria or their toxins (Siegrist 2001). However, in the case of several viral and other intracellular infections and cancer, the production of antibody is not sufficient to prevent viral replication and persistent infection and a cell-mediated response is needed. The need for secondary cell-mediated responses upon encounter with certain pathogens constitutes a barrier to the development of effective vaccines, as current vaccination protocols have been designed to result in enhanced antibody responses and whereby induction of immune reactions upon first vaccination primarily consists of humoral responses. Therefore the investigation into the mechanisms behind neonatal Th2 bias is critical for how to vaccinate and under what conditions a correct immune response can be elicited.

CHAPTER 2

2. HYPOTHESIS AND OBJECTIVES

As outlined in the introduction, there are many factors that can contribute to the Th1/Th2 phenotype of an immune response mounted upon challenge with antigen. As highlighted in section 1.1, the role that CD8 T cells have during the immune response can be diverse, and their presence is critical in the clearance of certain infections and to make sure that an appropriate immune response is made. The role of CD8 T cells in promoting a cell-mediated response is outlined in the sections above which discuss their role in response to allo-antigenic challenge as well as challenge with other antigens. The absence of CD8 T cells in *in vivo* transplantation models results in Th2 type responses and their presence is crucial for Th1 responses (Via et al. 1987). Also, as discussed in section 1.5, the ability to mount different kinds of immune responses changes throughout the development of the individual. As seen in chicks, challenge with MHC-disparate allogeneic cells at different stages of development results in differences in the allo-specific immune response made (Havele 1981). Tolerance toward the donor strain is seen in the early stages of development upon challenge with the MHC incompatible donor's cells, up to day 15 of development. Challenge on day 17 with donor strain cells does not result in tolerance, but an antibody response specific for the antigens of the donor. Challenge on day 21 results in a cell-mediated response made by the mature, developed immune system of the host. Similarly, outlined in section 1.5 is the inability of neonatal mice to mount strong cell-mediated responses to particular antigens under circumstances which result in cell-mediated responses in adult individuals.

Combining evidence from the experiments outlined above, showing that in the absence of CD8 T cells, Th2, antibody responses are generated, as well as evidence showing that newborn individuals generally have a bias toward similar Th2 type responses, we have formulated a hypothesis to account for these findings. We propose that the Th2 bias, seen in neonatal immune responses, is in part due to a lack of CD8 T cells present early in development resulting in a CD4:CD8 T cell ratio that is much higher than that seen in adult mice of the same strain. The previous evidence regarding the effect of the presence/absence of CD8 T cells during immune responses generated against MHC incompatible cells, as well as the Th2 bias of immune

responses made by newborn individuals, provided the basis for this hypotetical. The objectives of the project are fourfold, and will be briefly outline below.

2.1 Define the CD4:CD8 T cell Ratio in Adult and Neonatal C57BL/6 and BALB/c Mice

The first objective of my research project was to examine whether the ratio of CD4 to CD8 T cells in the spleens of adult mice, 6-12 weeks old, is significantly different from that of 8-10 day old neonatal mice. Since it is well known that neonatal mice respond to antigenic challenge with a predominant Th2 response, and that the presence of CD8 T cells can play a major role in influencing Th1 responses, mice could be relatively deficient in these cells early on in life. We wanted to examine whether or not there is an insufficient number of CD8 T cells in the spleens of neonatal mice in both BALB/c and C57BL/6 strains of mice to show a generalizability of the phenomenon across different strains. C57BL/6 mice are generally regarded as Th1 responding mice and BALB/c are regarded as Th2 responding mice. The use of these strains provides "extreme" examples of mice that make predominantly one type of immune response and are therefore appropriate to show that the phenomenon is generally applicable across different strains of mice.

2.2 Characterize the Antigen Specific Th1/Th2 Immune Response Made by Adult and Neonatal Mice of the Two Strains of Mice

Secondly, we wanted to examine whether there is a difference in the type of immune response made by adult and neonatal spleen cells of both strains and in particular to test the likelihood that while neonatal spleen cells preferentially produce a Th2 type response, adult spleen cells of the same strain produce a predominant Th1 response upon challenge with the same antigen. Upon *in vitro* challenge with MHC-incompatible allo-antigenic spleen cells, we set out to show that the type of response made by adult and neonatal mice differs dramatically, and that we can determine the type of response made by ELISPOT assay, which gives us the number of individual cytokine-producing cells present in the culture producing IFN-γ and IL-4.

2.3 Determine the Role of CD8 T Cells in Influencing the CD4, Th1/Th2 Nature of the Response

To show the role that CD8 T cells play in influencing the immune response of both adult and neonatal mice, we sought to show that the addition of age-matched CD8 T cells to neonatal cultures can alter the immune response made. We wanted to examine whether neonatal cultures that have been reconstituted with age-matched CD8 T cells isolated from pooled neonatal spleens to result in a lower CD4:CD8 T cell ratio, characterizing an adult splenic ratio, would produce a Th1 response similar to that of normal adult mice.

The figure illustrated below is our working model as to how CD8 T cells an affect the Th1/Th2 phenotype of activated CD4 T cells.
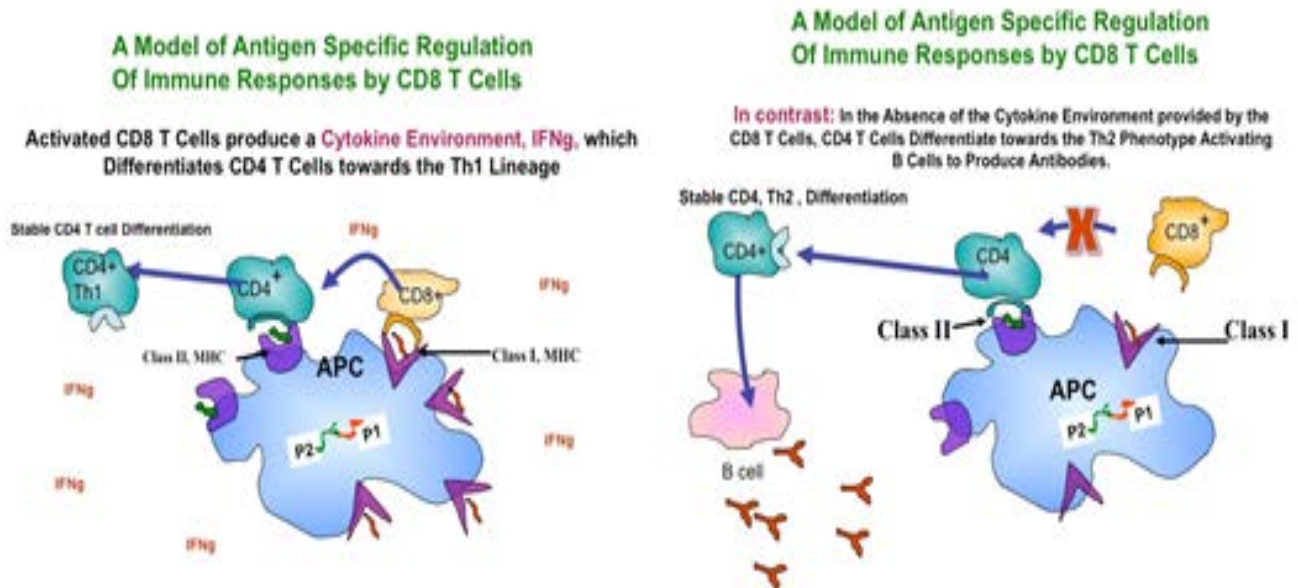
This model indicates that when an APC presents peptides to both CD4 and CD8 T cells coordinately via class I and class II MHC, this will result in the activation of both CD4 and CD8 T cells. The activation of both CD4 and CD8 T cells results in the production of IFN-γ by CD8 T cells, and the presence of this cytokine promotes the induction of T helper 1 type CD4 T cells. Conversely, if there are no CD8 T cells present at the initiation of the immune response, and the antigen presenting cells only encounter CD4 T cells without CD8 T cell "help", the CD4 cells become T helper 2 type cells leading to an antibody response.

2.4 Characterize the Phenotype of the Effector CD4 and CD8 T cells in Neonatal and Reconstituted Neonatal Cultures to Show the Influence of CD8 T cells on the activation of CD4 T Cells

Finally, we aim to examine whether the presence of CD8 T cells, that have been added to neonatal cultures to simulate an adult CD4:CD8 T cell ratio, have an effect on the CD4 T cells that are being activated and responding to the antigenic challenge. If there is a difference in the immune responses made between adult, neonatal, and reconstituted neonatal cultures, we wanted to know whether this difference is due to an influence of the CD8 T cells on the responding CD4 T cells resulting in different CD4 T helper phenotypes.

CHAPTER 3

Materials and Methods

3.1 MICE

BALB/c ($H-2^d$) and C57BL/6 ($H-2^b$) adult mice, at least six weeks of age, were obtained from the Jackson Laboratory (Bar Harbour, Maine, USA) and housed at the Health Sciences Laboratory and Animal Services Unit (LASU), University of Saskatchewan. F1 mice ($H-2^{b/d}$, BALB/c x C57BL/6) and neonatal BALB/c and C57BL/6 mice were bred and housed in the Laboratory and Animal Services Unit, University of Saskatchewan. Adult and F1 mice used were at least six weeks of age. Neonatal mice were between eight and ten days old at the beginning of each experiment. All mice were housed in specific pathogen free conditions and all experiments were conducted under a protocol approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

3.2 MEDIA AND SOLUTIONS

3.2.1 RPMI Complete Medium

RPMI 1640 medium (GIBCO Laboratories) was prepared from powder according the manufacturer's instructions. The medium was filtered through a 0.2µm filter into bottles and incubated overnight at 37° to check for sterility. Complete RPMI was obtained by additon of 10% heat-inactivated fetal bovine serum (FBS; Hyclone), 0.1% 0.5M β-mercaptoethanol, 0.8% 100mM sodium pyruvate, 100U/mL penicillin, and 100µg/mL streptomycin (GIBCO).

3.2.2 FACS Buffer

Buffer used for incubation and running of cells during flow-cytometry consisted of phosphate buffered saline (PBS) with 2% fetal bovine serum (FBS).

3.2.3 MACS Buffer

Buffer used for Magnetic Activated Cell Sorting isolation consisted of PBS with 2% FBS.

3.2.4 ELISPOT Coating Buffer

Enzyme Linked Immunosorbent Spot coating buffer was a carbonate/bicarbonate buffer used for coating 96-well, nitrocellulose bottomed plates. Carbonate/bicarbonate buffer was made by adding 16 mL 0.2 M Na_2CO_3 and 34 mL 0.2 M $NaHCO_3$ to 150 mL ddH_2O.

3.2.5 ELISPOT Substrate Buffer

ELISPOT substrate buffer consisted of a pH 9.5 solution of 0.1 M Tris-HCl, 0.1M NaCl, and 0.05 M $MgCl_2$.

3.2.6 PBS-Tween 20 (PBST)

PBST was prepared by adding 0.05% v/v Tween 20 (SIGMA) to PBS.

3.2.7 ELISPOT Plate Preparation

On day 4 of the experiment, ELISPOT plates (96-well, nitrocellulose-bottomed; Millipore) were coated overnight at 4°C with either anti-IL4 (2μL/mL of 0.5 mg/mL) or anti-IFNγ (1μL/mL of 1 mg/mL) antibody (BD Pharmingen) in carbonate/bicarbonate buffer.

3.2.8 Cell Culture Preparation

On day 5, cell cultures were harvested and centrifuged for 7 minutes and resuspended in 2 mL of RPMI complete media. Cells were counted and dilutions of the culture were prepared. For adult cultures, 0.1%, 0.05%, and 0.025% dilutions of the cultures were prepared for ELISPOT plating. For neonatal cultures, 1%, 0.5%, and 0.25% dilutions of the cultures were prepared for plating. 100 μL of the dilutions were plated in duplicate along with 10^6 F1 spleen cells as antigen per well along with a negative control containing no antigen. The plates were incubated at 37°C with 5% CO_2 overnight.

3.2.9 ELISPOT Plate Development

Following incubation, the plates were emptied and washed twice with ddH_2O, followed by four washes with PBST. Biotinylated anti-IFN-γ and IL-4 (2μL in 1 mL PBS; BD Pharmingen) were added to the appropriate wells and the plates were incubated at 37°C for 2 hours. The plates were then washed five times with PBST and twice with ddH_2O to remove excess secondary antibody. Streptavidin conjugated alkaline phosphatase (CedarLane) in PBS was added to the wells (0.2μg/mL; 100μL per well) and the plates were incubated for 1 hour at 37°C. Plates were washed extensively with ddH_2O. 200 μL NBT/BCIP substrate (Roche) per 10 mL subsrate buffer was prepared and 100 μL was added to each well. The plates were incubated at room temperature in the dark for ten minutes or until spots develop. The development is stopped by washing twice with ddH_2O. The plates were left to dry overnight.

The number of antigen dependent cytokine producing cells per well were counted manually using a dissecting microscope. Antigen dependent cytokine producing cells were enumerated by counting spots in antigen positive wells and substracting the number of spots in antigen negative wells. The number of cytokine producing cells per culture was obtained by multiplying the number of spots per well by the dilution factor used for plating the cells.

3.3 PREPARATION OF SPLEEN SINGLE CELL SUSPENSIONS

Mice were euthanized by cervical dislocation (adult) or by asphyxiation via CO_2 (neonates), and their spleens removed aseptically and placed in RPMI complete media. The spleens were then cut into small pieces using sterile scissors, and gently pushed through a sterile wire mesh using the rubber end of a sterile syringe. The cells were transferred into a 15 mL tube to allow for cellular debris to settle and to remove clumps. The cells present in the supernatant were transferred to a fresh 15 mL tube and centrifuged at 500 x g for 7 minutes. The cell pellet was resuspended in 5 mL of fresh RPMI complete media for adult spleens and 2 mL fresh RPMI complete media for neonatal spleens. The number of leukocytes present in the cell suspensison was determined by manual counting after a 1:4 dilution of cells in trypan blue via a hemocytometer under light microscopy. The cells were adjusted to be at 10^7 leukocytes per mL.

3.4 GAMMA IRRADIATION

F1 antigenic spleen cells were rendered incapable of replicating and then used as stimulators in one-way mixed lymphocyte reactions by gamma irradiation. Single spleen cell suspensions from F1 mice (BALB/c x C57BL/6) were irradiated with 800-1200 Rads of gamma irradiation.

3.5 ISOLATION AND DEPLETION OF CELL TYPES
3.5.1 Nylon Wool Separation of T Cells

The isolation of neonatal CD8 T cells for reconstitution employed nylon wool columns, used for the separation of T cells from the spleen cell suspension. These columns were prepared the day before the experiment. Nylon wool columns (Polysciences, Inc.), were washed with warm RPMI complete media and then the columns were filled with warm RPMI complete media to allow for soaking of the nylon wool. The columns were incubated overnight at 37°C. Pooled

neonatal spleen single cell suspensions were prepared as above and a fraction of the cells were placed into a fresh 15 mL tube. Each suspension of neonatal spleen cells was fractionated with a separate column. The neonatal single cell suspensions are centrifuged for 7 minutes to pellet the cells and the cells were resuspended in 2 mL of warm RPMI complete media. The nylon wool columns were washed once with warm RPMI complete media, and the neonatal cells were added to the columns. 7 mL of warm RPMI complete media was added to the columns to allow for the cells to enter the wool. The columns were incubated at 37°C for 1 hour. After incubation, the columns were washed twice with 5 mL of warm RPMI complete media. The cells were diluted 1:2 in trypan blue and counted via hemocytometer.

3.5.2 MACS

After nylon wool separation, the cells were centrifuged and resuspended in MACS buffer (90 μL per 10^7 cells). CD8 T cells were isolated from the cell suspension via CD4 negative selection using CD4 (L3T4) MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. The number of CD8 T cells isolated were counted via hemocytometer and the purity of the isolation was assessed via flow-cytometry by determining the absolute number of neonatal CD8 T cells present. The CD8 T cells were centrifuged and resuspended in 1 mL of RPMI complete media for addition to cultures.

3.5.3 Negative Selection of CD4 and CD8 T Cells

Negative selection of CD4 and CD8 T cells at day 5 was used in experiments examining the phenotype of activated CD4 T cells. At Day 5, the cultures were harvested, spun, and counted using a hemocytometer. CD4 T cells were isolated from the culture via negative selection using CD8 (Ly-2) Microbeads (Miltenyi Biotec) according to the manufacturer's instructions. CD8 T cells were obtained via CD4 negative selection using CD4 (L3T4) MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. The percentage of CD4 and CD8 T cells present after negative selection was determined by flowcytometry. The cultures were spun and reconstituted in 2 mL of RPMI Complete Media for preparation for plating in the ELISPOT assay.

3.6 *IN VITRO* CELL CULTURE

Cells were resuspended to achieve 10^7 cells per mL. Primary cultures of adult spleen cells contain 10^7 responder cells (BALB/c or C57BL/6) with 10^7 irradiated F1 spleen cells as antigenic stimulators plus 5 mL of RPMI complete media per well of a 6-well plate (9.5 cm^2 growth area).

For neonatal cultures, 10^7 responder cells (BALB/c or C57BL/6) were plated with 5 x 10^6 irradiated F1 spleen cells as antigenic stimulators plus 1.5 mL of RPMI complete media per well of a 12 well plate (3.8 cm^2 growth area).

The *in vitro* cultures were incubated at 37°C with 5% CO_2, for 5 days, while checking periodically on the growth of the cells.

For reconstitution of neonatal cultures with isolated CD8 T cells, the cells were plated as described above in 12-well plates, followed by the addition of the isolated CD8 T cells resulting in a CD4:CD8 T cell ratio resembling that of adult spleen cell suspensions.

3.7 FLOWCYTOMETRY

For the determination of CD4:CD8 T cell ratios at day 0, cells were suspended at 10^7 cells per mL. 10^6 cells were diluted 1:1 in FACS buffer and incubated with either 0.5 μg PE-conjugated anti-CD4 antibody or 0.1 μg FITC-conjugated anti-CD8 antibody (CedarLane) in 1.5 mL centrifuge tubes. The cells were incubated at 4°C for 30 minutes. The cells were washed twice with FACS buffer and spun at 850g for two minutes between washes. Cells were resuspended in 0.5 mL FACS buffer for analysis. The cells were run on BD Accuri C6 cytometer or BD EPIC XL Cytometer, and results analyzed using BD Accuri C6 software or Flojo software (Tree Star).

3.8 STATISTICAL ANALYSIS

All graphs and statistics were done using Prism Software 5.0 (GraphPad). Statistical significance was assessed by student's *t* test or by one-way ANOVA as applicable.

CHAPTER 4

Results

This chapter is divided into five sections, four of which correspond to a particular objective outlined above involving a particular type of experiment. The first two sections will report observations showing that the ratio of CD4 to CD8 T cells in adult and neonatal mice differs significantly, in both C57BL/6 and BALB/c strains of mice. The third reports observations on the distinct immune responses made by adult and neonatal mice of the same strain, both in terms of the total number of cells producing IFN-γ and IL-4 in culture, and the ratio of IFN-γ: IL-4 producing cells. The fourth reports on the responses generated by neonatal cultures, reconstituted to decrease the CD4:CD8 T cell ratio with pooled, isolated neonatal CD8 T cells, and these responses are compared with non-manipulated neonatal cultures as well as adult cultures. The fifth section presents data characterizing the phenotypes of the antigen specific cytokine producing CD4 T cells, so that we can infer the effect of the presence of CD8 T cells on the Th1/Th2 phenotype of the CD4 T cells generated in neonatal and reconstituted neonatal cultures.

4.1 CD4:CD8 Ratio of Adult and Neonatal C57BL/6 and BALB/c Mice

First we wanted to determine the CD4:CD8 T cell ratios found in the spleens of adult and neonatal mice to determine whether there is a significant difference between these two ratios, and that neonatal mice typically have a relatively reduced number of CD8 T cells. Spleens from naïve mice were removed aseptically after euthanization, and one million spleen cells were run through a flow-cytometer to determine the number of CD4 and CD8 T cells present.

As can been seen in Figures 1 and 2, adult C57BL/6 mice, greater than six weeks of age, have a splenic CD4 to CD8 T cell ratio close to 2:1. C57BL/6 mice, as mentioned in the introduction, genetically lack the I-E chain of the class II MHC, and thus have a lower number of CD4 T cells compared to other strains with fully intact MHC class II due to a lack of positively selected T cells during development. This lower number of total CD4 T cells present results in a CD4:CD8 T cell ratio that is lower on average than adult mice of different strains. This is typical of C57BL/6 mice, which are used in viral models to generate cell-mediated immune responses, and due to their ability to resist certain infections requiring a strong Th1 immune response. Despite the relative lower numbers of CD4 T cells generally seen in C57BL/6 mice, neonates

have very few CD8 T cells and thus a much higher ratio of CD4 to CD8 T cells, as depicted in Figures 3 and 4. When these ratios in adult and neonatal mice are compared in Figure 5, a significant difference is seen, with neonatal mice having a splenic ratio almost three-fold higher on average.

The same phenomenon was seen in BALB/c mice, shown in Figures 6 and 7, even though BALB/c mice have a fully intact repertoire of CD4 T cells. The average CD4:CD8 T cell ratio of BALB/c mice was slightly higher than the ratio of C57BL/6 mice, but a similar trend was seen when ratios of adult mice are compared to neonatal mice of the same strain, seen in Figures 8 through 10. The difference between the ratios of adult and neonatal mice seems to be consistent across different strains of mice, with the important point being that the lack of CD8 T cells in the spleens of newborn mice results in much higher ratio than that seen in adult mice of the same strain.

We wanted to determine how the CD4:CD8 T cell ratio at day 0 of culture would change after five days in culture in the presence of F1 stimulator cells. As naïve responder CD4 and CD8 T cells interact with APC of the F1 mouse, these cells will be activated to become effector and memory T cells. We sought to observe the change in the percentage of CD4 and CD8 T cells in the lymphocyte population to map how these populations expand in the presence of allogeneic cells. As seen in Figure 11, there is a large expansion of CD8 T cells in adult C57BL/6 mice with a lower number of CD4 cells than seen at day 0. In C57BL/6 neonatal mice, there are an approximately even number of CD4 and CD8 T cells. The comparison of day 0 and day 5 CD4 and CD8 T cell populations in Figure 13 shows a marked contrast in adult and neonatal C57BL/6 mice and this is reflected in the type of immune response produced by the cells upon re-stimulation with allogeneic cells, as seen later. In BALB/c mice, shown in Figure 14, there is an expansion of both CD4 and CD8 T cells, with approximately similar numbers of each in the lymphocyte population. Similar to C57BL/6 neonatal cultures, BALB/c neonatal cultures, shown in Figure 15, show an expansion of both CD4 and CD8 T cells to nearly even numbers. A comparison of the CD4 and CD8 T cells present at day 0 and day 5 of culture in BALB/c mice in Figure 16 shows that there is a relatively larger expansion of CD8 T cells in the adult and again this will be reflected in the type of immune responses made by these mice reported later.

The following figures are representative of multiple experiments. The flow-cytometry plots have been taken from a single experiment, and scatter plots represent the means of data taken from a single experiment representative of multiple experiments.
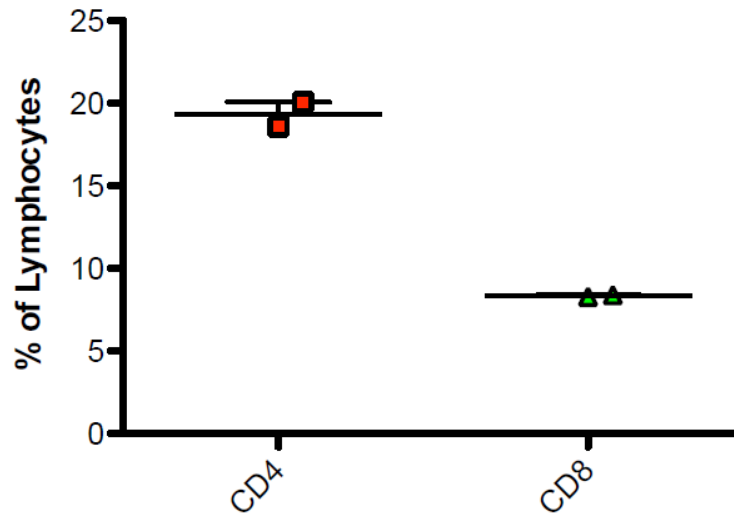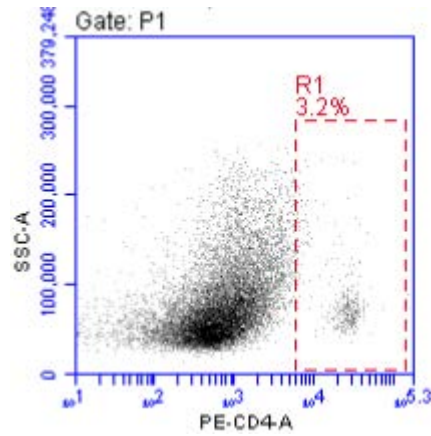
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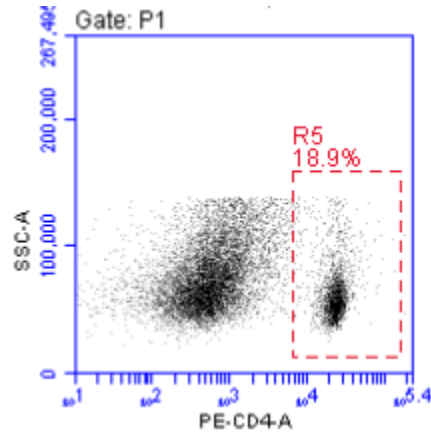
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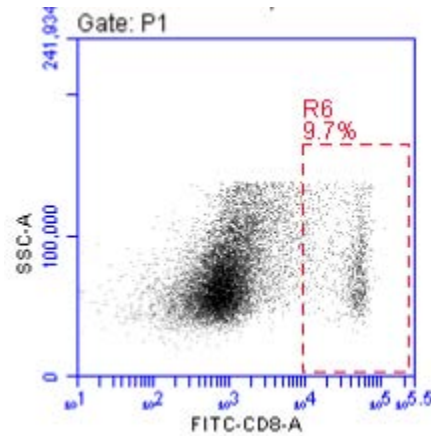
Figure 1: CD4 and CD8 T cell Percentages of Adult C57bL/6 Mice. Spleens of adult C57BL/6 mice (≥6 weeks old) were gated on lymphocytes and the percentages of A) CD4 and B) CD8 T cells were assessed via flow-cytometry. PE-anti-CD4 and FITC-anti-CD8 antibodies were used for detection of CD4 and CD8 T cells. Data are representative of ten independent experiments (n=2*). *Two adult mice per experiment, with greater than 20 adult mice tested over multiple experiments.*
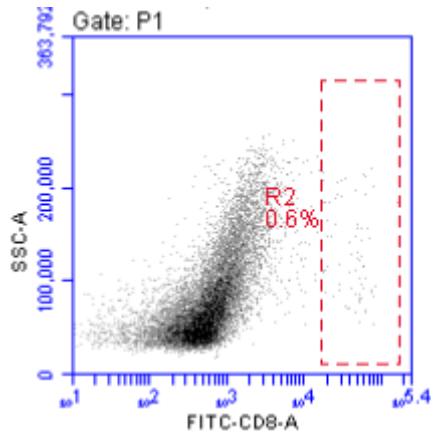
Figure 2: CD4:CD8 T cell Percentages of Adult C57BL/6 Mice. Spleens of adult C57BL/6 mice (≥6 weeks old) were gated on lymphocytes and the percentages of CD4 and CD8 T cells were assessed via flow-cytometry. The percentages of lymphocytes that are CD4 and CD8 in the spleen, respectively, are presented. Data are representative of ten independent experiments (n=2*). Data are mean + SEM. *Two adult mice per experiment, with greater than 20 adult mice tested over multiple experiments*
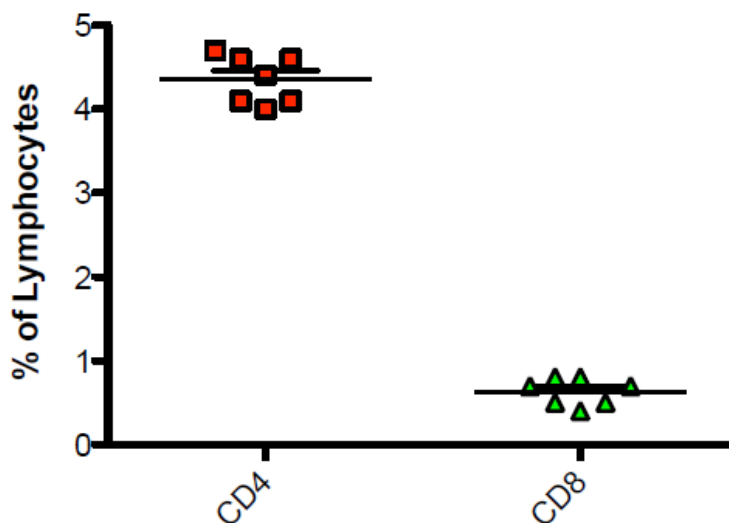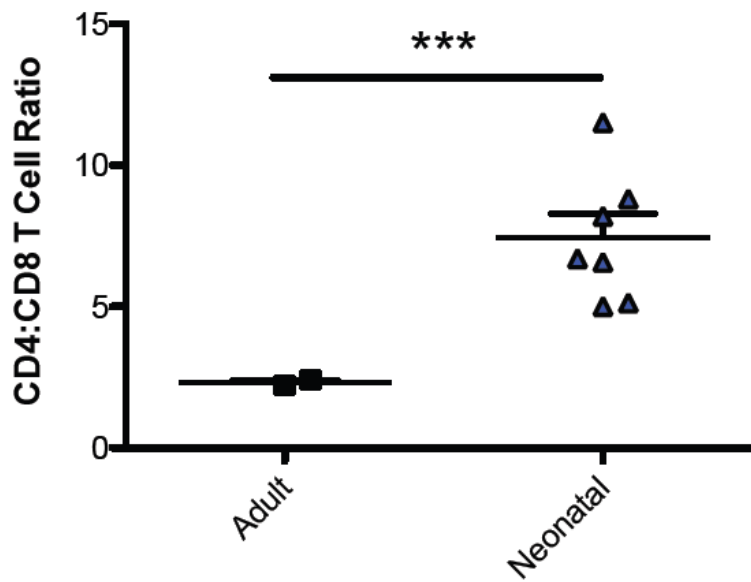
A.

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Figure 3: CD4 and CD8 T cell Percentages of Neonatal C57BL/6 Mice. Spleens of neonatal C57BL/6 mice (8-10 days old) were gated on lymphocytes and the percentages of A) CD4 and B) CD8 T cells were assessed via flow-cytometry. PE-anti-CD4 and FITC-anti-CD8 antibodies were used for detection of CD4 and CD8 T cells. Data are representative of seven independent experiments (n=7*). *At least four neonatal mice per experiment, with greater than 50 neonatal mice tested over multiple experiments.*
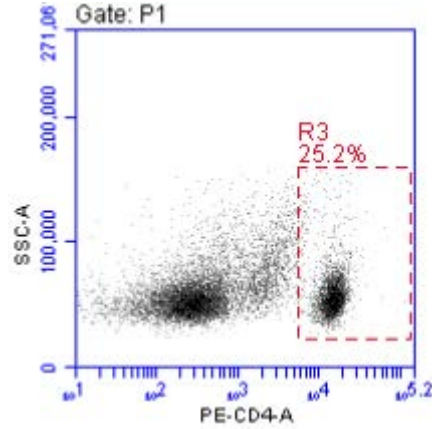
Figure 4: CD4:CD8 T cell Percentages of Neonatal C57BL/6 Mice. Spleens of neonatal C57BL/6 mice (8-10 days old) were gated on lymphocytes and the percentages of CD4 and CD8 T cells were assessed via flow-cytometry. The percentages of lymphocytes that are CD4 and CD8 in the spleen, respectively, are presented. Data are representative of seven independent experiments (n=7*). *At least four neonatal mice per experiment, with greater than 50 neonatal mice tested over multiple experiments*. Data are mean + SEM.
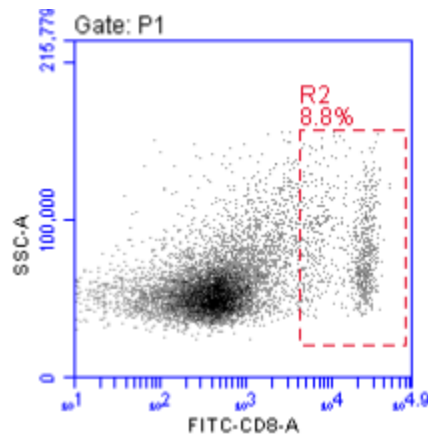
Figure 5: Comparison of Neonatal and Adult C57BL/6 CD4:CD8 T cell Ratio. The average CD4:CD8 T cell ratio found in adult and neonatal C57BL/6 mice were compared. Data are representative of ten independent experiments. Data are mean + SEM. Statistical significance assessed using students' *t* test. *** = p<0.001. Adults: n=2, neonates: n=7.
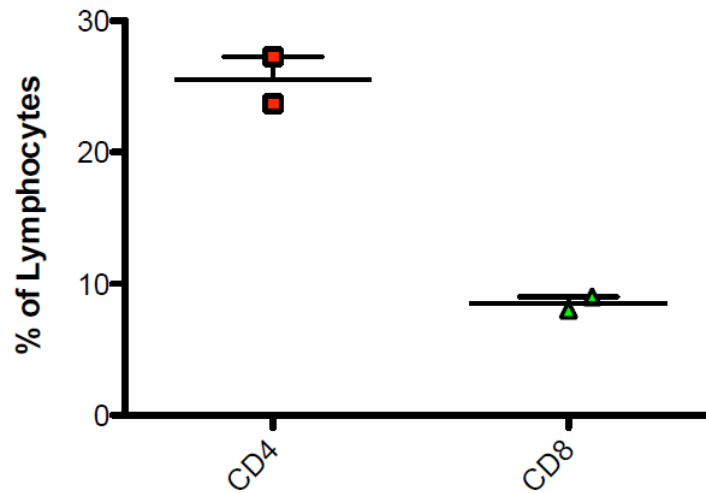
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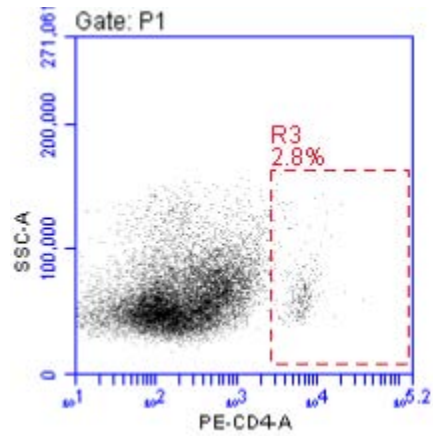**Figure 6: CD4 and CD8 T cell Percentages of Adult BALB/c Mice.** Spleens of adult BALB/c mice (≥6 weeks old) were gated on lymphocytes and the percentages of A) CD4 and B) CD8 T cells were assessed via flow-cytometry. PE-anti-CD4 and FITC-anti-CD8 antibodies were used for detection of CD4 and CD8 T cells. Data are representative of five independent experiments (n=2*). *Two adult mice per experiment, with greater than 10 adult mice tested over multiple experiments.*

Figure 7: CD4:CD8 T cell Percentages of Adult BALB/c Mice. Spleens of adult BALB/c mice (≥6 weeks old) were gated on lymphocytes and the percentages of CD4 and CD8 T cells were assessed via flow-cytometry. The percentages of lymphocytes that are CD4 and CD8 in the spleen, respectively, are presented. Data are representative of five independent experiments (n=2*). *Two adult mice per experiment, with greater than 10 adult mice tested over multiple experiments.*). Data are mean + SEM.
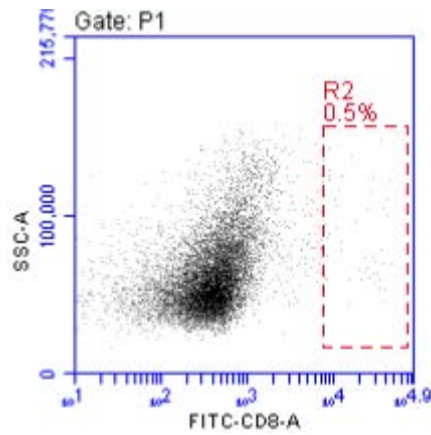
A.

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Figure 8: CD4 and CD8 T cell Percentages of Neonatal BALB/c Mice. Spleens of neonatal BALB/c mice (8-10 days old) were gated on lymphocytes and the percentages of A) CD4 and B) CD8 T cells were assessed via flow-cytometry. PE-anti-CD4 and FITC-anti-CD8 antibodies were used for detection of CD4 and CD8 T cells. Data are representative of at least five independent experiments (n=4*). *At least four neonatal mice per experiment, with greater than 20 neonatal mice tested over multiple experiments.*
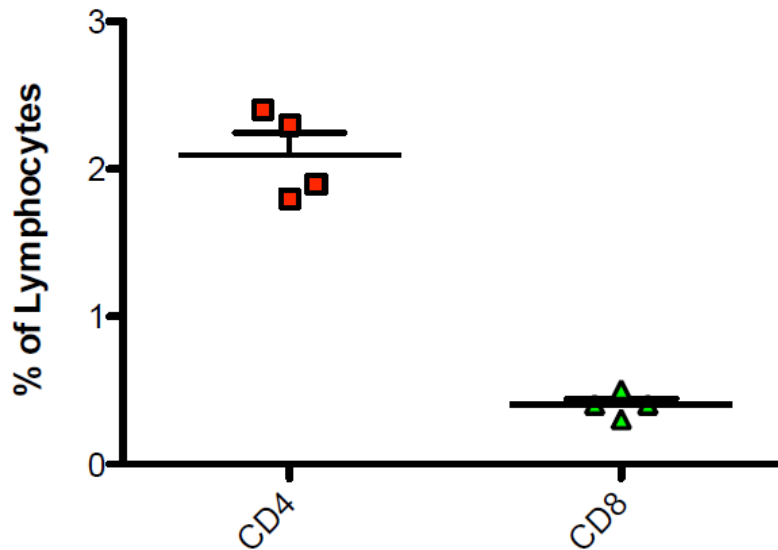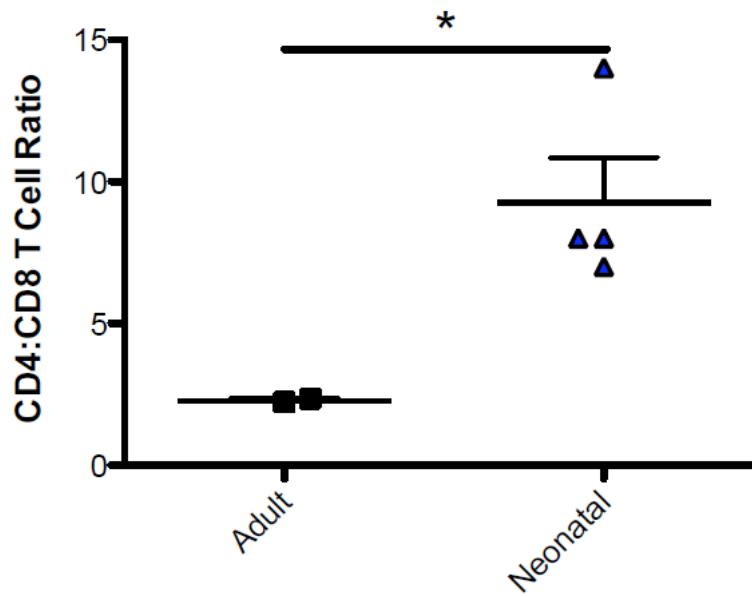
Figure 9: CD4:CD8 T cell Percentages of Neonatal BALB/c Mice. Spleens of neonatal BALB/c mice (8-10 days old) were gated on lymphocytes and the percentages of CD4 and CD8 T cells were assessed via flow-cytometry. The percentages of lymphocytes that are CD4 and CD8 in the spleen, respectively, are presented. Data are representative of at least five independent experiments (n=4*). *At least four neonatal mice per experiment, with greater than 20 neonatal mice tested over multiple experiments.* Data are mean + SEM.

Figure 10: Comparison of Neonatal and Adult BALB/c CD4:CD8 T cell Ratio. The average CD4:CD8 T cell ratios of adult and neonatal BALB/c mice were compared. Data are representative of five independent experiments. Data are mean + SEM. Statistical significance assessed using students' *t* test. * = p<0.05. Adults: n=2, neonates: n=4.

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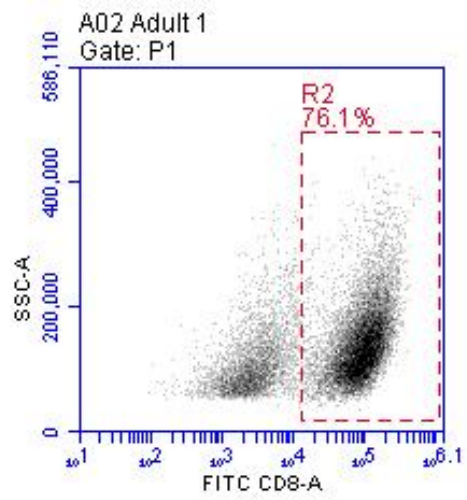
C.

Figure 11: CD4 and CD8 T cell Percentages in Day 5 Cultures of Adult C57BL/6 Spleen Cells Stimulated with Irradiated F1 Cells. Day 5 Cultures of adult C57BL/6 mice spleen cells were gated on lymphocytes and the percentages of A) CD4 and B) CD8 T cells were assessed via flow-cytometry. C) The percentages of lymphocytes that are CD4 and CD8 in the culture, respectively, are presented. PE-anti-CD4 and FITC-anti-CD8 antibodies were used for detection of CD4 and CD8 T cells. Data are mean + SEM. Data are representative of ten independent experiments (n=2*). *Two adult mice per experiment, with greater than 20 adult mice tested over multiple experiments.*
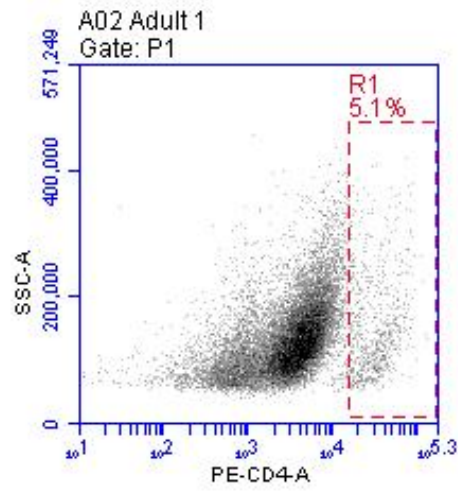
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Figure 12: CD4 and CD8 T cell Percentages in Day 5 Cultures of Neonatal C57BL/6 Spleen Cells Stimulated with Irradiated F1 Cells. Day 5 cultures of neonatal C57BL/6 spleen cells were gated on lymphocytes and the percentages of A) CD4 and B) CD8 T cells were assessed via flow-cytometry. C) The percentages of lymphocytes that are CD4 and CD8 in the culture, respectively, are presented. PE-anti-CD4 and FITC-anti-CD8 antibodies were used for detection of CD4 and CD8 T cells. Data are mean + SEM. Data are representative of ten independent experiments (n=7*). *Seven neonatal mice per experiment, with greater than 50 neonatal mice tested over multiple experiments.*

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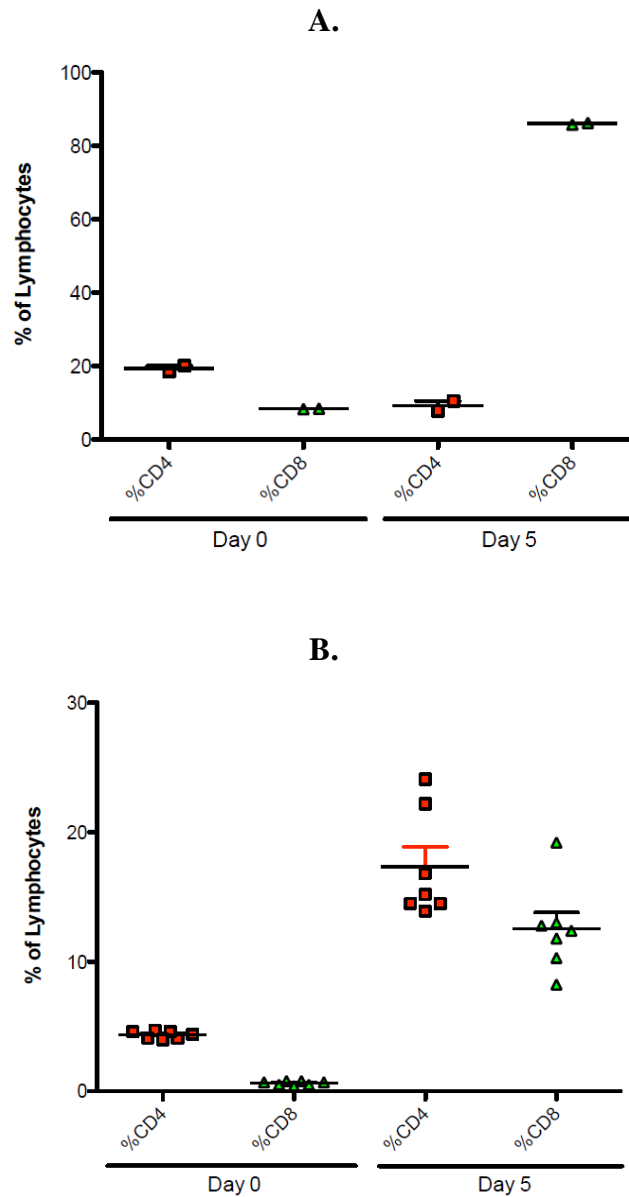
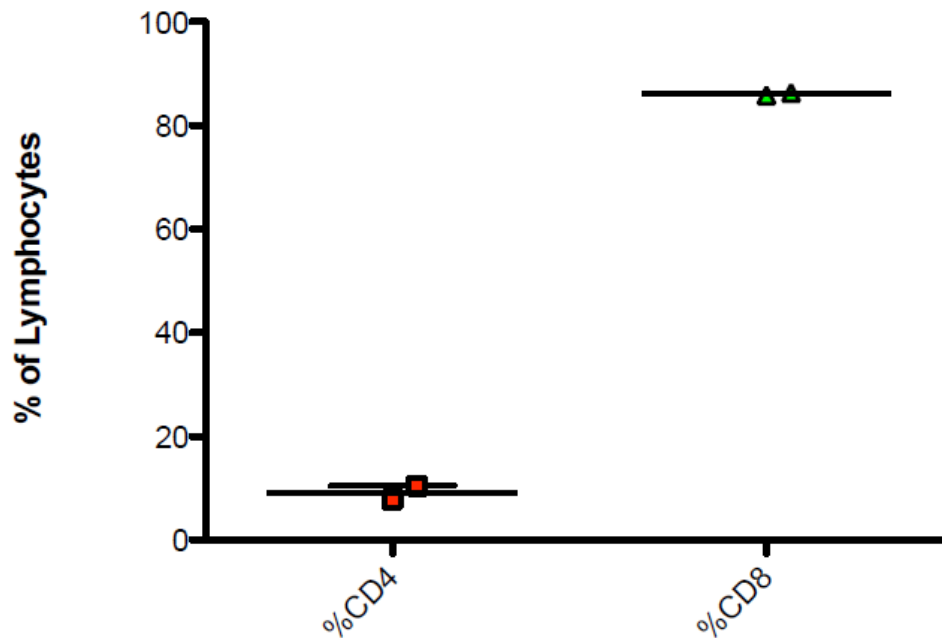**Figure 13: CD4 and CD8 T cell Percentages of Adult and Neonatal C57BL/6 Mice at Day 0 and Day 5.** Day 0 spleen cells and day 5 cultures of adult and neonatal C57BL/6 spleen cells were gated on lymphocytes and the percentages of CD4 and CD8 T cells were assessed via flow-cytometry. The percentages of lymphocytes that are CD4 and CD8 at day 0 and day 5 in A) adult cultures and B) neonatal cultures, respectively, are represented. PE-anti-CD4 and FITC-anti-CD8 antibodies were used for detection of CD4 and CD8 T cells. Data are mean + SEM. Data are representative of ten independent experiments. Adults: n=2, neonates: n=7.
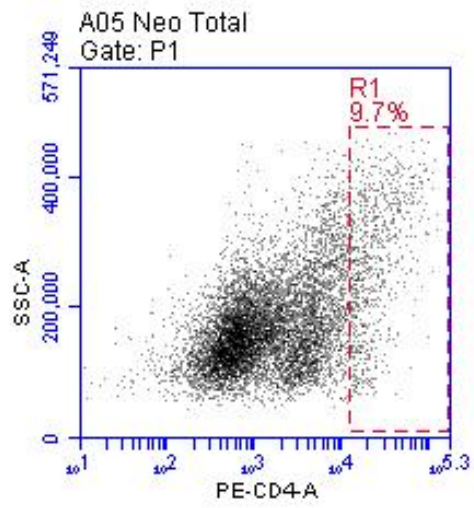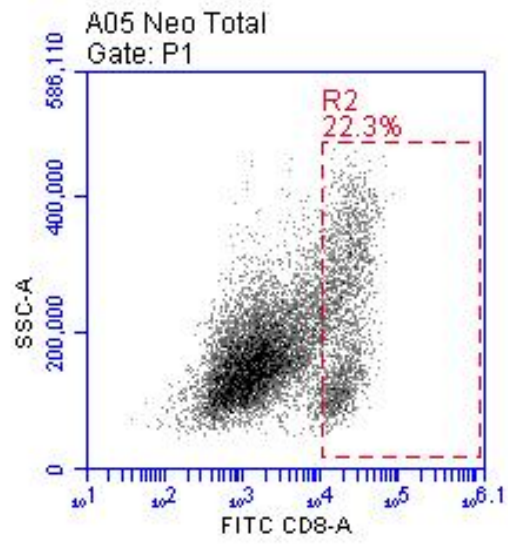
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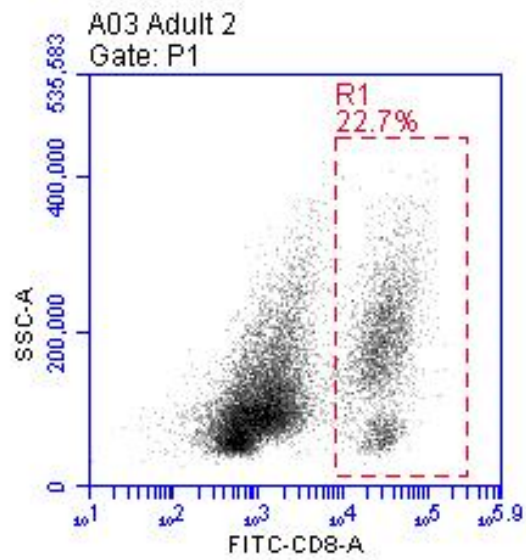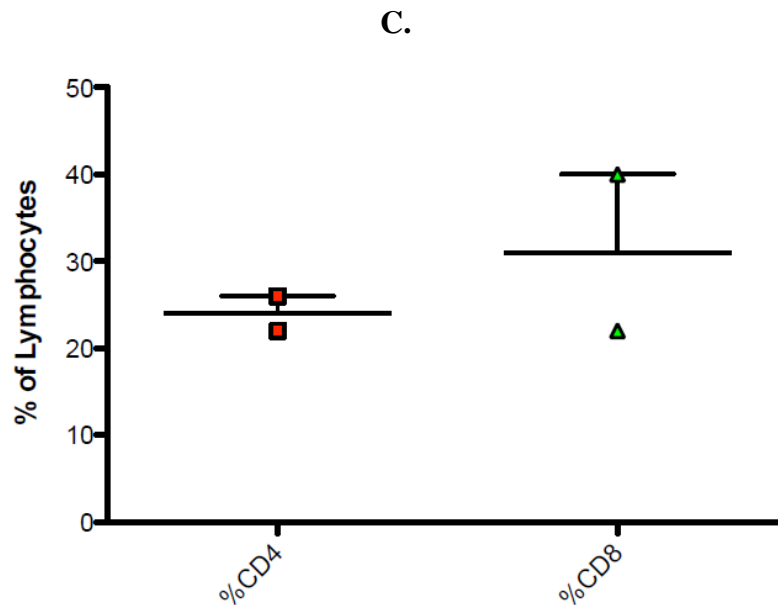
Figure 14: CD4 and CD8 T cell Percentages in Day 5 Cultures of Adult BALB/c Spleen Cells Stimulated with Irradiated F1 Cells. Day 5 Cultures of adult BALB/c mice spleen cells were gated on lymphocytes and the percentages of A) CD4 and B) CD8 T cells were assessed via flow-cytometry. C) The percentages of lymphocytes that are CD4 and CD8 in the culture, respectively, are presented. PE-anti-CD4 and FITC-anti-CD8 antibodies were used for detection of CD4 and CD8 T cells. Data are mean + SEM. Data are representative of five independent experiments (n=2*). *Two adult mice per experiment, with greater than 10 adult mice tested over multiple experiments.*
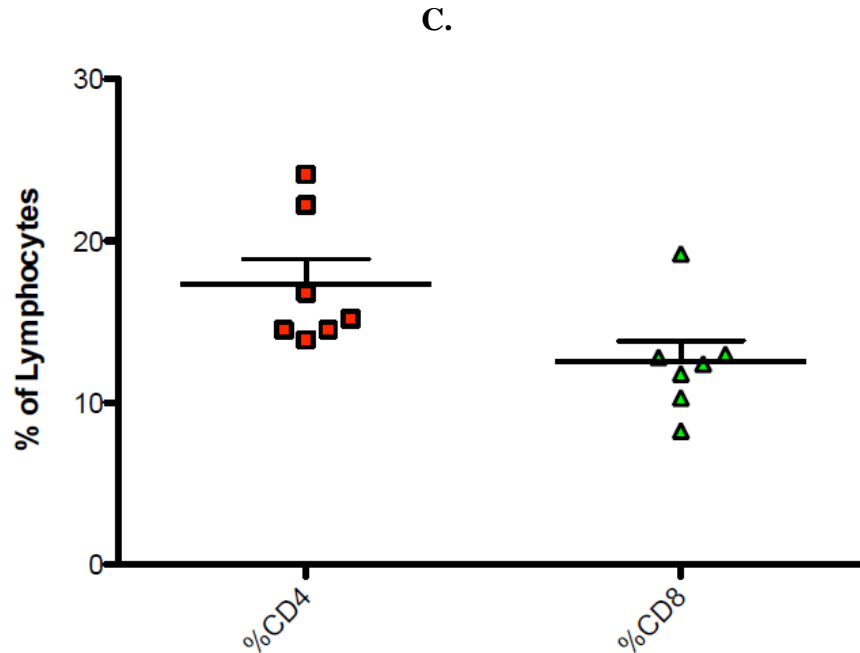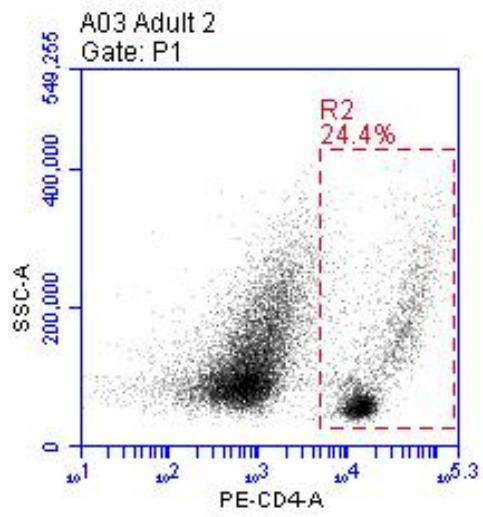
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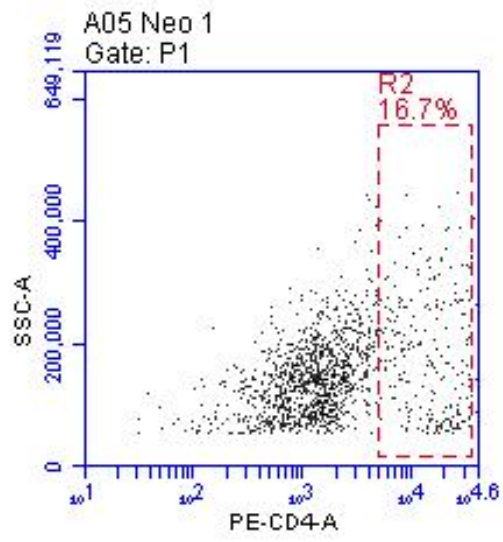
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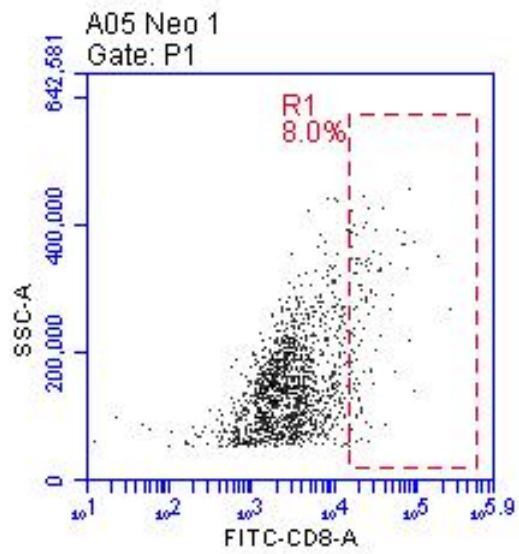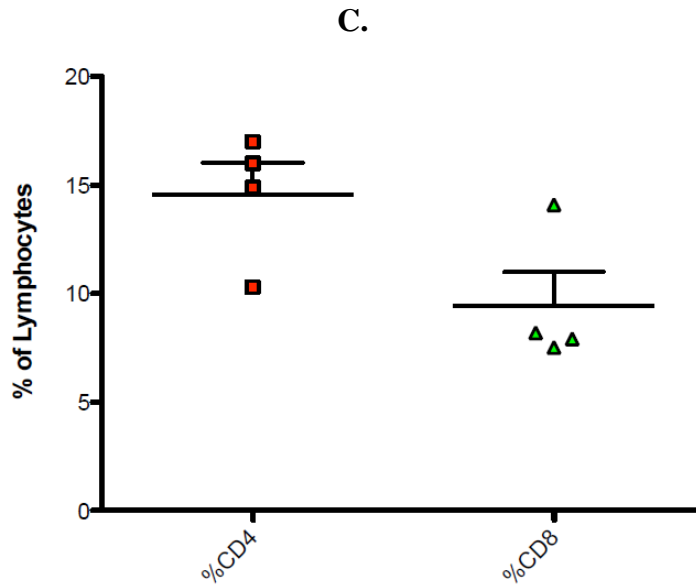
Figure 15: CD4 and CD8 T cell Percentages in Day 5 Cultures of Neonatal BALB/c Spleen Cells Stimulated with Irradiated F1 Cells. Day 5 cultures of neonatal BALB/c spleen cells were gated on lymphocytes and the percentages of A) CD4 and B) CD8 T cells were assessed via flow-cytometry. C) The percentages of lymphocytes that are CD4 and CD8 in the culture, respectively, are presented. PE-anti-CD4 and FITC-anti-CD8 antibodies were used for detection of CD4 and CD8 T cells. Data are mean + SEM. Data are representative of five independent experiments (n=4*). *Four neonatal mice per experiment, with greater than 20 neonatal mice tested over multiple experiments.*

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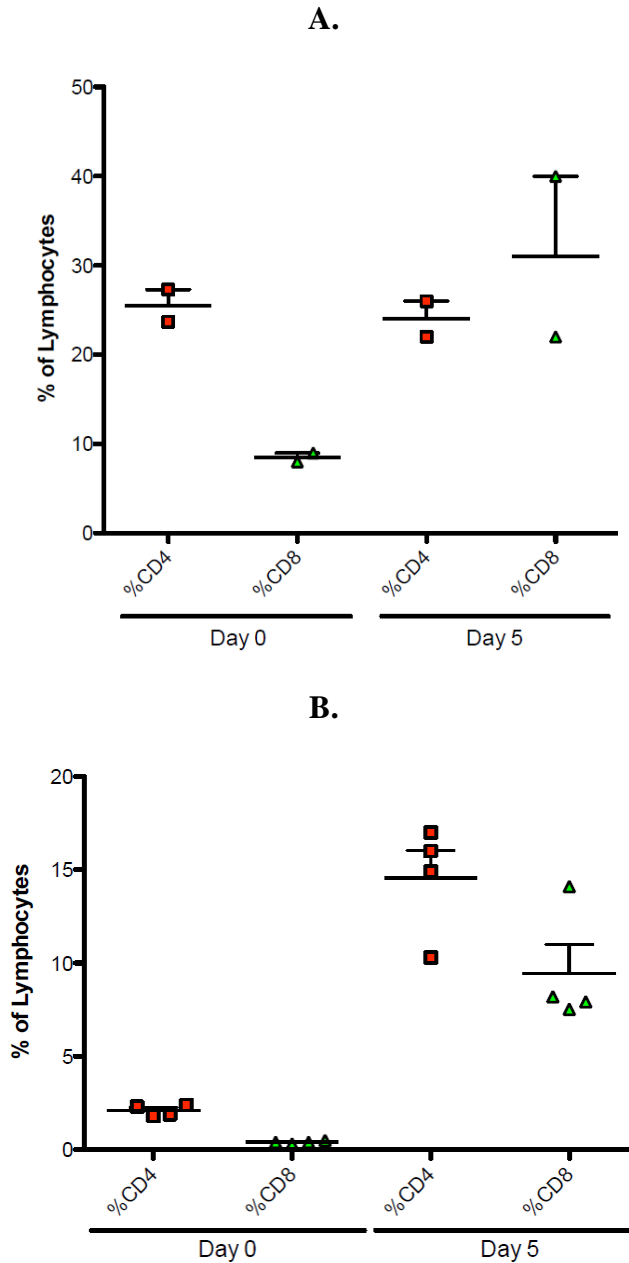
Figure 16: CD4 and CD8 T cell Percentages of Adult and Neonatal BALB/c Mice at Day 0 and Day 5. Day 0 and day 5 cell suspensions of adult and neonatal BALB/c spleen cells were gated on lymphocytes and the percentages of CD4 and CD8 T cells were assessed via flow-cytometry. The percentages of lymphocytes that are CD4 and CD8 at day 0 and day 5 in A) adult cultures and B) neonatal cultures, respectively, are represented. PE-anti-CD4 and FITC-anti-CD8 antibodies were used for detection of CD4 and CD8 T cells. Data are mean + SEM. Data are representative of five independent experiments. Adults: n=2, neonates: n=4.

4.2 Pooled Adult and Neonatal Cultures Have Similar Percentages of CD4 and CD8 T cells and Produce the Same Type of Immune Response as Cultures Containing Spleen Cells from Individual Mice

Individual adult and neonatal mouse spleen cells were used to assess the CD4 and CD8 T cell ratio as well as the immune response generated by these cells. Subsequently, experiments were performed in which neonatal cells were cultured either in the presence or absence of isolated, age-matched CD8 T cells. After the establishment that different types of immune responses are generated by adult and neonatal spleen cells as well as the role that CD8 T cells play in altering the immune response in reconstitution experiments, we decided, for logistical purposes, to use pooled spleen cells for the remaining reconstitution experiments, as well as experiments whereby the CD4 phenotype is assessed. For this, we needed to test whether the CD4 and CD8 T cell percentages differed between individual and pooled spleen cells in both adult and neonatal cultures and also whether pooled spleen cells would generate a different type of immune response when challenged with the same antigen. As seen in Figures 17 and 18, the number of CD4 and CD8 T cells, as well as the CD4:CD8 T cell ratio, are not significantly different between individual mouse spleens and pooled spleens. Additionally, as reported in Figure 19, the type of immune response generated by pooled spleen cells is not significantly different from the immune response generated by cells from individual mice. This confirmation allowed us to go ahead and use pooled spleen cells for our experiments.

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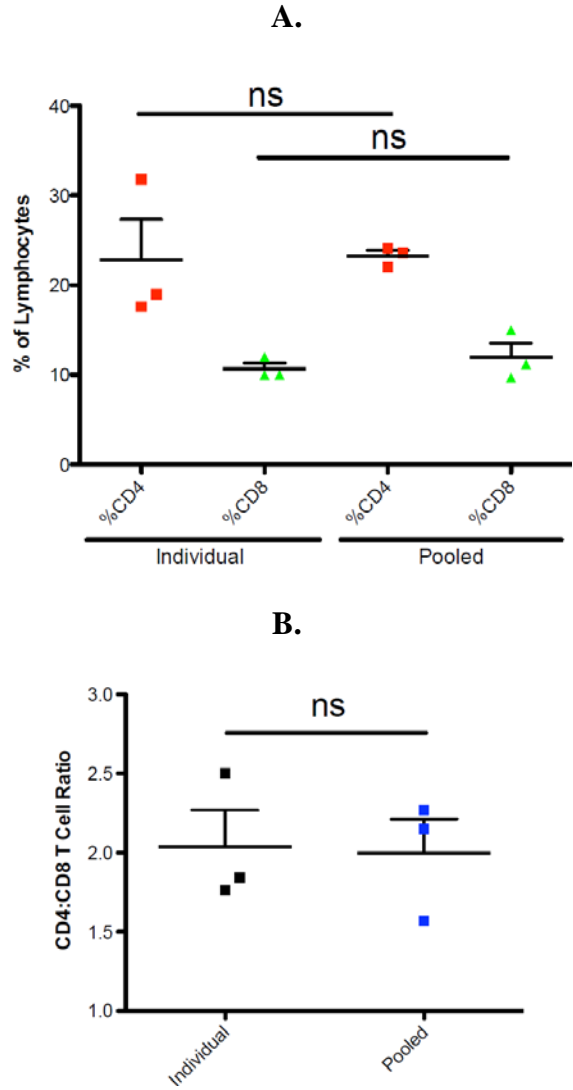
Figure 17: CD4 and CD8 T cell Percentages of Individual and Pooled Adult C57BL/6 Mice. Spleens of adult C57BL/6 mice (≥6 weeks old) were gated on lymphocytes and the percentages of CD4 and CD8 T cells were assessed via flow-cytometry. A) Adult C57BL/6 spleen cells were tested for the percentage of CD4 and CD8 T cells either individually or by pooling multiple spleens. B) The average CD4:CD8 T cell ratios of individual and pooled adult C57BL/6 mice were compared. Data are mean + SEM. Data are representative of 2 independent experiments (n=3). ns = not significant
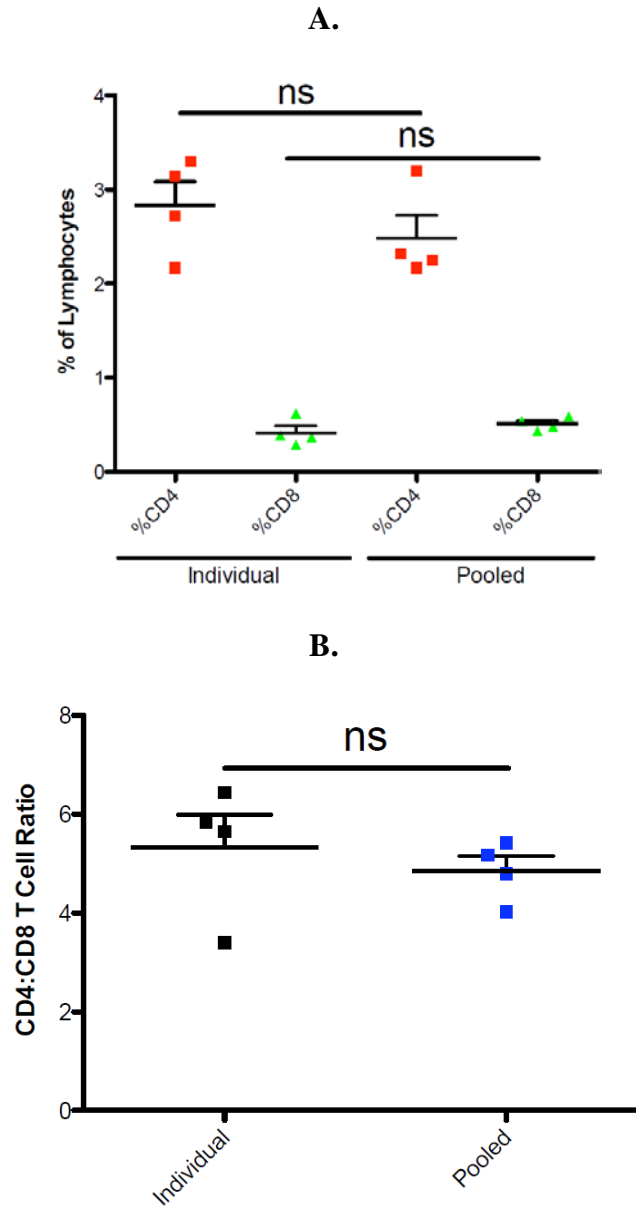
Figure 18: CD4 and CD8 T cell Percentages of Individual and Pooled Neonatal C57BL/6 Mice. Spleens of neonatal C57BL/6 mice (8-10 days old) were gated on lymphocytes and the percentages of CD4 and CD8 T cells were assessed via flow-cytometry. A) Neonatal C57BL/6 spleen cells were tested for the percentage of CD4 and CD8 T cells either individually or by pooling multiple spleens. B) The average CD4:CD8 T cell ratios of individual and pooled neonatal C57BL/6 mice were compared. Data are mean + SEM. Data are representative of 2 independent experiments (n=4). ns = not significant
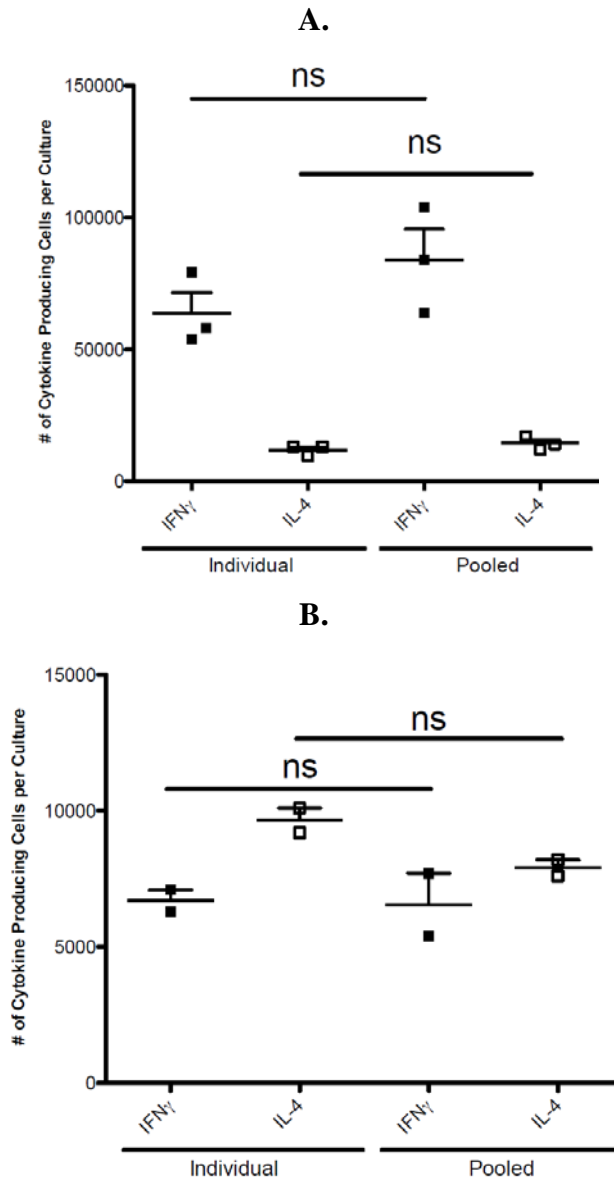
Figure 19: Immune Responses of Individual and Pooled Adult and Neonatal C57BL/6 Mice. Cytokine producing cells in adult and neonatal C57BL/6 cultures were assessed using ELISPOT assay. A) The cells producing IFN-γ and IL-4 in individual and pooled adult C57BL/6 cultures are shown. B) The cells producing IFN-γ and IL-4 in individual and pooled neonatal C57BL/6 cultures are shown. Data are mean + SEM. Data are representative of 2 independent experiments. Statistical significance assessed using students' t test. ns = not significant. Adults: n=3, Neonates: n= 2

4.3 Distinct Immune Responses Generated by Adult and Neonatal Mice

We wanted to show that the immune responses generated by adult and neonatal mice differed, so we utilized an ELISPOT assay to assess the number of antigen-specific T cells generated upon *in-vitro* stimulation of adult and neonatal spleen cells. After challenge with allo-antigen for five days *in vitro,* cells were harvested and again stimulated with allo-antigen, and the number of antigen-dependent cytokine producing cells assessed.

There were initial difficulties with the culturing of neonatal spleen cells and the detection of cytokine-producing cells when these spleen cells were cultured under similar conditions as adult cells. In previous work in the lab involving adult spleen cell cultures, six well plates were utilized with an area of 9.5 cm^2. Culturing of neonatal spleen cells at the same concentration in the same size wells did not result in enough lymphocyte activation to detect cytokine-producing cells upon secondary challenge with antigen, likely due to a far lower percentage of spleen cells being lymphocytes in the neonatal spleen, resulting in far lower lymphocyte densities when using six-well plates. Using similar concentrations of cells in twelve-well plates with an area of 3.8 cm^2 was sufficient to be able to detect cytokine-producing cells after secondary transfer in the ELISPOT assay.

We saw a significant difference in the CD4:CD8 T cell ratio of adult and neonatal mice, with adults having a higher number of CD8 T cells than neonatal mice in both strains. We wanted to know if there is a difference in the immune responses made when adult and neonatal cells are challenged with the same antigen. Adult C57BL/6 mice challenged with allo-antigen (C57BL/6 x BALB/c F1 mice), produced a strong Th1 or cell-mediated response, with a predominance of IFN-γ production, with relatively little IL-4, as seen in Figure 20, while neonatal mice of the same strain produced a predominant Th2 or a mixed response, as seen in Figure 21. The high amounts of IFN-γ production are expected in C57BL/6 adult mice, as they have been utilized for their propensity to make cell-mediated responses and be resistant to a number of pathogens. What is interesting is the bias toward a Th2 response when spleen cells of newborn C57BL/6 mice are stimulated *in vitro*, in contrast to the predominant Th1 response generated by adult spleen cells of this strain. Similar results can be seen when the response of BALB/c mice are examined. When spleen cells of BALB/c adult and neonatal mice are stimulated with the same allo-antigen, the adult spleen cells produce a stronger Th1 response, while neonatal spleen cells produce a predominant Th2 response, seen in Figures 23 and 24.

Thus we see a similar trend across both strains of mice, whereby adult cells are more proficient in producing IFN-γ and making cell-mediated responses, while neonatal cells produce much more IL-4 relative to IFN-γ, and so make a predominant Th2 response.

The side-by-side comparison of adult and neonatal cytokine producing cells is a difficult one statistically due to the much larger numbers seen in adult cultures. Statistically significant differences are readily observed when the ratio of the number of IFN-γ to the number of IL-4 producing cells is calculated. When looking at the IFN-γ: IL-4 ratio of adult and neonatal mice in Figures 22 and 25, it is clear that adults of both strains produce much more IFN-γ compared to IL-4 and are capable of making a cell-mediated response while neonates are biased towards Th2 type responses to the same antigen.

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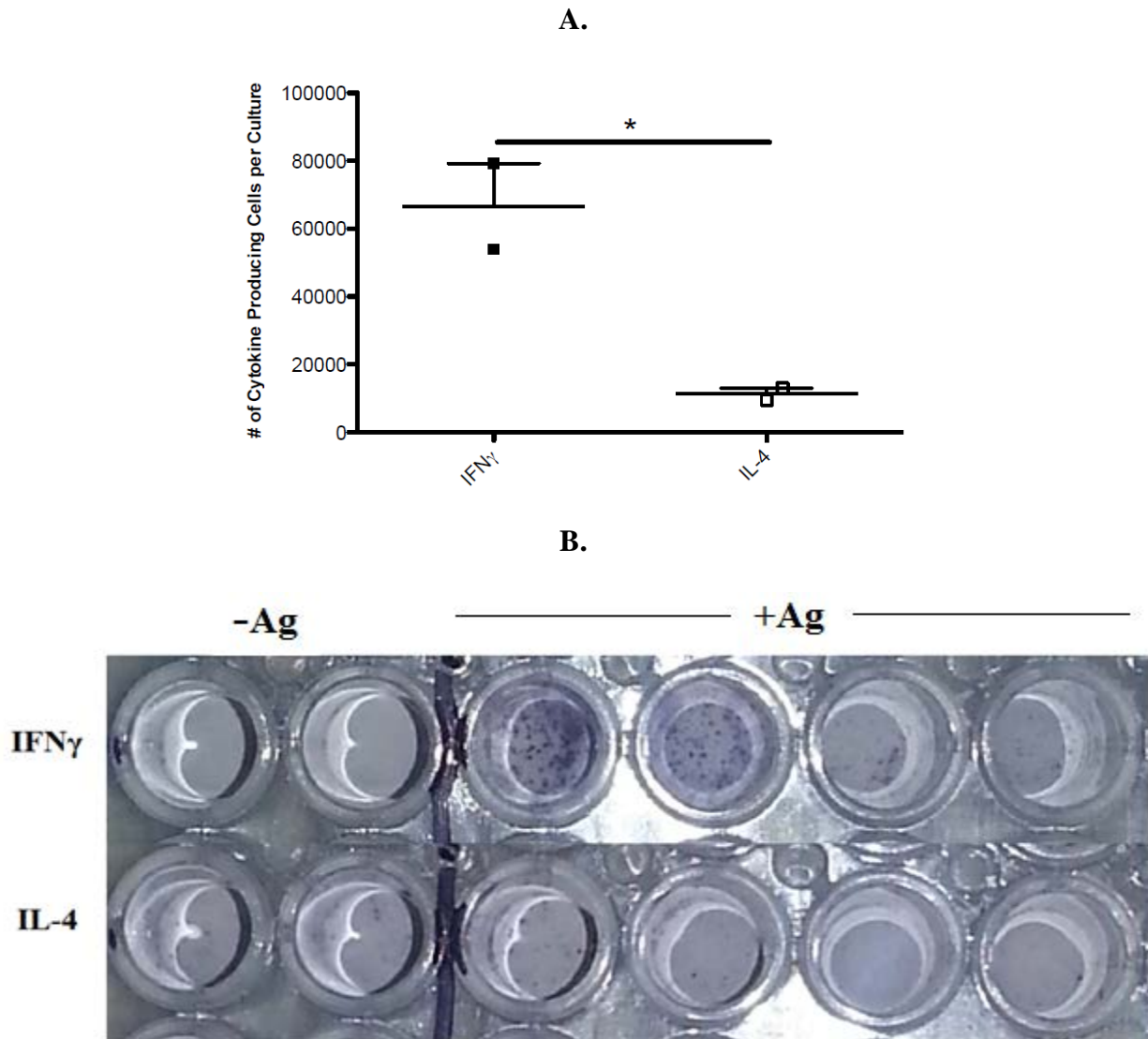
Figure 20: Adult C57BL/6 Mice Produce a Th1 Type Immune Response. Cytokine producing cells in Adult C57BL/6 cultures were assessed using ELISPOT assay. A) Averages of adult immune responses are shown. B) Representative photos of ELISPOT wells of adult C57BL/6 cultures are shown. Data are mean + SEM. Data are representative of 5 independent experiments (n=2). Statistical significance assessed using students' *t* test. * = p< 0.05

A.

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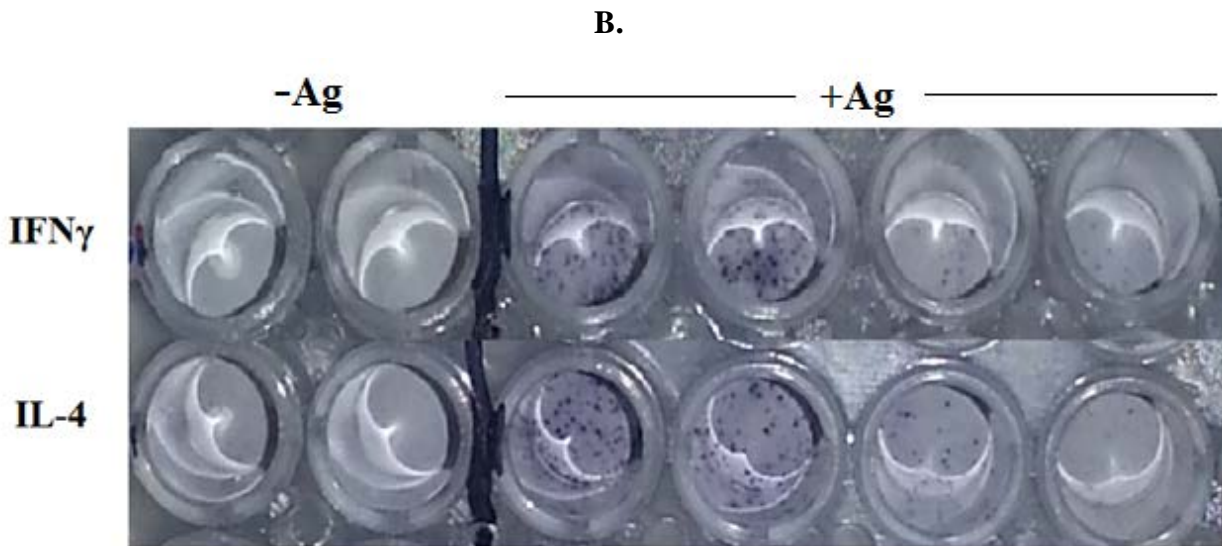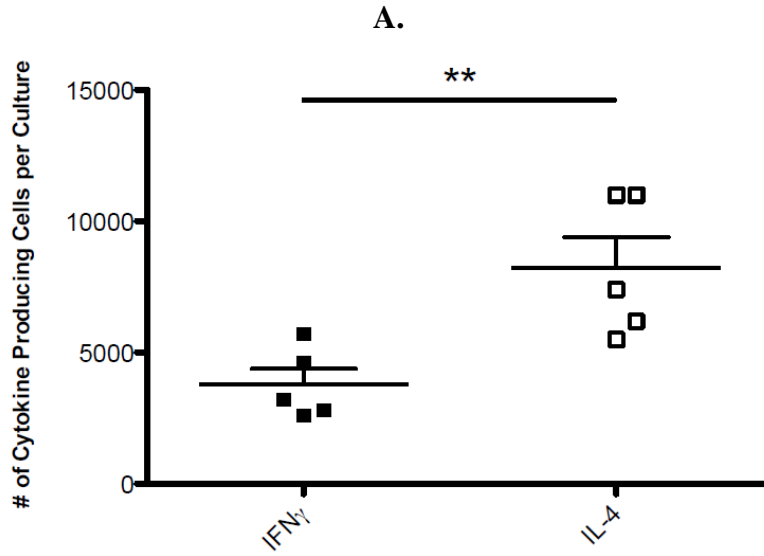
Figure 21: Neonatal C57BL/6 Mice Produce a Predominantly Th2 or Mixed Type Immune Response. Cytokine producing cells in neonatal C57BL/6 cultures were assessed using ELISPOT assay. A) Averages of neonatal immune responses are shown. B) Representative photos of ELISPOT wells of neonatal C57BL/6 cultures are shown. Data are mean + SEM. Data are representative of four independent experiments (n=5). Statistical significance assessed using students' *t* test. ** = p< 0.01
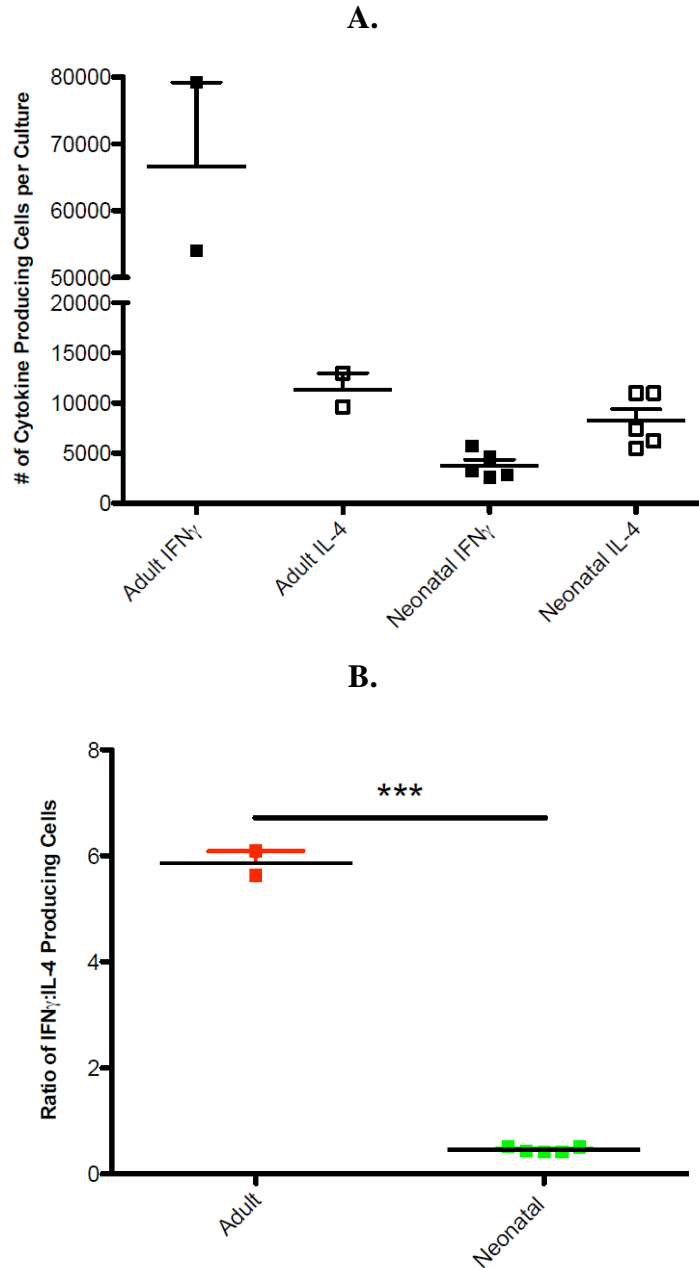
Figure 22: Comparison of IFNγ: IL-4 Producing Cells in C57BL/6 Adult and Neonatal Mice. A) The number of cytokine producing cells in both adult and neonatal cultures was compared. B) The ratio of IFN-γ: IL-4 producing cells in adult and neonatal cultures were compared. Data are representative of at least four independent experiments. Data are mean + SEM. Statistical significance was assessed by students' *t* test. *** = p<0.00. Adults: n=2, Neonates: n=5.

A.

B.
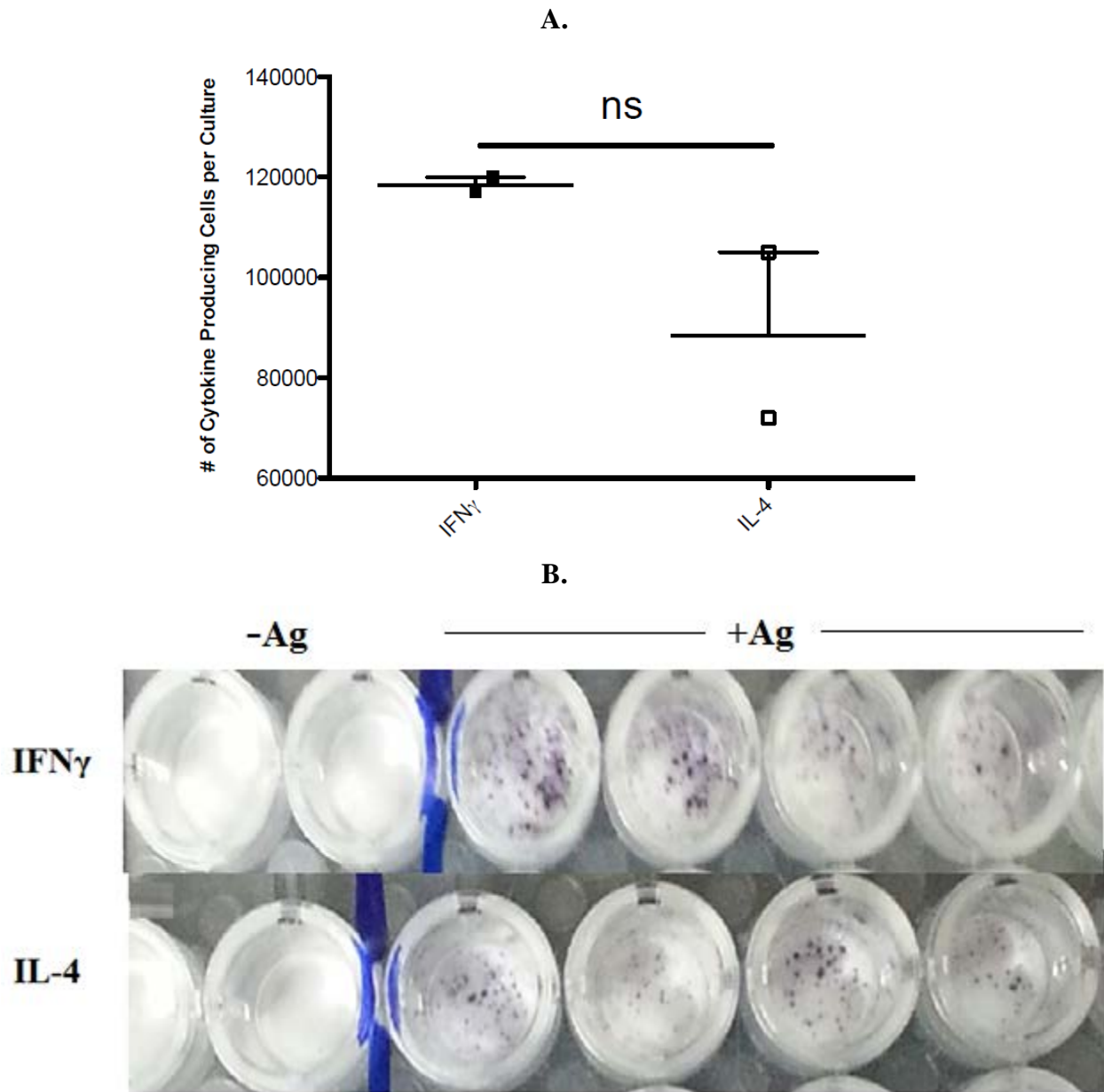
Figure 23: Adult BALB/c Mice Produce a Mixed Type Immune Response. Cytokine producing cells in Adult BALB/c cultures were assessed using ELISPOT assay. A) Averages of adult immune responses are shown. B) Representative photos of ELISPOT wells of adult BALB/c cultures are shown. Data are mean + SEM. Data are representative of 5 independent experiments (n=2). Statistical significance assessed using students' *t* test. ns = not significant

Figure 24: Neonatal BALB/c Mice Produce a Predominantly Th2 Type Immune Response. Cytokine producing cells in neonatal BALB/c cultures were assessed using ELISPOT assay. A) Averages of neonatal immune responses are shown. B) Representative photos of ELISPOT wells of neonatal BALB/c cultures are shown. Data are mean + SEM. Data are representative of 5 independent experiments (n=4). Statistical significance assessed using students' t test. ** = $p < 0.01$
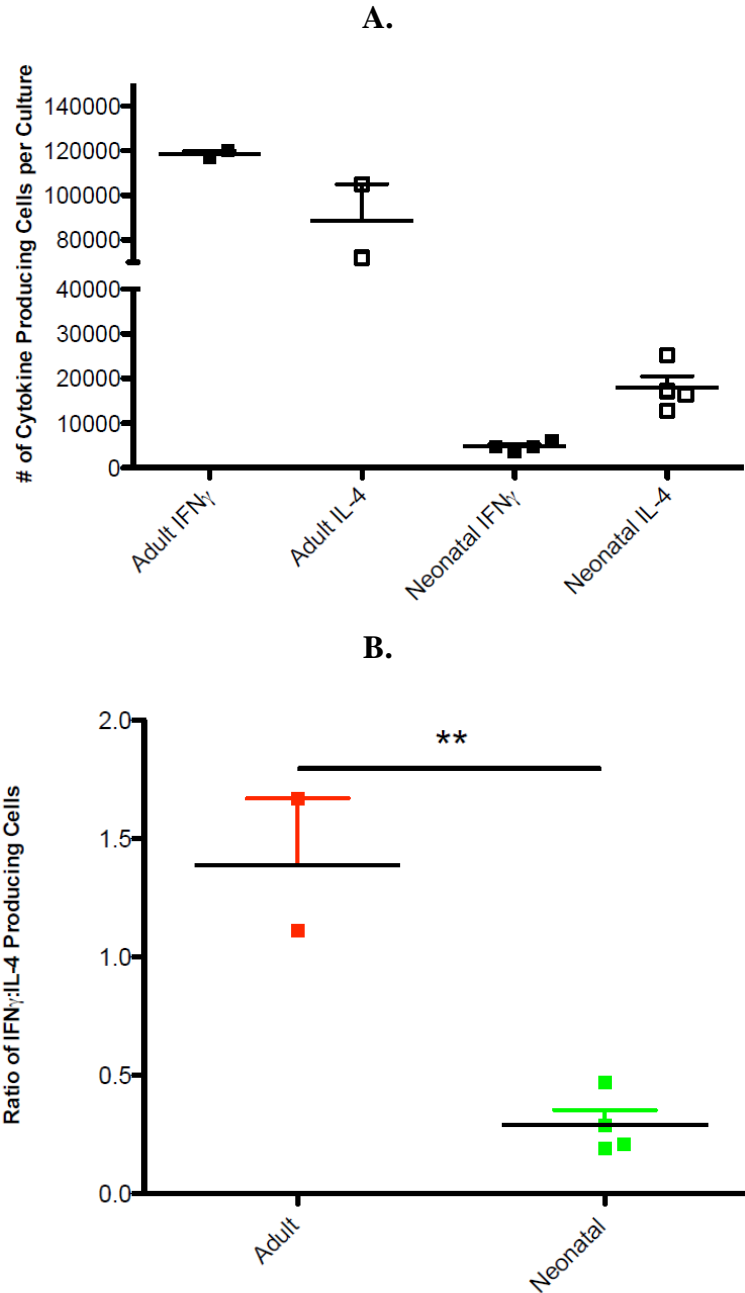
Figure 25: Comparison of IFNγ: IL-4 Producing Cells in BALB/c Adult and Neonatal Mice. A) The number of cytokine producing cells in both adult and neonatal cultures was compared. B) The ratio of IFN-γ: IL-4 producing cells in adult and neonatal cultures were compared. Data are representative of five independent experiments. Data are mean + SEM. Statistical significance was assessed by Students' *t* test. ** = p<0.01. Adults: n=2, neonates: n=4.

4.4 Reconstitution of Neonatal Cultures with Pooled, Isolated, Age-matched CD8 T Cells Results in Th1 Type Responses.

We sought to determine whether or not the addition of CD8 T cells at the initiation of culture to neonatal spleen cells could switch the response generated to a Th1 mode. The addition of CD8 T cells isolated from pooled adult mouse spleens could modulate the immune response generated from a Th2 to a Th1 mode, in experiments I performed that are not shown here. We wanted to know if the presence of CD8 T cells from age-matched neonatal spleens could similarly significantly modulate the immune response. I faced early on methodological difficulties in isolating CD8 T cells from pooled neonatal mouse spleens. Due to their low numbers, it was difficult to isolate sufficient numbers to alter the CD4:CD8 T cell ratios of the neonatal cultures. Pooled spleen cells are passed through nylon wool columns to remove B cells, macrophages, and dendritic cells, followed by MACS negative selection of CD8 T cells by removal of CD4 T cells. Flowcytometry is employed to enumerate the number of CD8 T cells that have been isolated. Knowing the CD4:CD8 T cell ratios of the neonatal cultures by flow-cytometry, one can calculate how many additional CD8 T cells are needed to lower the CD4:CD8 T cell ratio to one similar to that seen in adult cultures. My early trials at this involved reconstituting a larger number of cultures, and thus not having enough CD8 T cells to sufficiently lower the ratio of the cultures down to adult levels, but these experiments resulted in lower ratios nonetheless and produced interesting results. Reconstituting fewer cultures with more CD8 T cells reduced the CD4:CD8 T cell ratio enough to significantly alter the Th1/Th2 nature of the immune response. As seen in Figures 26 through 32, the reconstruction of neonatal cultures with CD8 T cells to produce adult-like CD4:CD8 T cell ratios results in altered immune responses, similar to those made by adult mice of the same strain. As seen in Figures 29 through 32, an intermediate ratio results in a mixed type response, with a lower ratio resulting in significantly more IFN-γ production and more predominant Th1 responses. These results validated our idea that the presence of CD8 T cells at initiation of culture can significantly modulate a Th2 immune response toward a Th1 type response.
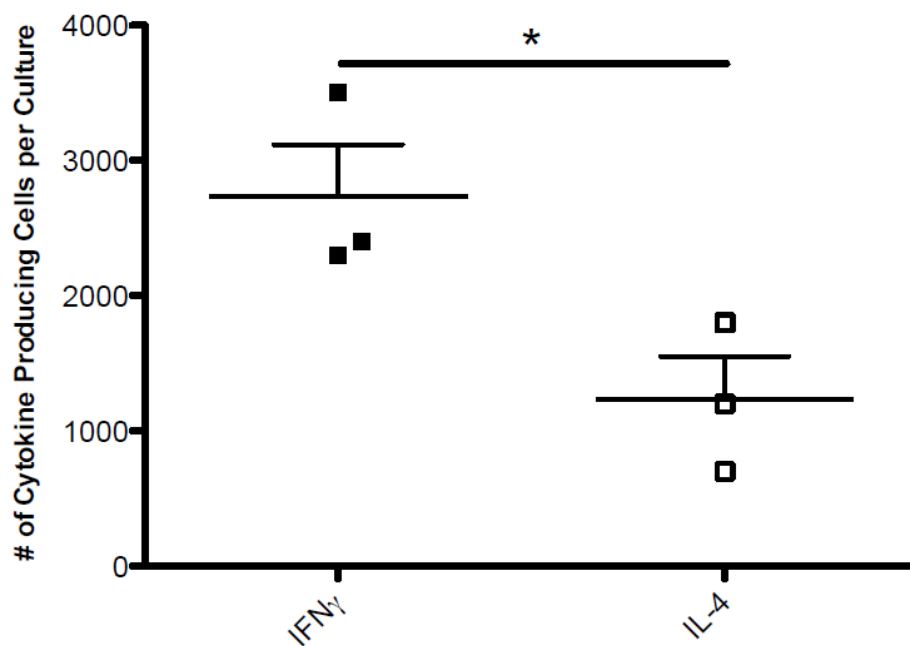
Figure 26: Neonatal C57BL/6 Cultures Reconstituted with Isolated, Age-Matched CD8 T Cells Resulting in an Average CD4:CD8 T Cell Ratio Similar to Adult Mice Produce a Th1 Immune Response Similar to Adult Mice. Neonatal C57BL/6 spleen cells were cultured in the presence of pooled, age-matched, isolated CD8 T cells to produce a lower CD4:CD8 T cell ratio, around 2, and their immune response assessed using ELISPOT assay. Averages of reconstituted immune responses are shown. Data are representative of four independent experiments (n=3). Data are mean + SEM. Statistical significance was assessed by student's *t* test. * = p< 0.05

A.

B.

C.
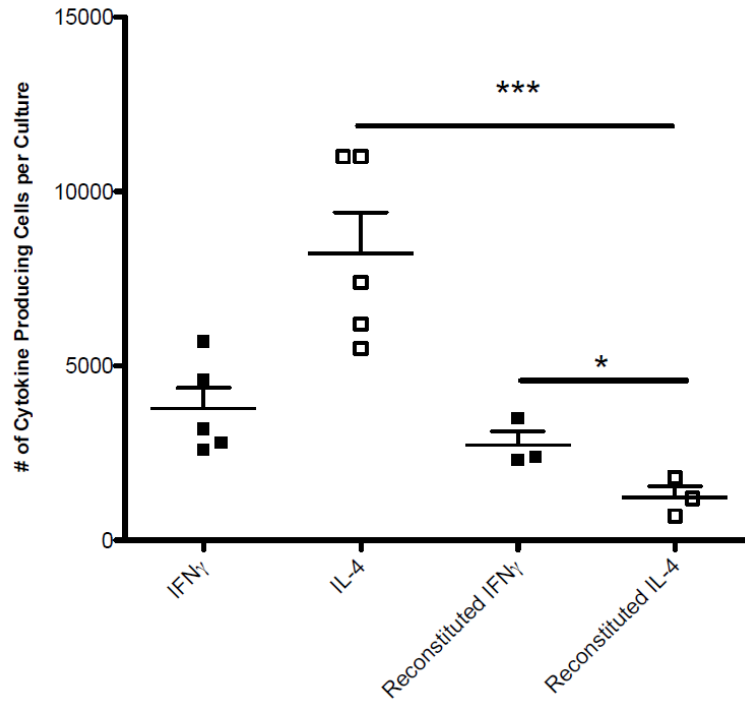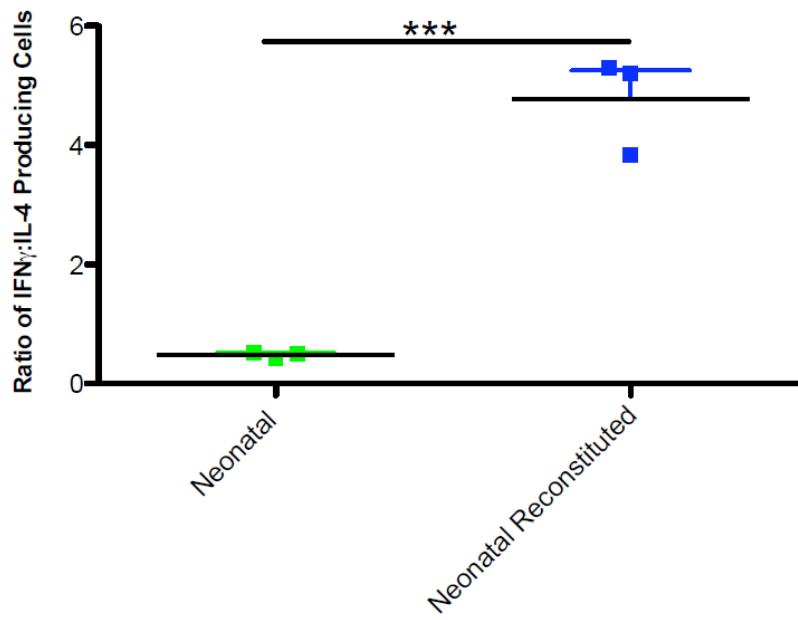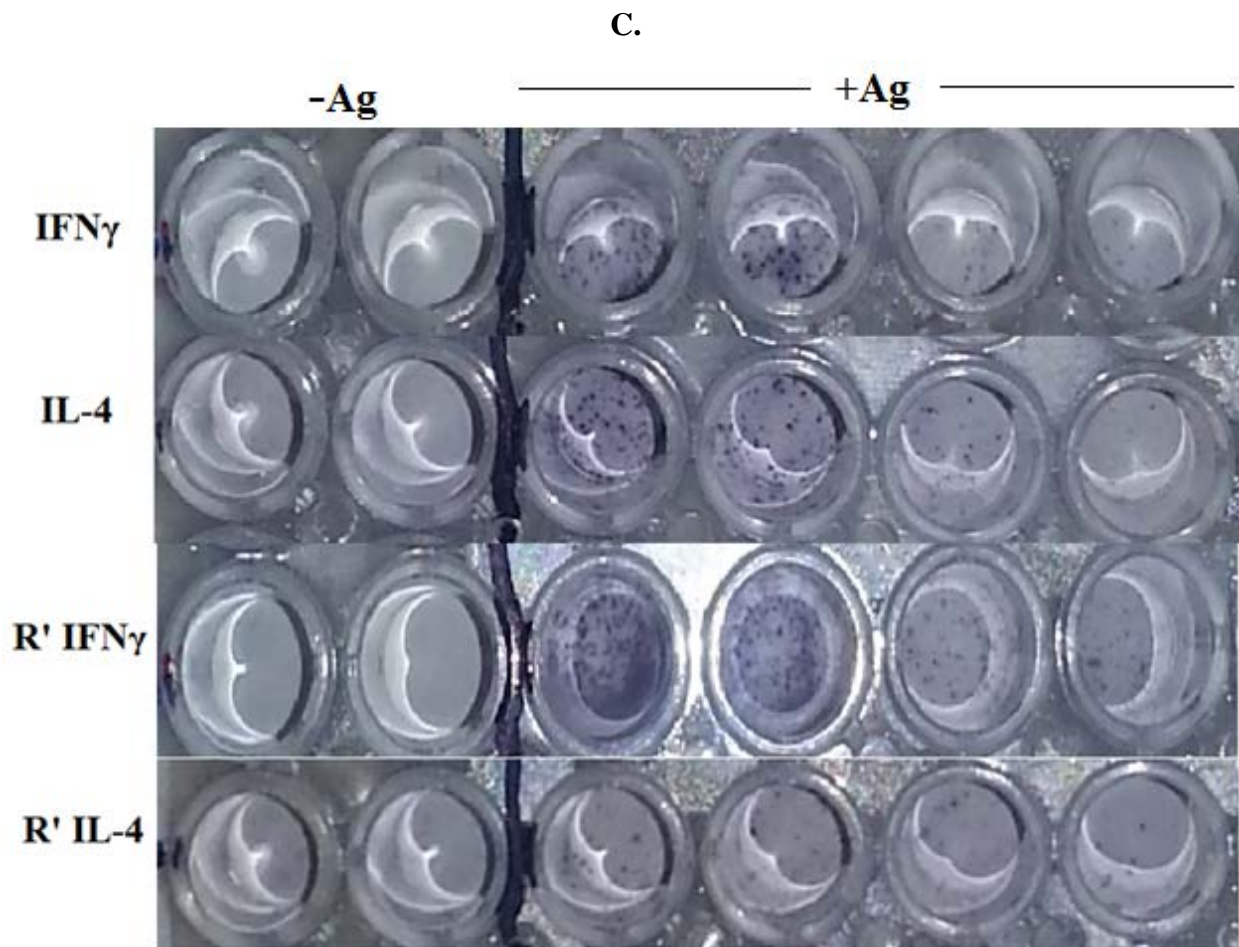
Figure 27: Neonatal C57BL/6 Cultures Reconstituted with Isolated, Age-Matched CD8 T Cells Produce a Th1 Immune Response Similar to Adult Mice. Neonatal C57BL/6 spleen cells were cultured in the presence of pooled, age-matched, isolated CD8 T cells to produce a lower CD4:CD8 T cell ratio, around 2, and their immune response assessed using ELISPOT assay. A) Averages of neonatal and reconstituted immune responses were compared. B) The ratio of IFN-γ: IL-4 producing cells in neonatal and reconstituted cultures were compared. C) Representative photos of ELISPOT wells of neonatal and reconstituted cultures are shown. Data are representative of four independent experiments (n=3). Data are mean + SEM. Statistical significance was assessed by one-way ANOVA or student's *t* test. * = p< 0.05, *** = p<0.001

A.

B.

C.

Figure 28: Neonatal C57BL/6 Cultures Reconstituted with Isolated, Age-Matched CD8 T Cells Produce a Th1 Immune Response Similar to Adult Mice. Neonatal C57BL/6 spleen cells were cultured in the presence of pooled, age-matched, isolated CD8 T cells to produce a lower CD4:CD8 T cell ratio, around 2, and their immune response assessed using ELISPOT assay. A) Averages of adult, neonatal, and reconstituted immune responses were compared. B) The ratio of IFN-γ: IL-4 producing cells in adult, neonatal, and reconstituted cultures were compared. C) Representative photos of ELISPOT wells of adult, neonatal, and reconstituted cultures are shown. Data are representative of four independent experiments (n=3). Data are mean + SEM. Statistical significance was assessed by one-way ANOVA. *** = p<0.001

A.

B.

Figure 29: Neonatal BALB/c Cultures Reconstituted with Isolated, Age-Matched CD8 T Cells Produce a Th1 Immune Response Similar to Adult Mice. Neonatal BALB/c spleen cells were cultured in the presence of pooled, age-matched, isolated CD8 T cells to produce a lower CD4:CD8 T cell ratio and their immune response assessed using ELISPOT assay. A) The reconstituted immune response with CD4:CD8 T cell ratio of ~3.6 is shown. B) The reconstituted immune response with CD4:CD8 T cell ratio of ~1.2 is shown. Data are representative of three independent experiments, n=5 for the 3.6 ratio experiments, and four independent experiments, n=3 for the 1.2 ratio experiments. Data are mean + SEM. Statistical significance was assessed by student's *t* test. *** = p<0.001

Figure 30: Neonatal BALB/c Cultures Reconstituted with Isolated, Age-Matched CD8 T Cells Produce a Th1 Immune Response Similar to Adult Mice. Neonatal BALB/c spleen cells were cultured in the presence of pooled, age-matched, isolated CD8 T cells to produce a lower CD4:CD8 T cell ratio and their immune response assessed using ELISPOT assay. A) Averages of the reconstituted immune response with CD4:CD8 T cell ratio of ~3.6 and neonatal cultures were compared. B) The reconstituted immune response with CD4:CD8 T cell ratio of ~1.2 and neonatal cultures were compared. Data are representative of three independent experiments, n=5 for the 3.6 ratio experiments, and four independent experiments, n=3 for the 1.2 ratio experiments. Data are mean + SEM. Statistical significance was assessed by one-way ANOVA. * = $p < 0.05$, *** = $p < 0.001$
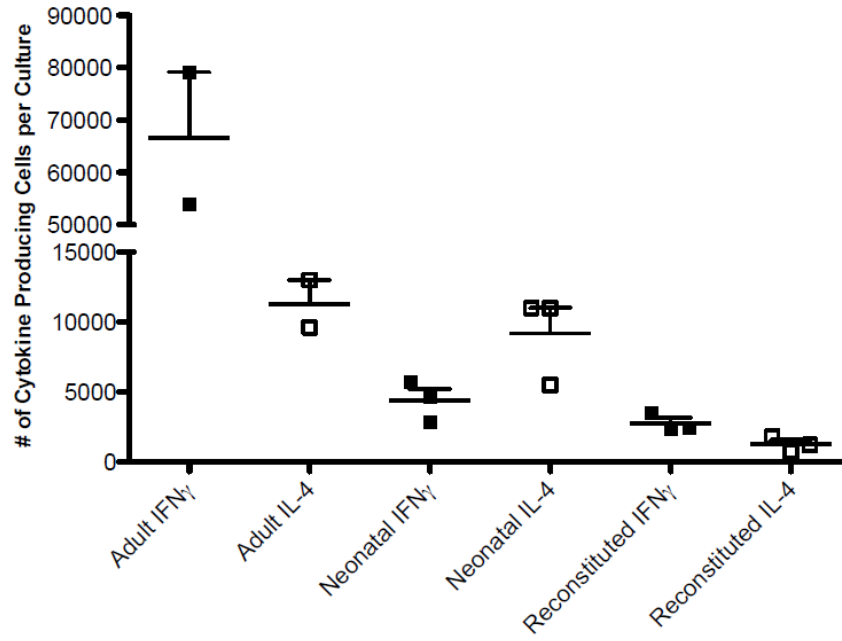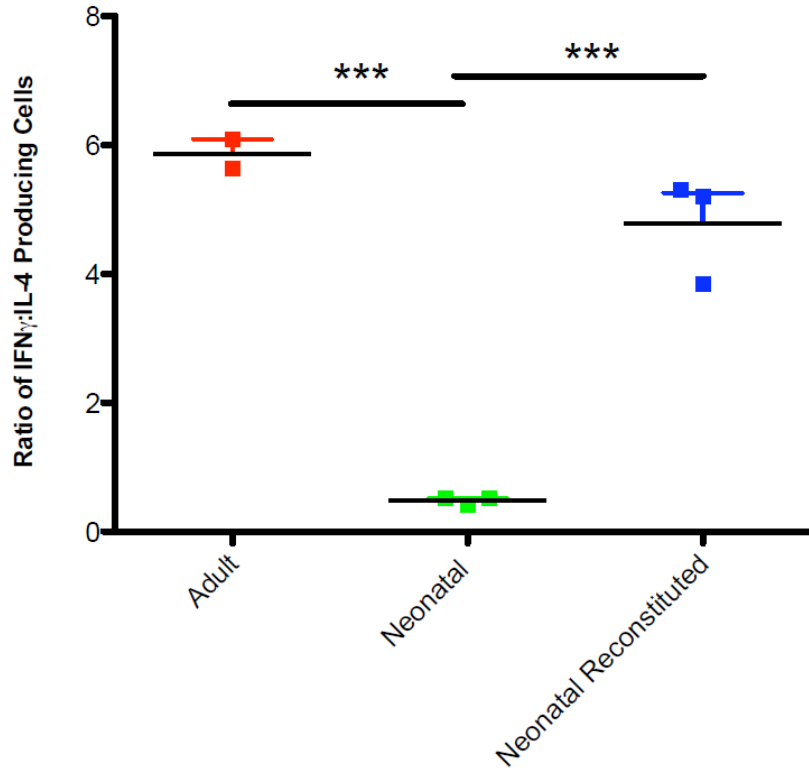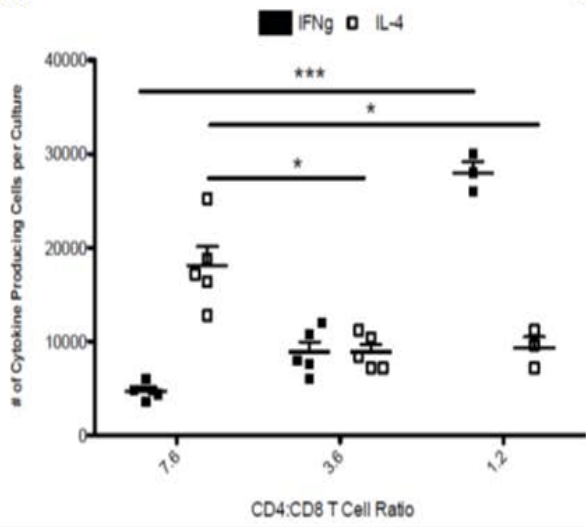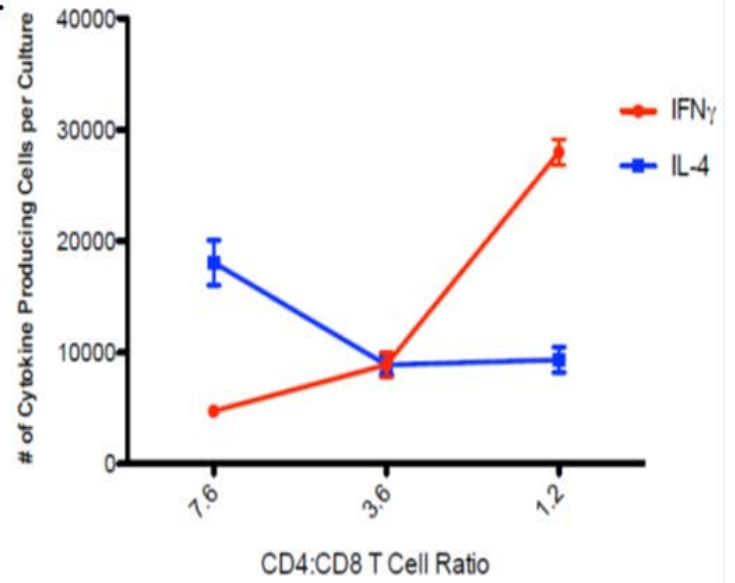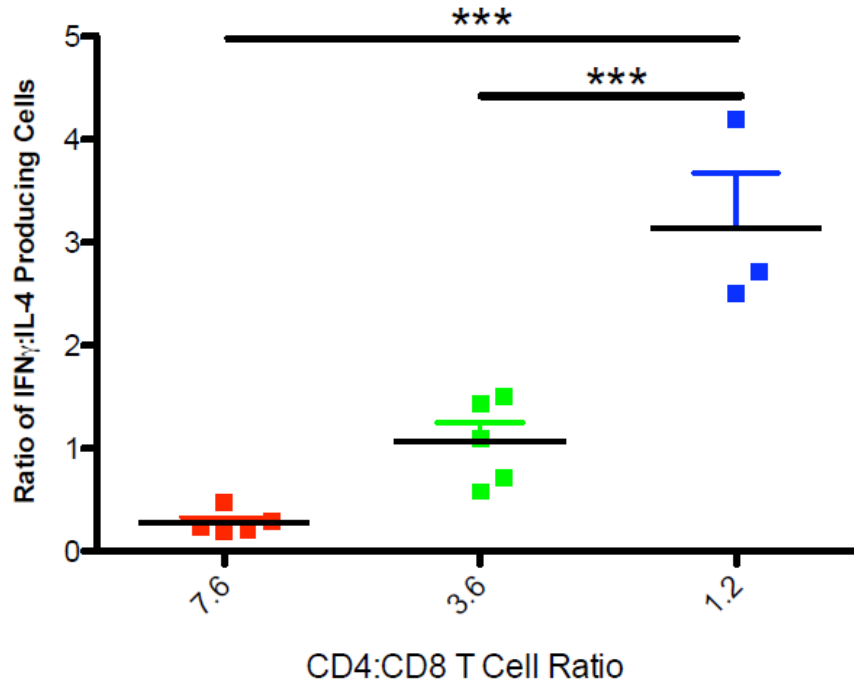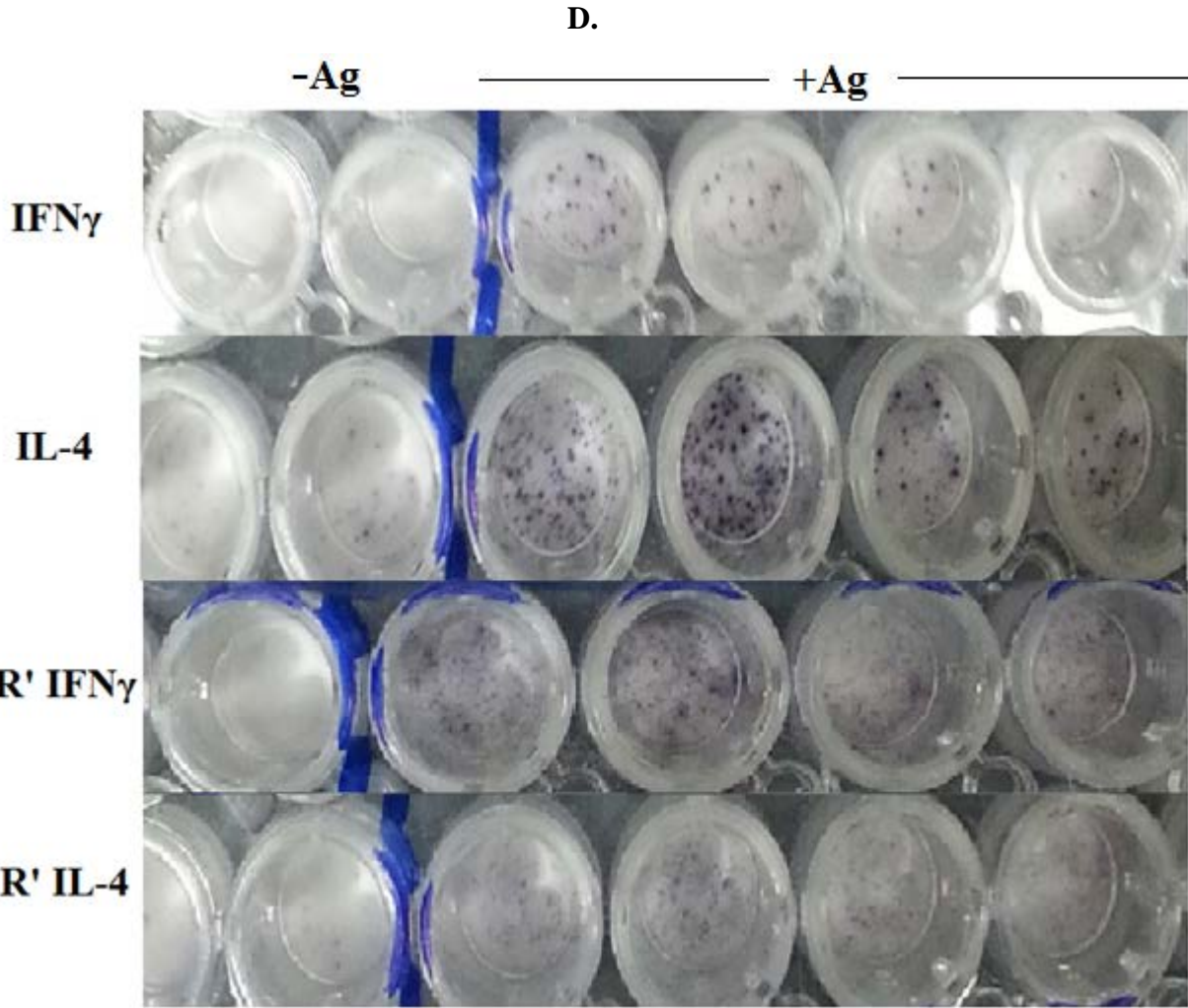
Figure 31: Neonatal BALB/c Cultures Reconstituted with Isolated, Age-Matched CD8 T Cells Produce a Th1 Immune Response Similar to Adult Mice. Neonatal BALB/c spleen cells were cultured in the presence of pooled, age-matched, isolated CD8 T cells to produce a lower CD4:CD8 T cell ratio and their immune response assessed using ELISPOT assay. A and B) Averages of the reconstituted immune response with CD4:CD8 T cell ratio of ~3.6 and ~1.2 were compared to neonatal immune responses. C) The average IFN-γ: IL-4 producing cells in neonatal and reconstituted cultures were compared. D) Representative photos of ELISPOT wells of neonatal and reconstituted cultures are shown. Data are representative of three independent experiments, n=5 for the 3.6 ratio experiments, and four independent experiments, n=3 for the 1.2 ratio experiments. Data are mean + SEM. Statistical significance was assessed by one-way ANOVA. * = p<0.05, *** = p<0.001

A.

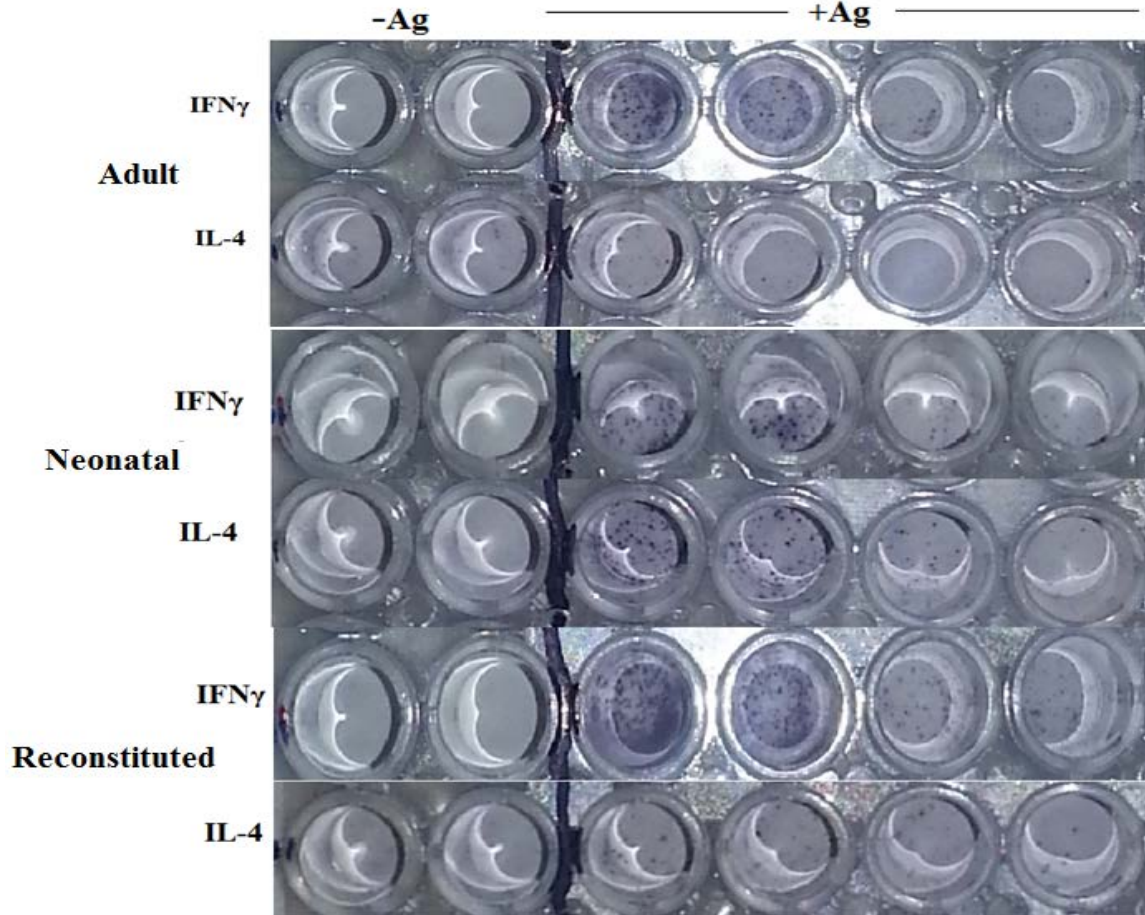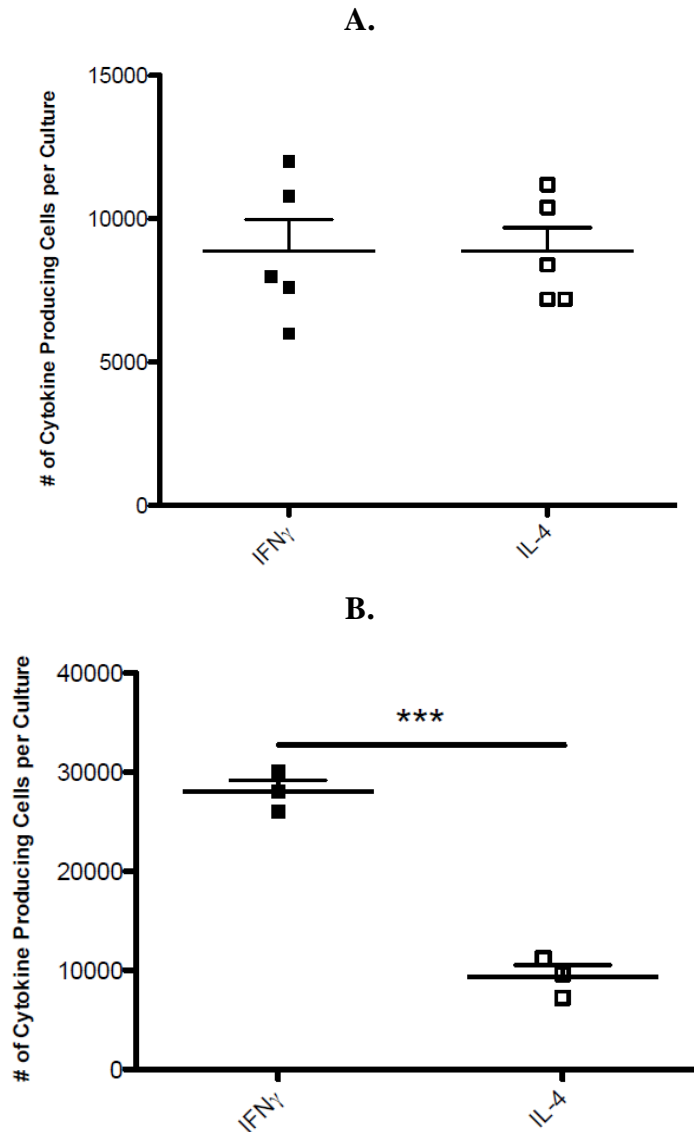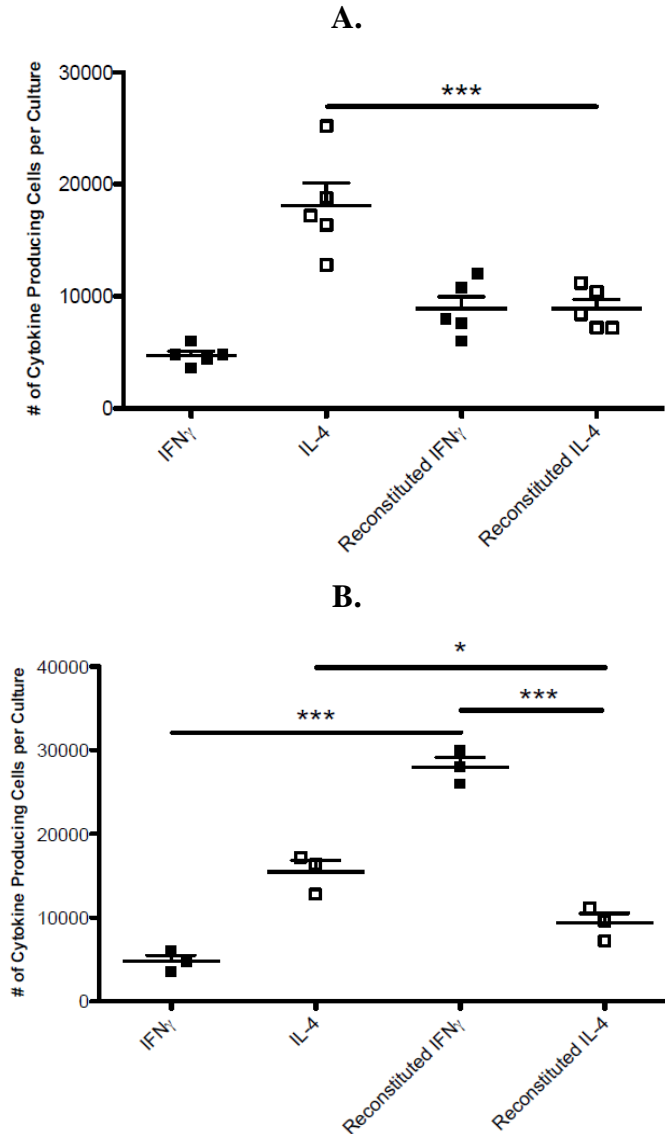**B.**
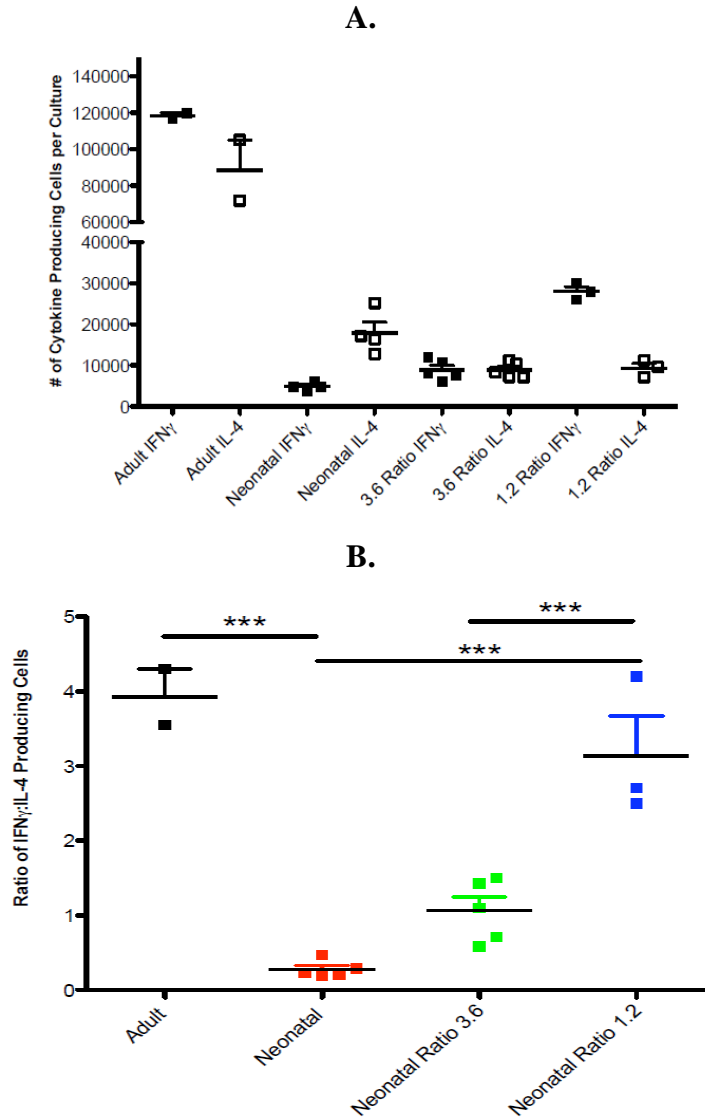
Figure 32: Neonatal BALB/c Cultures Reconstituted with Isolated, Age-Matched CD8 T Cells Produce a Th1 Immune Response Similar to Adult Mice. Neonatal BALB/c spleen cells were cultured in the presence of pooled, age-matched, isolated CD8 T cells to produce a lower CD4:CD8 T cell ratio and their immune response assessed using ELISPOT assay. A) Averages of adult, neonatal, and reconstituted immune responses were compared. B) The ratio of IFN-γ: IL-4 producing cells in adult, neonatal, and reconstituted cultures were compared. Data are representative of three independent experiments, n=5 for the 3.6 ratio experiments, and four independent experiments, n=3 for the 1.2 ratio experiments. Data are mean + SEM. Statistical significance was assessed by one-way ANOVA. * = $p<0.05$, *** = $p<0.001$

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4.5 Reconstitution of Neonatal Cultures with Pooled, Isolated, Age-matched CD8 T cells Alters the Phenotype of Responding Antigen-Specific CD4 T Cells.

The addition of CD8 T cells to neonatal cultures resulted in a switch from a predominant Th2 to a Th1 response. The question that remained was whether or not the addition of CD8 T cells into culture was having an influence on the responding, antigen-specific CD4 T cells according to our model, or was the presence of more CD8 T cells in reconstituted cultures solely responsible for increasing the number of IFN-γ producing cells as assessed by the ELISPOT assay. If the presence of CD8 T cells in culture at the initiation of the immune response has an effect on CD4 T cells, we would expect to see a difference in the phenotype of CD4 T cells from neonatal cultures and those that have been reconstituted. After 5 days of stimulation in culture, neonatal or reconstituted cultures were harvested and the CD4 and CD8 T cells isolated, for secondary challenge with antigen in the ELISPOT assay to assess their cytokine production. As shown in Figure 33, neonatal CD4 T cells produced a mixed or Th2 response, but when reconstituted with isolated, age-matched CD8 T cells, the CD4 T cells produced a Th1 response, showing that the presence of CD8 T cells at the initiation of the immune response can alter the phenotype of responding CD4 T cells.
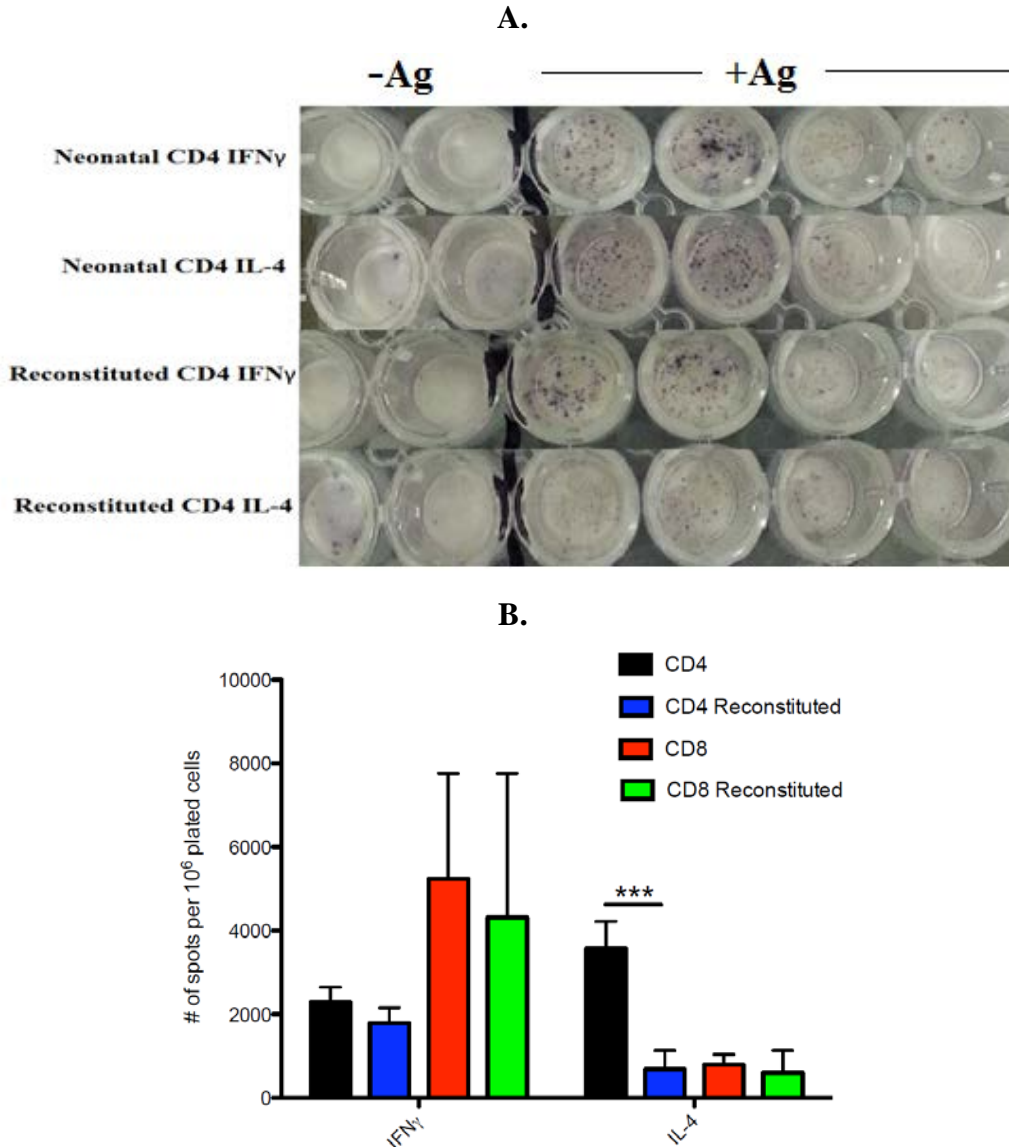
Figure 33: The Phenotype of the Responding Neonatal CD4 T cell Changes when Stimulated in the Presence of Additional Isolated, Age-Matched CD8 T Cells. Neonatal C57BL/6 spleen cells were cultured in the presence of pooled, isolated, age-matched CD8 T cells to produce a lower CD4:CD8 T cell ratio. At day 5, the CD4 and CD8 T cells were separated and challenged with antigen in an ELISPOT assay. A) Representative photo of ELISPOT wells of isolated CD4 T cells from neonatal and reconstituted cultures producing IFNγ and IL-4 are shown. B) The cytokine producing CD4 and CD8 T cells per 10^6 plated cells in both neonatal and reconstituted cultures are shown. Data are representative of two independent experiments (n=2). Data are mean + SEM. Statistical significance was assessed by students' *t* test. *** = p<0.001

Chapter 5

Discussion

5.1 Adult and Neonatal Mice have a Distinct CD4:CD8 T cell Ratio Leading to Differential Immune Responses upon Challenge with the Same Antigen

Neonatal mice, upon challenge with antigen produce a predominant Th2 response, characterized by strong antibody production, predominantly of IgG1 and IgE isotypes. For many years, immunologists thought that neonatal exposure to antigen resulted in tolerance. The work of Billingham, Brent, and Medawar in the 1950's showed that fetal animals could be rendered tolerant to allogeneic cells and would not produce an immune response characteristic of adult individuals challenged with the same allogeneic cells as assessed by skin graft rejection (Billingham et al. 1953). Later it was discovered that the same type of phenomenon was seen when newborn, and not just fetal, individuals were challenged with allogeneic cells. Individuals challenged with allogeneic skin grafts were tolerant of these grafts if they were given a challenge of the same allogeneic cells sufficiently early in life (Billingham and Brent 1959; Billingham et al. 1962; Schwarz 1968).

This work involving newborn animals made tolerant upon challenge with different antigens began to shift after the much later discovery that these individuals were not tolerant toward the antigens in question, but in fact had an abundance of lymphocytes present that were capable of responding to the antigen. An inability of the lymphocytes specific for the antigen with which neonatal individuals were challenged to home to lymph nodes resulted in a lack of a sufficiently strong response which suggested tolerance (Forsthuber et al 1996). The lack of homing to the lymph nodes led to an accumulation of lymphocytes in the spleens of individuals challenged with antigen. These cells were capable of producing a strong immune response upon secondary challenge with antigen, and this was found to be a strong Th2 response, with significant production of IgG1 antibody (Forsthuber et al 1996). Other experiments by Matzinger led to similar findings, specifically, that neonatal females challenged with male donor dendritic cells could produce a Th2 response against the H-Y antigens of the donor cells (Ridge et al. 1996). Additionally, neonatal mice challenged with several logs lower doses of antigen

could produce Th1 responses with CTL induction, however the "normal challenge" led to a strong Th2 response with a lack of cell-mediated immunity (Sarzotti et al. 1996).

Since the discovery that neonates are capable of mounting immune responses, with a bias toward Th2 type responses, there has been a vast amount of work to determine the cause of this bias. The experiments we have performed show that adult and neonatal individuals have distinct splenic CD4:CD8 T cell ratios. Adult mice of both C57BL/6 and BALB/c strains have a ratio of typically about 2-3 (Figures 1-2, 6-7), while neonatal mice, 8-10 days old, have very few CD8 T cells present in the spleen resulting in a ratio that is much higher (Figures 3-4, 8-9). They also have very few CD4 T cells present in the spleen when compared to adults of the same strain. The extreme lack of CD8 T cells present in neonatal spleens has a drastic effect on the ratio of CD4 to CD8 T cells present in the spleen, with adults typically having a ratio approximately 2 to 3 fold lower (Figures 5 and 10). We have performed multiple experiments examining the CD4:CD8 T cell ratio in individual and pooled adult and neonatal mice in both C57BL/6 and BALB/c strains. In total, well over 50 individual neonatal mice of each strain have been tested and compared with adult mouse spleens to give us a statistically significant result, showing that newborn mice have very low CD8 T cell numbers and that this results in a much higher CD4:CD8 T cell ratio in the spleens of these mice.

CD8 T cells are important for the clearance of intracellular pathogens, and their main, recognized, immunological role is the cytotoxic killing of infected cells after recognition of their cognate antigens in the context of Class I MHC. They are important for killing of infected cells through the secretion of cytolytic granules and are major players in Th1, cell-mediated immune responses. They also produce large amounts of IFN-γ which contributes to the production and maintenance of cell-mediated responses. Aside from this central role of CD8 T cells in the lysis of infected cells, an additional role for CD8 T cells as a regulatory cell has been shown under various circumstances. Kondo showed that the antibody response made by mice challenged with SRBCs could be eradicated if mice were given a dose of thymocytes at the time of challenge (Gershon and Kondo 1970; Gershon and Kondo 1971). The idea that T cells could suppress the antibody response became a well-studied phenomenon. Basten expanded upon previous experiments, showing that the antibody response, in most cases, required B and T cells, by showing that T cells could also have a suppressive effect on B cells (Basten et al. 1974). The suppressive effect seen could be eliminated by the addition of anti-θ antibody. Additionally, it

was demonstrated that the supernatants from T cells stimulated with Con A could inhibit the antibody response *in vitro* (Rich and Pierce 1974; Dutton 1975). In later experiments researchers were able to distinguish between different cells types, and show that T cells that had different surface marker expression were responsible for helping or suppressing antibody responses. This work showed that T cells which had Ly1 were primarily helper T cells and did not generate "killer responses", while Ly2 and Ly3 T cells became suppressor or killer cells upon stimulation with Con A and that these cells were capable of the suppression of antibody production (Okumura et al. 1976). The establishment of the now modern nomenclature of CD4 and CD8 T cells occurred several years later, but the idea that "CD8 T cells" were indeed responsible for the suppression of antibody responses was confirmed (Ramshaw et al. 1977). The presence of CD8 T cells has been shown to play a regulatory role in the inhibition of allergic responses and IgE production (McMenamin and Holt 1993; Kemeny et al. 1995), as well as in productive Th1 responses to viral proteins which normally results in a Th2 response and eosinophilia (Srikiatkhachorn and Braciale 1997).

In terms of the regulatory role of CD8 T cells in response to allo-antigens, it has been shown that they play an important role in the development of disease and disease progression in certain models. In a murine model of autoimmunity that resembles SLE, the injection of parental lymphocytes in to F1 recipients leads to the development of donor T cell mediated disease. If there is a lack of CD8 T cells in the donor inoculum given to F1 recipients in this model, such as with DBA/2 donor cells, this results in characteristic SLE like disease and a predominant Th2 type autoimmune response. In contrast, if there are CD8 T cells present in the lymphocyte population, as seen in cells taken from C57BL donors, and these are injected into F1 recipients, a Th1 phenotype is seen in the autoimmune reaction, and this Th1 response is dependent upon the presence of CD8 T cells (Via et al. 1987; Rus et al. 1995). In an allogeneic model, CD8 cells present during the immune response are critical for the generation of Th1 type responses.

From Figures 1-10, we see that there is a severe lack of CD8 T cells in the spleens of neonatal mice, and that this results in a much higher ratio of CD4 to CD8 T cells than the ratio seen in adult mice. In accordance with previous models of autoimmunity and transplantation, a challenge with allogeneic cells is expected to generate a robust immune response *in vitro* in a MLR. Upon challenge with allogeneic F1 stimulator cells *in vitro*, we have shown that spleen cells from adult mice of both C57BL/6 and BALB/c strains produce a predominant Th1 response

when responding in a MLR characterized by the production of large amounts of IFN-γ (Figures 20 and 23). The Th1 type responses seen in adult cultures upon challenge with allo-antigen correlate with the relatively greater number of CD8 T cells seen in adult spleens, and their subsequently lower CD4:CD8 T cell ratio. In comparison, the immune responses seen in neonatal cultures upon challenge with the same antigen are drastically different, with a Th2 or mixed response being generated in both C57BL/6 and BALB/c neonates (Figures 21 and 24). Again this mixed or Th2 response is correlated to the lack of CD8 T cells in the spleen of neonatal mice and their much higher CD4:CD8 T cell ratio. These results are consistent with other transplantation models involving lymphocyte populations responding against allogeneic cells in the presence or absence of CD8 T cells. The neonatal spleen cell cultures mimic that of a lymphocyte population that is devoid of CD8 cells, and thus produces a similar Th2 response. When the immune responses of adult and neonatal mice are compared, it is clear that there is a significant difference in the type of response generated. The adult Th1 response is characterized by many cells producing IFN-γ, with very few producing IL-4, resulting in a ratio of IFN-γ to IL-4 producing cells much greater than that seen in neonatal cultures (Figures 22 and 25).

5.2 The Addition of Isolated, Age-Matched CD8 T cells to Neonatal Cultures Leads to a Change in the Immune Response

The regulatory role of CD8 T cells discussed above provided the basis for the next set of experiments. In the experiments done by Via *et al* employing the injection of parental spleen cells into F1 recipients, it was shown that if CD8 T cells were added or removed from the donor inoculum, the immune response was altered significantly. If further DBA/2 CD8 T cells were added to DBA/2 splenocytes and transferred to the recipient, a Th1 response and acute GVH disease was seen, while, in contrast, when CD8 T cells were depleted from the C57BL donor inoculum, a Th2 response and SLE-like disease occurred. These observations provided the basis for the idea that if we can manipulate the CD4:CD8 T cells ratio in culture by adding CD8 T cells, we may be able induce a different type of response than that seen under normal conditions. After many attempts at isolating CD8 T cells from pooled neonatal spleens, we managed to add sufficient numbers of isolated CD8 T cells to result in a ratio similar to that seen in adult spleens.

The number of CD8 T cells isolated could be assessed using flow-cytometry, and a known number of CD8 T cells was added to neonatal *in vitro* cultures.

As shown in Figures 26, 27, 29, 30, and 31, in both C57BL/6 and BALB/c strains of mice, addition of CD8 T cells in "reconstituted" cultures resulted in a shift of the immune response from a mixed or predominant Th2 to a Th1 mode. Additionally, in Figures 29, 30, and 31, the addition of slightly more or fewer CD8 T cells resulting in an average CD4:CD8 T cell ratio of approximately 3.6 and 1.2, resulted in a mixed response and a strong Th1 response, respectively. These experiments provided evidence that the presence of CD8 T cells *in vitro* in this model could change a Th2 into a Th1 response.

5.3 The Presence of Isolated, Age-Matched CD8 T Cells Alters the Immune Phenotype of Responding Antigen-Specific CD4 T Cells

Once we showed that the presence of CD8 T cells in culture could influence the immune response overall, it was important to show that the effects were due to an influence of the CD8 T cells on the responding CD4 T cells in accordance with our three-cell model of T cell activation, mentioned in chapter 2. The presence of CD8 T cells led to an increased number of IFN-γ producing cells, but the question remained whether this increase was due simply to the presence of more CD8 T cells, or a change in cytokine production in responding CD4 cells? There was some indirect evidence for the latter possibility in that the number of cells producing IL-4 in reconstituted cultures was significantly reduced. Assuming that very few CD8 T cells are responsible for IL-4 production, it would follow that the IL-4 producing cells in the ELISPOT assay are predominantly CD4 T cells specific for antigen. The large reduction in IL-4 producing cells between neonatal and reconstituted cultures provided indirect evidence that there is some influence on the phenotype of responding CD4 cells by CD8 cells. We wanted to confirm this by showing that CD4 T cells that have been cultured in the presence or absence of CD8 T cells, i.e. neonatal and reconstituted cultures, show different immune phenotypes as assessed by an acute assay that enumerates effector T cells. In Figure 33, the number of cytokine-producing CD4 T cells in neonatal and reconstituted cultures is shown. Similar to total neonatal cultures, neonatal CD4 T cells from non-manipulated cultures display a mixed response, with both IFN-γ and IL-4

production. The CD4 T cells isolated from reconstituted cultures produce much less IL-4, and slightly more IFN-γ, indicative of a Th1 response. These results confirmed our idea that the presence of CD8 T cells in culture not only changes the immune response from a Th2 to a Th1 type response, but the CD8 T cells changed the fate of the responding CD4 T cells as well. Also of importance in Figure 33, is the observation recorded that the CD8 T cells producing cytokines in both neonatal and reconstituted cultures are present in similar numbers, the large majority of which produce IFN-γ. That the number of CD8 T cells producing IFN-γ is not significantly different confirmed our thoughts regarding the results from reconstitution experiments. The increase in the number of IFN-γ producing cells in the ELISPOT assay was not simply due to the presence of more CD8 T cells in reconstituted cultures.

5.4 Conclusion

After the discovery of the role of CD8 T cells as mediators of cellular killing and clearance of virally infected as well as cancer cells, their role as a suppressor of the antibody response was largely left behind. Additionally, despite vast amounts of literature on the mechanisms behind the Th2 bias of neonatal immune responses, there is little consensus on this issue despite its importance in terms of vaccination and early childhood disease. The hypothesis and the objectives presented in this thesis aimed to address the former issue regarding neonatal Th2 bias in the context of the CD4:CD8 T cell ratio in these individuals.

The literature on the direct impact of the CD4:CD8 T cell ratio on immune responses is limited. However, there have been some studies in humans that have shown the CD4:CD8 ratio is important in the context of certain diseases and aging. In a study of humans with ovarian cancer, those who had a higher proportion of CD8 T cells present in the population intraepithelial lymphocytes had a greater disease prognosis and higher mean survival time, both associated with a strong Th1 and CTL response (Sato et al 2005). A study on the homeostatic regulation of T cell numbers in humans, under either steady state conditions or during viral infection, noted that a "normal" CD4:CD8 T cell ratio is around 2:1, before the expansion of the CD8 T cell population in response to viral infection (Stockinger et al 2004). The CD4:CD8 T cell ratio in the context of aging has been sparsely studied, but there is evidence from human studies that aging is associated with an increase in CD4:CD8 T cell ratio (Amadori et al 1995; Adriaensen et

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al 2015). Analysis on the factors that determine the CD4:CD8 T cell ratio in humans showed that this ratio is under genetic control and that environmental factors have a very limited role in determining a person's CD4:CD8 T cell ratio (Amadori et al 1995). The same study examined healthy humans aging from 20 to greater than 60 years of age, with average CD4:CD8 T cell ratios ranging from 1.88:1 in individuals less than 30, to 2.79:1 in individuals greater than 60 years of age (Amadori et al 1995).   A recent paper examining the CD4:CD8 T cell ratios in elderly humans who were either seropositive or seronegative for Cytomegalovirus (CMV) showed a positive correlation between aging and increased CD4:CD8 T cell ratio, and that a CD4:CD8 T cell ratio of greater than 5:1 resulted in a diminished capacity to response effectively against CMV, as well as further physical impairments in those infected with CMV (Adriaensen et al 2015). Studies on the ratio of CD4:CD8 T cells in mice have been less common. A recent study on the age dependent levels of CD4 and CD8 T cells in both BALB/c and C57BL/6 mice showed that the splenic CD4:CD8 T cell ratio is higher, with a higher proportion of CD4 T cells in BALB/c mice, and that the ratio changes with age, but not to a significant degree (Pinchuk and Filipov 2008). This study on CD4:CD8 T cell ratio and aging in mice dealt with mice having already reached maturity, charting the percentages of CD4 and CD8 T cells after one month of age, rather than in newborn mice. These experiments do provide evidence that the CD4:CD8 T cell ratio can play a significant role in the developing immune response and disease outcome.

Studies on CD4:CD8 T cell ratios in neonates are not common, but there have been some on the numbers of different types of lymphocytes early in life. A study on age-related changes in lymphocyte populations in humans showed that there was a small change in CD4:CD8 ratio between subjects aged 2 days to 11 months and subjects in the three groups over 1 year of age (Erkeller-Yuksel 1992). A longitudinal survey of lymphocyte populations in a group of infants found that in the first year of life the peripheral CD4:CD8 T cell ratio is higher than that found in adults (de Vries 2000). In mice, it has been shown that the absolute number of CD4 and CD8 T cells and their percentages of the spleen and lymph nodes are significantly lower in neonates than in adult mice (Garcia et al 2000). Also, the CD4:CD8 T cell ratio through the first 16 days of life if different than the ratio seen in adult mice (Garcia et al 2000).

Despite the limited literature on the influence of the CD4:CD8 T cell ratio on the type of immune response generated, there has been a large number of studies in the influence of CD8 T

cells in controlling infection in certain models, and their role as regulator as well as effector cells during infection. If the presence of CD8 T cell is important for protective immunity in certain models and their absence is correlated with disease progression, this would be indirect evidence supporting the idea that a lower CD4:CD8 T cell ratio is important for controlling certain infectious diseases. In an altered HIV-1 model in pigtailed macaques, animals that had been depleted of CD8 T cell *in vivo* and infected with the altered HIV-1 virus capable of infecting certain simian strains had a rapid progression to AIDS, while untreated animals had an elite controller phenotype and did not succumb to the disease (Hatziioannou et al 2014). Another early study involving macaques showed that animals that had been treated with an anti-CD8 monoclonal antibody for CD8 T cell depletion *in vivo* were not able to control SIV infection, while animals treated with a control antibody could control virus infection and did not have viremia (Jin et al 1999). Another study involving HIV in humans showed that non-progressors have highly functional HIV-specific CD8 T cells and disease progressors are limited in their CD8 T cell functionality and this is inversely correlated with viral load (Betts et al 2006). CD8 T cells have also been shown to play a role in protection against *M. tuberculosis.* Rhesus macaques immunized with BCG or with *M. tuberculosis* and subsequently depleted of CD8 T cells lost the ability to control infection with the bacteria (Chen et al 2009). Mice that had been immunized and depleted of CD8 T cells had compromised immunity against the bacteria. Additionally, mice that had been previously infected and cleared the infection and then depleted of CD8 T cells lost the ability to control a subsequent infection (Chen et al 2009). These are examples in both bacterial and viral models that show the importance of CD8 T cells in controlling infections and for generating the appropriate type of immune response. The presence of more CD8 T cells and thus a lower CD4:CD8 T cell ratio therefore could be critical for protection against disease in certain circumstances.

In addition to bacterial and viral models where CD8 T cells are important for protection against disease progression, CD8 T cells can play a role in various autoimmune disorders. In experiment autoimmune glomerulonephritis in rats, an animal model of Goodpasture's Disease, T cell-mediated immunity is crucial for disease progression. If CD8 T cells are depleted from rats *in vivo,* autoimmunity is inhibited (Reynolds et al 2001). This reflected the importance of CD8 T cells in generating cell-mediated immunity that is necessary for disease progression. Another study found that CD8 T cells producing IFN-γ present in the synovium of rheumatoid

arthritis (RA) patients are at least partially responsible for inflammatory lesions, and that depletion of CD8 T cells in a human-SCID mouse model of RA reduced the incidence of inflammation, lymphotoxin production, and immunoglobulin in inflammatory lesions (Kang et al 2002). These examples, as well as the examples stated above in the experiments by Via et al and Rus et al showing the importance of CD8 T cell in autoimmunity induced via injection of parental lymphocytes into F1 hosts demonstrate the importance of CD8 T cells in the context of autoimmunity. The presence of a sufficient number of CD8 T cells and a subsequently low CD4:CD8 ratio can be critical in terms of disease outcome in bacterial, viral, and autoimmune models.

The knowledge that newborns respond with a predominant Th2 response coupled with evidence that an absence of CD8 T cells also results in a predominant Th2 response led to the hypothesis that newborn individuals likely do not possess large enough numbers of CD8 T cells to mount Th1 responses. This relative lack of CD8 T cells thus would result in a significantly different CD4:CD8 T cell ratio between newborn and mature individuals with the latter possessing a much lower splenic ratio, which we have shown is the case consistent with previous literature. These same mature and newborn individuals also responded differently when exposed to the same antigen, producing vastly different immune responses, Th1 made by the former, Th2 by the latter. According to our model, this difference in immune responses is due to differences in CD4:CD8 T cell ratios, with CD8 T cells playing a regulatory, as well as effector role in Th1 responses. After showing a difference in neonatal and adult immune resposnes, our goal was, specifically, to assess whether an alteration in the CD4:CD8 T cell ratio at the initiation of the immune response could result in a change in the Th1/Th2 phenotype of the response generated. We used isolated CD8 T cells to show that their addition to cultures of neonatal spleen cells, thus lowering the CD4:CD8 ratio, changes the immune response from Th2 to Th1. These experiments produce results similar to those seen in experiments testing the opposite phenomenon, whereby CD8 T cells are depleted from adult splenic cultures resulting in Th2 type responses. In addition, the presence of these CD8 T cells, in accordance with our hypothetical model, alters the phenotype of CD4 T cells specific for the antigen during the priming of the immune cells. Thus we have shown a regulatory role for CD8 T cells during the immune response, similar to that shown in parent into F1 models from greater than 30 years ago. The

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relative absence of CD8 T cells and thus their diminished regulatory function is most probably, in part, a reason for the predominant Th2 nature of immune responses made by neonates.

Chapter 6

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