MECHANISMS AND FUNCTIONAL CONSEQUENCES OF CD4 T CELL COOPERATION

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in the Department of Microbiology and Immunology

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ABSTRACT

CD4 "helper" T cells are central players in the regulation of immune responses. These T cells affect the magnitude and phenotype of immune responses, and indeed, affect whether or not the host responds, at all, to a given antigen. Thus, knowledge of the cellular and molecular requirements for the activation, differentiation, and survival of CD4 T cells is critical for understanding immune responses in general; this understanding may lead to advances in rational vaccine design and effective immunotherapy.

Evidence supports a role for CD4 T cells in the activation and acquisition of effector function of other CD4 T cells. In this thesis I set out to develop experimental systems to directly observe, and explore the mechanisms involved in, cooperative interactions between CD4 T cells, "CD4 T cell cooperation".

I developed two major experimental systems wherein cooperative effects in the activation and differentiation of CD4 T cells were observed. Firstly, we show that simultaneous administration, in incomplete Freund's adjuvant, of multiple peptides, but not single peptides, known to bind host class-two major histocompatability antigens (MHCII) and to stimulate endogenous naive CD4 T cells, results in the cooperative generation of cytokine-producing effector CD4 T cells, *in vivo*, as assessed by cytokine ELISpot assay. We demonstrate that these cooperative interactions depend on the presence of CD4 T cells with specificity for the administered peptides in the context of host MHCII. In the second experimental system, syngeneic, splenic antigen-presenting cells (APC), loaded *in vitro* with exogenous peptides, that bind the distinct MHCII molecules, I-A and I-E, mediate cooperation between peptide-specific CD4 T cell populations, *in vivo*, upon adoptive transfer. Furthermore, we find that, in order for cooperative interactions to occur, these peptides must be simultaneously presented by the same APC. In addition it appears that B cells, and not splenic dendritic cells, efficiently mediate this cooperation. We demonstrate that cooperation between CD4 T cell populations depends on OX40L, as blocking this molecule abrogates cooperative effects. Enhanced primary effector CD4 T cell generation, influenced by cooperation, results in enhanced secondary effector CD4 T cell responses, indicating that cooperation between CD4 T cells plays a role in the establishment of immunological memory.

A third experimental system allowed us to address the role of CD4 T cell cooperation in immunological self-tolerance. We find that, in general, activated CD4 cells are susceptible to inactivation by systemically administered peptides. However, under conditions that normally result in inactivation of effector CD4 T cells, the systemic ligation of CD40 or OX40 by agonistic antibodies, but not the administration of isotype-matched control antibodies, rescues cytokine production by these cells. Given the role of these molecules in CD4 T cell cooperation, our findings lead us to propose that cooperation between autoreactive CD4 T cells may, occasionally, result in sustained autoimmune responses.

The findings presented in this thesis allow us to make comprehensive models of the role of CD4 T cell cooperation in the activation, differentiation and, the maintenance of self-tolerance in CD4 T cells. Our findings have advanced our understanding sufficiently to allow for further experimentation in models of disease or immunological misregulation.

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The environment that is fostered in the department of Microbiology and Immunology at the University of Saskatchewan is unique. I knew that I had found a scientific home the first time that I tilted a bottle of pilsner with a room full of both professors and students. I hope that this atmosphere can endure the onslaughts of administrators and lawyers and that others will be able to experience the camaraderie that I felt during my time here.

Three scientists were key to my development over the years. Firstly, I would like to thank Popi Havele, who was the first to encourage me to pursue academics. The confidence that I received from this made me believe that I did have something to offer scientifically. I am grateful for this kindness as it is becoming increasingly rare.

I would like to acknowledge the contribution of Christopher Rudulier, one time fellow student and one of my best friends. Chris is a perennial source of optimism and an excellent sounding board for theoretical speculation. I will truly miss the opportunity to show him my latest results and discuss them for a number of hours, neglecting most other things.

Finally, the contribution of Peter Bretscher to both my scientific and philosophical development cannot be overstated. In his laboratory Peter cultivates a relaxed but serious atmosphere. He allows you to learn your own lessons and motivates you with stories of Francis Crick and Fred Sanger. Peter believes that his science addresses big issues, and he can convince you of that when at times you loose perspective. I realize it is standard to thank one's supervisor in the acknowledgements of a thesis. In this case I am also thanking a friend.

DEDICATION

I dedicate this thesis to Erica Kroeger, my best friend and wife, who celebrated the highs and endured the lows on my roller coaster called grad school.

PREFACE

In writing this thesis, I hoped that I could paint a picture of the study of CD4 T cell biology that would emphasize what I feel are important issues. Therefore, while the following chapters do not give a fully comprehensive view of the field, I hope that the reader will still be left satisfied that the remaining issues could be fitted into the models presented.

I have decided to write the experimental chapters in, what I have been told, is a somewhat unconventional way. I have given a description of how the experimental systems described were developed. Often this description depends, as did the research itself, on pilot experimentation. I have included this preliminary type of data in my thesis. I believe that it not only makes the experimental chapters more easily comprehensible, but also honestly reflects how the science was originally undertaken. Too often, published works are so highly polished that it is difficult to see how anyone could have conceived of the experiments as presented.

I hope that the reader can derive even a fraction of the pleasure from these experiments that I did.

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LIST OF ABBREVIATIONS

2A3	Isotype-matched control antibody for HRPN
AIRE	Autoimmune regulator
Akt	See PKB
ALUM	Aluminum hydroxide adjuvant
anti-μ	Anti-IgM antibody
APC	Antigen presenting cell
B Cell	Bursa, or bone marrow-derived lymphocyte
B7/B7.1/B7.2	Co-stimulatory molecules (CD80/CD86)
BCL-2	B cell lymphoma antigen 2
BCL-Xl	B cell lymphoma-extra large
BCR	B cell receptor
BH3	Third BCL-2 homology domain
BRBC	Burro red blood cell
BSA	Bovine serum albumin
CD_ antigen	Cluster of differentiation antigen
cDNA	Copy DNA
CDR3	Complimentarity determining region 3
CFA	Complete Freund's adjuvant
CNS	Central nervous system
conA	Concanavillin A
CpG	Poly deoxycytidine-deoxyguanosine DNA
CsA	Cyclosporin A
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTLA-4-Ig	Cytotoxic T lymphocyte antigen 4-Ig fusion protein
DC	Dendritic Cell
DCSIGN	DC-Specific Intercellular adhesion molecule-3 Non-integrin
DEC205	DC and eptihelial cell cell surface glycoprotein, 205 kDa
DNA	Deoxyribonucleic acid

DNP	Dinitrophenyl- (hapten)
DO11.10	T cell clone or mouse strain bearing an $OVA_{323-339}$-specific TCR
DTH	Delayed-type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethlyend diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISpot	Enzyme-linked Immunospot assay
FACS	Fluorescence-activated cell sorting
Fas	Fas death receptor (TNFR)
FasL	Fas ligand
FGG	Fowl gamma globulin
FGK45	Agonistic anti-CD40 monoclonal antibody
foxP3	Forkhead box P3
H-2	Murine MHC region
H-2D	H-2 D-encoded MHCI
H-2K	H-2 K-encoded MHCI
HA	Haemagglutanin
hCC	Human cytochrome C
HEL	Hen egg lysozyme
HGG	Human gamma globulin
HRPN	Isotype-matched control monoclonal antibody for OX86
I-A	I-A encoded MHCII
I-E	I-E encoded MHCII
IDDM	Insulin-dependent diabetes mellitus
IFA	Incomplete Freund's adjuvant
IFNγ	Interferon gamma
Ig	Immunoglobulin
IgG	Immunoglobulin with γ-encoded heavy chain
IgL	Immunoglobulin light chain
IgM	Immunoglobulin with μ-encoded heavy chain
IL-	Interleukin-

IP3	Inositol triphosphate
Ir	Immunity region
JHD	B cell deficient mouse strain with targete mutation in the J region of IgH
KLH	Keyhole limpet haemocyanin
LACK	Leishmania analog of activated C kinase
LP	*Lamina propria*
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting™ (Miltenyi Biotech)
mCC	Mouse cytochrome C
MCC	Moth cytochrome C
MD4	Strain of mouse transgenic for HEL-specific BCR
MDP	Muramyl dipeptide
MHCI	Major histocompatability antigen class I
MHCII	Major histocompatability antigen class II
mIgM	Strain of mouse having only membrane-bound IgM
MLR	Mixed lymphocyte reaction
MOG	Myelin oligodendracyte protein
MS	Multiple sclerosis
MUC.1	Cell surface associated mucin 1
μMT	B cell deficient mouse strain with IgHM targeted mutation
NFATc1	Calcineurin-dependent nuclear factor of activated T cells 1
NFκB	Nuclear factor kappaB
NOD	Non-obese diabetic (mouse strain)
OVA	Chicken ovalbumin
OX40	OX40, CD134
OX40L	Natural ligand for OX40
OX86	Agonistic OX40 monoclonal antibody
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with twean-20
PCC	Pigeon cytochrome C

PI3K	Phosphoinositor 3 kinase
PIP2/3	Phosphoinositol di-/tri-phosphate
PKC	Protein kinase C
PLL	Poly L-lysine
PMA	Phorbol myristate acetate
PTK	Protein tyrosine kinase
QRTPCR	Quantitative real-time polymerase chain reaction
RA	Retinoic Acid
RAG	Recombinase activating gene
RF	Rheumatoid factor
RM134L	OX40L blocking monoclonal antibody
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institue Medium 1640
S+	Positive co-stimulatory function
SLE	Systemic lupus erythramatosis
SRBC	Sheep red blood cell
T cell	Thymus derived cell
TCR	T cell receptor
TGFβ	Transforming growth factor beta
Th1	Type 1 CD4 T-helper cell
Th2	Type 2 CD4 T-helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNFSF	Tumor necrosis factor superfamily
TNP	Trinitrophenyl- (hapten)
TRAF	Tnf receptor associated factor
Treg	Regulatory CD4 T cell
Vbeta17a	Variable TCR region 17 a

1.0 CHAPTER 1 – INTRODUCTION

1.1 General Introduction to Immunology

The beginning of scientific study of the immune system dates to roughly the mid 1700s. However, long before this, one of the major hallmarks of the adaptive immune system, immunity, was recognized. Observations of Greek historians, amongst others, suggest that ancient folk knowledge held that those who suffered sickness, associated with a given plague, and recovered, were free to tend to the sick, without fear of further infection (Silverstein 1989; Silverstein 1999). This essential feature of the vertebrate immune system, specific immunological memory, and how it is induced, remains one of the most studied phenomena in immunology.

The cellular and molecular mechanisms surrounding how immunity is achieved were the subject of heated debate for decades in the 1800s. One of the first mediators of adaptive immunity identified was antibody, a substance present in the serum of immune animals that was found to specifically bind antigen and mediate its destruction or removal from solution. Some believed that antibody was the sole mediator of antigen clearance. Others, like Metchnikoff, argued that phagocytic cells were the main effectors of immunity.

The beginnings of modern immunology can be traced back to the theories of Paul Ehrlich who advanced a particularly interesting theory for the formation of antibody (Silverstein 2005). Ehrlich believed that antibody was a manifestation of increased production of receptors present on the surface of a cell. To Ehrlich, these receptors were nutrient receptors that would be blocked by a toxin. Thus, when a toxin bound to a given receptor, the cell would respond to this toxin by producing excess receptor, including secreted forms, which neutralized the toxin. While it was subsequently demonstrated that

Ehrlich's theory could not be generally true, the essential features of his model bear a close resemblance to modern models for antibody production. In this model antigen selects the antibody from a pre-existing repertoire.

Those immunologists who believed that antibody was the sole effector mechanism mediating immunity eventually became dominant. Immunological researchers in the early 1900s were consumed by biochemical analysis of antibody-antigen interactions and the functional consequences of this. However, in 1946 Merrill Chase demonstrated that a particular form of immune reaction, delayed type hypersensitivity (DTH), could be transferred between mice, not with serum from sensitized animals, but with leukocytes (Chase 1946). Cellular immunology was again brought into the consciousness of immunologists. In the following years, the cells responsible for antibody production, the bursa of fabricius-derived or, B cells, were identified in chickens due to the work of Bruce Glick (Ribatti, Crivellato et al. 2006). Subsequently, similar cells were identified in mammals. Investigations on the requirements for antibody production led to the identification of thymus-derived cells, T cells, as "helpers" for antibody production, and as the cells that mediate DTH (Claman, Chaperon et al. 1966; Miller and Mitchell 1968; Mitchell and Miller 1968; Mitchell and Miller 1968).

The mechanistic requirements for antibody production remained poorly understood throughout the early 1900s. Many believed that antigens instructed the formation of complimentary antibody. Jerne suggested that the existence of "natural antibodies", which pre-exist antigen exposure in unmanipulated animals, was evidence that antigens *selected* antibodies for production (Jerne 1955). In some ways this idea was rather similar to Ehrlich's theory. Talmage extended this idea, suggesting that antigen selected a cell, bearing antibody, for multiplication leading to increased antibody production by multiple clones (Talmage 1957). Talmage's ideas were borrowed by Burnet (Burnet 1957) and their "clonal selection theory" was subsequently summarized and modified to accommodate accumulating evidence on the role of DNA in protein synthesis by Lederberg (Lederberg 1959).

The clonal selection theory has become a pillar of immunology. The basic tenets of the theory, paraphrased from a modern review by Silverstein (Silverstein 2002) are: 1)

the entire immunological repertoire (antibody molecules) is generated in the host (i.e. it is not germline encoded), 2) each antibody is uniquely expressed by a single cell, 3) when these antibodies interact with antigen, the antibody-bearing cells proliferate and differentiate, and 4) some of these expanded clones produce antibody while others become "memory" cells. These basic principles were demonstrated to be generally true. Both B cells and T cells express single antigen receptors that are encoded by somatically re-arranged genes. The need for an extensive immune repertoire means that clones are particularly rare and therefore selection of these cells as the multiplying unit, leading to effector functions in immunity, is essential.

The idea that immune cells develop their repertoire somatically poses a potential problem. If this repertoire is randomly generated, as is more or less true, then often cells will be produced that bear antigen receptors specific for self-components. Burnet was particularly aware of this problem, and as early as 1949 proposed that a mechanism for removing reactivity to self, or for maintaining "self-tolerance" must exist (Burnet and Fenner 1949; Silverstein 2002). As I will describe below, intricate mechanisms have evolved to ensure that the immune system has the capacity to respond to virtually any pathogen, while at the same time remaining tolerant to the host's antigens.

1.2 Activation and Inactivation of CD4 T cells and the Maintenance of Self-Tolerance

1.2.1 Introduction

It has long been known that the immune system has the capacity to recognize self-components. In order to avoid pathology it is important that checkpoints exist to control the activation of the powerful effector mechanisms of the immune response against the host. Paul Ehrlich's famous coining of the term *horror autotoxicus*, translated as "the horror of autotoxicity", to describe the body's tendency to avoid producing antibodies that react to self, exemplifies this (Silverstein 1989). Some of the originators of the clonal selection theory also bore this idea in mind and they later proposed various models for how autoreactive lymphocyte clones could be removed during development. While

autoimmunity does exist, it is a rare phenomenon. In cases where control mechanisms fail, autoimmunity ensues. As I will describe, much that is encapsulated in the ideas of early immunologists has been demonstrated, while some findings, that would be surprising to them, demonstrate the sophisticated mechanisms that have evolved to limit autoimmunity.

The prolific Joshua Lederberg realized that naive precursors to antibody-producing cells, that had specificity for self-components, should be removed to avoid the production of autoantibody (Lederberg 1959). Implicit in this idea was his proposition that the genes that encoded antibody molecules were capable of encoding autoreactive molecules as a result of the random mutations in the chromosomal DNA that lead to antibodies of more-or-less random specificity. Having previously reported that the cells that produce antibody are monospecific (Nossal and Lederberg 1958), he proposed that immature precursor cells, upon detection of antigen, for which their single receptor has affinity, are eliminated. Thus, it is both the specificity and the maturity of the precursor cell that determined whether it was sensitive to removal/inactivation, or induction to produce secreted antibody. According to this model, mature precursor cells, upon interaction with antigen, can only be induced. As I will describe below, this proposition was found to be inconsistent with experimental observation. Nevertheless, much of what is found in Lederberg's elegant theoretical paper, *Genes and Antibodies* (1959), proved to be true, including his description of how small numbers of precursor cells divide upon antigen exposure, enhancing the number of cells that are available to produce the massive quantities of antibody seen upon immunization, and his description of how persistence of these "clones" leads to enhanced secondary immune responses.

Talmage and Pearlman offered their own model by which autoreactive antibody (autoantibody) production could be limited. According to their hypothesis, the binding of antigen alone, to antibody on the cell surface, would be insufficient to result in significant antibody production. However, a distinct stimulus could be delivered to the precursor cell by the degree of antigen aggregation when bound to the cell; appropriate delivery of the second stimulus results in antibody production (Talmage and Pearlman 1963). Supposedly, self-antigens would not be present in the appropriate concentration, or in a certain physical state, to cause appropriate aggregation of the antibody receptor. While

this model supposed, erroneously, that inactivation of autoreactive cells is not a major mechanism by which self-tolerance is maintained, the assertion that more than the simple binding of antigen to the antibody receptor, borne by a precursor cell, was needed to result in antibody production was prophetic.

1.2.2 The Two Signal Model for Lymphocyte Activation

By the end of the 1960's it became increasingly clear that binding of antigen alone to an antibody-bearing precursor cell was insufficient to result in the production of antibody. The two-signal model for lymphocyte activation, first put forward by Bretscher and Cohn, attempts to reconcile difficulties of a minimal model for antibody induction, the binding of antigen alone, and a number of experimental observations regarding the requirements for induction and inactivation of antibody producing lymphocytes (Bretscher and Cohn 1968; Bretscher and Cohn 1970; Bretscher 1972). According to the more polished 1970 version, interaction of a lymphocyte with antigen through the antigen receptor (signal 1) results in death of the cell unless it also receives another signal (signal 2), that is provided by a T cell. This model was the first to give an essential role to the T cell (or T cell dependent factors), in the activation of the B cell, beyond simply an accessory function in facilitating the binding of antigen to B cells.

Three major types of observation are cited in the original papers describing the two-signal model for the primary activation of lymphocytes that are consistent with that model, but inconsistent with a simple antigen-binding model, for how antibody production is induced.

Macromolecular haptens, such as poly-L-lysine (PLL), were employed by some to demonstrate that the mere presence of a substance that can bind antibody is often insufficient to result in antibody production (McDevitt and Benacerraf 1969). Individuals of certain strains of guinea pig do not respond to PLL alone, by producing anti-PLL-antibody. However, the same guinea pigs have the immunological capacity to produce antibody towards PLL as, upon immunization with PLL coupled to bovine serum albumin (BSA), but not when PLL is coupled to guinea pig serum albumin, anti-PLL antibody is made (Benacerraf, Green et al. 1967). Thus PLL perfectly fits the definition

of a hapten in certain strains of guinea pig; it binds antibody but is not itself immunogenic. The finding that coupling a foreign protein to PLL, but not a homologous serum protein, results in antibody production, suggests that simultaneous recognition of this foreign protein is required for anti-PLL antibody to be produced, a proposition that is inconsistent with the idea that the simple binding of PLL, by an antibody-bearing cell, should induce antibody production.

Secondly, observations were made on the role of carrier proteins in generation of secondary anti-hapten antibody responses, which suggested that these responses involve simultaneous recognition of more than one foreign determinant. For example, it was found in rabbits that in order for a secondary anti-hapten antibody response to occur, the rabbits must be previously immunized with both the hapten and the carrier (Rajewsky, Schirrmacher et al. 1969). The suggestion that antibodies may bind both the hapten and part of the carrier protein together, leading to efficient responses, was shown to be unlikely by the results of experiments of the following type: rabbits immunized with a hapten (h)-X (where X is a carrier protein like human gamma globulin), will respond with secondary-type antibody production when challenged with h-X, but not when challenged with h-Y, where Y is an unrelated, non-crossreacting carrier. However, if these rabbits are primed with h-X and Y, secondary antibody production is observed whether they are challenged with h-X or h-Y. Rajewsky makes reference to similar results that were originally reported by Mitchison in mice (Mitchison 1969). This "carrier-effect" was eventually seen as evidence supporting cellular collaboration (Mitchison 1971). Clearly, the results of experiments of this type, including others made with isozymes of lactate dehydrogenase (Rajewsky, Rottlander et al. 1967) are at odds with an antigen-binding model of antibody production. Simultaneous recognition of both hapten and carrier is needed to result in enhanced secondary immune responses.

Finally, studies on the breaking of the unresponsive state, by immunization with cross-reacting proteins, in rabbits given heterologous serum proteins at birth, were cited as evidence that simple binding of antigen, to cell-bound receptors, was insufficient to induce antibody production. Classical studies had shown that rabbits could be made immunologically unresponsive to certain proteins if given around the time of birth (Hanan and Oyama 1954). The tolerance was fairly stable. Rabbits given BSA, HSA, or

human gamma globulin (HGG) remained unable to immunologically clear these antigens for months after the initial induction of unresponsiveness (Humphrey 1964). Even upon challenge with the same protein with the adjuvant ALUM, most rabbits fail to make robust antibody responses (Humphrey 1964). Thus, the results of experiments reported by Weigle are somewhat surprising (Weigle 1964). Rabbits made unresponsive to BSA by administration of high doses of the protein, shortly after birth, remain tolerant upon challenge with BSA. However, when the cross-reacting protein HSA, containing some epitopes that are common to BSA and HSA and some epitopes unique to HSA, was employed as a challenge, previously tolerant rabbits responded by making antibody that reacted with both HSA and BSA. Thus, immunization with an antigen containing both epitopes to which the rabbits were supposed to be tolerant of, and epitopes that were "foreign", allowed antibody production against both types of epitope. These results could not be readily understood under the simple antigen-binding model.

In all cases above, the precursor cells that can produce antibody, upon appropriate immunization, are present in the experimental animals but are not induced to produce antibody. That is, the simple presence of the hapten, or antigen, does not induce antibody production. In all cases, it appears that the recognition of other antigenic determinants is required to result in antibody production. This observed requirement led to Bretscher and Cohn's suggestion that cooperation between multiple cells bearing antibody (or the antibodies themselves) is required for antibody production.

Citing the experimental evidence above, in the formulation of the two-signal model for lymphocyte activation, Bretscher and Cohn make the following general predictions for the generation of immune responses. Firstly, induction of antibody production requires a second signal; this signal comes from the presence of "associative" antibodies (or cells bearing these antibodies) specific for the carrier protein. In contemporary terms, B cells require the presence of T cells, which recognize physically linked determinants of the same antigen, to produce antibody. Secondly, if no second signal, deriving from the recognition of other parts of the antigen, is received, paralysis, or inactivation, of the precursor antibody-bearing cell ensues. The authors also suggest that the induction of carrier-specific antibody, or the cells that produce it, follows the above two-signal rules. Given the fairly recent discovery of collaboration between bone

marrow-derived and thymus-derived cells in the induction of antibody responses (Claman, Chaperon et al. 1966; Miller and Mitchell 1968; Mitchell and Miller 1968; Mitchell and Miller 1968; Nossal, Cunningham et al. 1968), it was suggested that carrier-specific antibody is produced by T cells, while the true hapten-binding antibody is derived from bone marrow cells. Since recognition of the carrier is required for antibody production, under this model, all antibody responses should be thymus dependent. Finally, tolerance to self-components results from inactivation of both B cells and T cells, that react to self antigens (signal 1), but do not collaborate with other autoreactive cells (signal 2), as these cells should be eliminated, one by one, as they are generated. Lymphocytes specific for foreign antigens may accumulate in the absence of these antigens and, upon antigen impingement, may collaborate, resulting in antibody production.

The two-signal model for lymphocyte activation accounted for many experimental observations better that a simple antigen binding model. However, this model failed to stimulate much experimentation until nearly two-decades after its initial proposal. It seems that a conceptual block had occurred, possibly due to the ambiguity of the nature of antigen recognition by T cells, that limited immunologists' ability to critically test this model. However, when people began to test some of the predictions of this model, many of its essential features were found to be correct. In general, as we will see below, signal one, delivered to lymphocytes by their interaction with antigen, results in inactivation, while a second signal is required for their activation to enable them to give rise to descendants that provide effector functions.

1.2.3 Activation of CD4 T cells

The main cellular effectors of the adaptive immune response are B cells, which produce antibody, and cytotoxic $CD8^+$ T cells which kill self-cells under appropriate circumstances. Most antibody responses require the presence of thymus-derived cells, called $CD4^+$ helper T cells (Claman, Chaperon et al. 1966; Miller and Mitchell 1968). Keene and Foreman found that the activation of CD8-bearing T cells, specific for the murine MHCI-like antigen Qa1, required help from CD4 T cells specific for a linked

antigenic determinant (Keene and Forman 1982). Extending these findings Guerder and Matzinger showed that, in the absence of CD4 T cell help, exposure to antigen can inactivate CD8 T cells (Guerder and Matzinger 1992). Thus, all the major effector cell populations of the adaptive immune system abide by the rules of the two-signal model. However, in the early 1970s, the issue of how T lymphocytes are activated remained unresolved. The issue was complicated by the unknown nature of the antigen receptor of T cells (later known as the T cell receptor; TCR), and also by findings, discussed below, on the role of MHC in antigen recognition by T cells.

In the late 1960s Hugh McDevitt made a remarkable discovery, the ability of an animal to respond to a given antigen can be genetically controlled. The ability to respond to synthetic polypeptide antigens, by the production of potent antibody responses, depended on the existence of certain "immune response" (Ir) genes in mice (McDevitt and Sela 1965). The Ir locus mapped to the same segment of the germline DNA as H-2, the mouse major histocompatability gene complex (MHC) (McDevitt and Tyan 1968; McDevitt and Chinitz 1969). Interactions between the helper T cell, bearing the CD4 surface antigen, (CD4 T cell) and antigen-specific B cells, resulting in antibody production, were found to require compatibility at the H-2 "locus" (Kindred and Shreffler 1972; Kindred and Weiler 1972). Furthermore, it appeared that the proliferation in culture of previously primed T cells, in response to antigen in culture, depended on the presence of syngeneic "peritoneal exudate macrophages" and that proliferation could by blocked by antisera to H-2 encoded antigens (Shevach, Paul et al. 1972; Rosenthal and Shevach 1973; Shevach and Rosenthal 1973). Taken together, these studies led to the suggestion that the germline DNA-encoded MHC antigens played an essential role in the initiation of T cell-dependent immune responses.

The surprising finding of Zinkernagel and Doherty (Zinkernagel and Doherty 1974), leading to their Nobel Prize, that cytotoxic T cells kill virally infected target cells only when they share identity at the MHC, tied the above observations together. T cells, it appeared, recognize antigen only in association with MHC-encoded cell surface molecules, which, according to Zinkernagel and Doherty, were altered by the presence of foreign antigen. CD8+ CTL were found to recognize antigen in an MHC class I (MHCI) restricted fashion, requiring identity at only the H-2K or H-2D loci (Zinkernagel and

Doherty 1974). This finding was independently confirmed by Shearer who showed that CTL-mediated lysis of trinitrophenyl- (TNP-) modified splenocytes is restricted by the presence of the appropriate H-2K or H-2D allele on these target cells (Shearer, Rehn et al. 1975). Miller demonstrated that CD4+ T cells, the helper cell for antibody production, and the cell responsible for delayed-type hypersensitivity, respond to antigen in association with Ir gene/MHCII encoded I-A and I-E gene products (Miller, Vadas et al. 1975). Thus, MHC controls the activation and effector function of T cells.

The discovery of the MHC restriction of T cell antigen recognition had a dramatic impact on the field of immunology. At first, it was unclear what the implications of these findings were for the generation of T cell responses. For example, it was unclear whether antigen was recognized in complex with MHC or whether the MHC and antigen were detected as separate molecules bound on the surface of antigen-presenting cells (APC) that were recognized separately by different T cell receptors. However, in the absence of a clear description of the TCR, these possibilities could not be resolved.

While some immunologists made progress on the nature of the T cell-antigen interaction, some remained interested in the mechanistic rules governing the activation of T cells. In 1975, Lafferty and Cunningham published a half theoretical, half experimental paper in which they proposed a new two-signal model for the activation of alloreactive T cells (Lafferty and Cunningham 1975). The authors noted that in mixed lymphocyte reaction (MLR) cultures, only certain cell types were able to effectively stimulate T cells. The cells were called stimulator cells and thus possessed a positive stimulatory (S+) phenotype. The authors drew heavily on the proposals of Bretscher and Cohn, but placed the responsibility for delivering signal 2 to T cells on the shoulders of S+ cells. Thus, S- cells deliver only signal one to T cells, resulting in their inactivation, while antigen-presenting cells that have, or acquire, the S+ phenotype, deliver both signal one and signal two, resulting in activation of T cells.

Subsequent studies supported the propositions of Lafferty and Cunningham. Yano, Schwartz, and Paul confirmed the findings of Shevach (1973), originally made in a guinea pig-based system, that the proliferation of previously activated T cells required macrophages of the same H-2 haplotype; moreover, the authors showed that these macrophages must be viable to induce proliferation (Yano, Schwartz et al. 1977).

Presumably these macrophages provided additional signals to induce T cell proliferation. Work by others, employing the mitogenic lectin, concanavalin A (conA) as an analog of antigen stimulation, lent further weight to the notion that macrophages, or "accessory cells", provided essential second signals for the activation of T cells. Habu and Raff showed that conA stimulation of highly purified T cells was insufficient to induce proliferation (Habu and Raff 1977). However, the proliferation of T cells, by conA, could be induced by also supplementing these cultures with spleen-derived plastic-adherent accessory cells, presumably macrophage-like cells. Larsson took this system further, confirming the requirement for accessory cells, but also showed that conA stimulation alone made T cells receptive to stimulation by growth factors present in the supernatant of culture of truly activated T cells (Larsson and Coutinho 1979). The presence of such T cell growth factors in the supernatants of conA-stimulated T cells had been demonstrated a few years previously (Paetkau, Mills et al. 1976). From a modern standpoint, these growth factors are likely to be cytokines such as interleukin-2 (IL-2). Thus, antigen-mediated signals alone appear to be insufficient for T cell activation, but do affect these cells, making them receptive to second signals.

Studies involving the culture of T cells with macrophage-like accessory cells provided another great insight into how activation of T cells is achieved. Following the lead of others who showed that processing of antigen may be involved in presentation to T cells (Weinberger, Herrmann et al. 1981; Ziegler and Unanue 1982), Scala and Oppenheim demonstrated that catabolism by human macrophages was essential for the priming of antigen specific T cells, but not alloreactive T cells, in culture (Scala and Oppenheim 1983). Moreover, inhibition of antigen processing by chemical fixation did not have any effect if done longer than two hours after the initial addition of antigen to culture, suggesting that processing of antigen occurs prior to presentation. Later that year, Shimonkevitz et al. demonstrated that a T cell clone specific for ovalbumin (OVA) would release IL-2 when fragments of artificially "digested" OVA were added exogenously to cultures of this clone and fixed APC (Shimonkevitz, Kappler et al. 1983). The authors concluded that digestion of antigen is both necessary and sufficient to enable antigen presentation to T cell clones in association with MHC molecules.

The isolation of the T cell receptor protein complex by Haskins et al. (Haskins, Kubo et al. 1983), and the elegant identification of the genes encoding of the TCR by Hedrick, Cohen and Davis in 1983 (Hedrick, Cohen et al. 1984), revealed that T cells recognize antigens via a single antigen receptor. Conceptually, this posed a problem for immunologists. How could a single receptor be responsible for both antigen recognition and MHC restriction? The solution was provided by Babbitt et al. working with Emil Unanue and confirmed and extended by Buus et al. in the laboratory of Howard Grey (Babbitt, Allen et al. 1985; Buus, Colon et al. 1986; Buus, Sette et al. 1987). Peptides, derived from digested protein antigens, bind hydrophobic clefts in MHC molecules. The antigenic peptides are presented to T cells as a single complex with the MHC-encoded surface molecules. Antonio Lanzavecchia demonstrated that antigens, presented by B cells to CD4 T cells, resulting in antibody production, must also be processed and presented in fragments via MHCII molecules. Thus, the role that the MHC gene products play, in the activation of all the major T cell effector functions, was resolved.

These major discoveries in immunology revealed nature's beautiful mechanism for ensuring antigen recognition by T cells is restricted by the highly polymorphic MHC. They further opened up the field, for the investigation of the biology of T cells, by removing the conceptual block surrounding the nature of T cell recognition of antigen. As we will discuss below, progress on the rules for T cell activation and inactivation was made rapidly in the following years.

1.2.4 Dendritic Cells, the True S+ cell?

Throughout the time that the above studies were undertaken, the biology of dendritic cells (DCs) was being worked out. At the time of the initial identification of the DC, Steinman could not have known how big the field of DC biology would become (Steinman and Cohn 1973; Steinman 2003). DCs were characterized as a distinct population of cells, present within the population of adherent lymphoid cells, based on morphological and cell-surface-antigen phenotypes. These were shown to be non-B, non-T, and non-macrophage cells with a branching morphology, as reviewed by Steinman and Nussenzweig (Steinman and Nussenzweig 1980).

The first evidence of a functional role for DCs came from MLRs carried out with various APC populations, or purified DCs, as stimulators (Steinman and Witmer 1978). It was demonstrated that the ability of various lymphoid populations, including splenocytes, to stimulate allogeneic T cells in MLR cultures, correlated with the numbers of DCs that were present in these populations. The authors concluded that DCs are at least 100-fold more potent in stimulating T cells in culture than are B cells or macrophages. Moreover, purified macrophages were very poor stimulators after thorough depletion of DCs. The finding that B cells, macrophages, and DCs express the I-A (MHCII) antigen at similar levels, led Steinman to suggest that molecules other than antigen alone are recognized, in association with DCs, in order for maximal proliferation to occur. It was suggested that the discrepancy between this finding and those of others, like Rosenthal and Shevach (1973), that macrophages are potent stimulators in MLR cultures, might be due to contamination of macrophages cultures with DCs. The importance of DCs as APC in the MLR was underscored by a later study wherein DCs were selectively depleted from allogeneic stimulator populations. Depletion of DCs by provision of the 33D1 antibody and complement, removed between 75 and 90% of the stimulatory capacity of allogeneic spleen (Steinman, Gutchinov et al. 1983). Thus, DC appeared to be very potent APC.

Another functional role of the DC was demonstrated by Nussenzweig when he showed that DC are able to stimulate syngeneic T cells to divide in culture (Nussenzweig and Steinman 1980). This capacity was not due to the antigen specificity of T cells and required T cell-DC contact. Thus, DCs constitutively induce T cell proliferation in culture. It is possible that this proliferation is driven by the presentation of antigens "carried over" by DCs from the animals from which they are isolated. However, the possibility that this proliferation was influenced by the presentation of heterologous serum proteins, present in the culture medium, was excluded by employing only isologous serum in the DC preparation and culture procedures. Allogeneic stimulation by DCs was still found to be at least ten-fold more potent than syngeneic stimulation, and syngeneic cultures did not yield CTL, whereas allogeneic cultures did. DCs are also able to generate CTL specific for TNP-modified self cells, a common antigen-specific target (Nussenzweig, Steinman et al. 1980). However, the syngeneic MLR, as it was termed, appeared to have a functional role in inducing antibody responses. Inaba et al.

demonstrated that DC-T cell association allows for the production of "helper factors" for antibody production by B cells. Helper factors are presumably cytokines such as IL-4. According to Inaba, DCs activate the T cells to produce IL-2, which in turn stimulates the production of helper factors by these T cells. The culture supernatants from syngeneic MLR have the ability to stimulate antibody production when supplied to cultures of B cells and xenogeneic erythrocytes. While no antigen-specificity was found in syngeneic MLRs, the production of B cell helper factors was found to be dependent on MHC gene products, as antibodies to these blocked the ability of DC-T cell culture supernatants to stimulate antibody production (Inaba, Granelli-Piperno et al. 1983). The syngeneic MLR is a curious phenomenon that is no longer discussed in DC biology. Later studies demonstrated a potent, antigen-dependent APC role for the DC.

Through the 1980s great strides were made in uncovering the biology of T cells. The discovery of MHC-restriction and the isolation of the TCR resolved long-standing questions about how T cells "see" antigen. All the known major functions of T cells could finally be understood in the context of MHC restriction. Proliferation to allogeneic MHC, transfer of DTH, and help for the production of antibody and in the generation of CTL, were now understood as requiring recognition of peptide antigens, in association with the MHC gene products, by CD4 T cells. Significantly, DC have been shown to be involved in all of these phenomena. Though it took many years for the consequences of the work of Steinman and his colleagues to be thoroughly appreciated, these have become central to current immunological paradigms. The awarding of the 2011 Nobel Prize in Physiology and Medicine to Ralph Steinman makes this point.

1.2.5 Shaping of the CD4 T cell Repertoire in the Thymus

Thymus derived cells are known to be essential for the development of antibody responses. It turns out that the thymus organ itself, is essential for the development of thymus-derived cells, as evidenced by classical studies on neonatal thymectomy. However, how the thymus is involved in the generation of T cells, and what its role is in shaping the repertoire of these cells, was not well established until well after the discovery of MHC restriction. Today it is known that the thymus plays an essential role

in ensuring that maturing T cells can recognize peptides in the context of self-MHC molecules while affecting elimination of those thymocytes whose T cell receptors (TCRs) react strongly with self peptide-MHC complexes. These processes have been called positive and negative thymic selection, respectively.

The thymus positively shapes the T cell repertoire, ensuring that T cells have affinity for self-MHC. Some of the first evidence that demonstrated a specific function of the thymus in positively determining the specificity of T cells came from Michael Bevan's work with irradiation bone marrow chimeras (Bevan 1977). Irradiated parental strain mice were engrafted with bone marrow from F_1 offspring derived from a cross between the parental strain and an H-2 disparate strain. Mature F_1 T cells from chimeric mice, having developed in the presence of only one H-2 haplotype, efficiently killed virally infected target cells possessing the MHC molecules of the host, but not the other parent. Thus, the specificity of developing T cells is determined by the H-2 haplotype of the host. Zinkernagel confirmed this finding a year later employing a very similar experimental system. CTL of F_1 into parental mouse bone marrow chimeras only lysed vaccinia infected target cells of the parental strain in which they developed (Zinkernagel, Althage et al. 1978). Studies like these led to the notion that thymocytes, that develop a TCR that interacts with self-MHC molecules, are given survival signals, the "kiss of life", whereas those that do not are left to undergo "death by neglect". Thus the thymus positively selects the affinity of the TCR.

Later experiments confirmed a role for the thymus in positive selection and extended our knowledge of how this process occurs. Kisielow et al. took advantage of transgenic mouse technology to formally address this issue. In radiation chimera experiments, MHC congenic mice were engrafted with bone marrow from mice bearing a transgene-encoded TCR with affinity for a peptide of the male specific antigen, H-Y, in the context of H-2D^b. Host mice that expressed H-2D^b supported the generation of high numbers of mature CD8$^+$ single positive thymocytes bearing the transgenic TCR, whereas no cells bearing the transgene-encoded TCR could be found in the thymus or periphery of host mice lacking H-2D^b. However, the less mature CD4$^+$CD8$^+$ "double positive" thymocytes did show evidence of transgene-encoded TCR expression, suggesting that positive selection depends exquisitely on the presence of the correct restricting element

during the maturation of thymocytes from the double positive stage to the CD8 (or CD4) single positive stage (Kisielow, Teh et al. 1988). More recently, Chmielowski et al. have shown that a normal repertoire of TCRs is expressed in mice that express only a single peptide-MHC complex in the thymus (Chmielowski, Muranski et al. 1999). CD4 T cells from these mice respond well to peptides that are not related to the positively selecting peptide and there is no evidence that these cells have enhanced reactivity towards the positively selecting peptide, or derivatives thereof. These results reinforce the ideas that positive selection functions mainly to ensure that T cells bear TCRs with affinity for self-MHC, and that positive selection is not dependent on the peptides that are presented.

At the time that thymic positive selection was being investigated, the first findings that the thymus had a role to play in maintenance of T cell self-tolerance were emerging. Shimon Sakaguchi made important discoveries on the role of the thymus in generating regulatory T cells that help maintain tolerance to self in a dominant fashion. However, the implications of these findings were not fully appreciated until much later, as will be discussed below. Rammensee and Bevan, and independently, Matzinger and Waldman, showed, by the use of sophisticated experimental systems, that tolerance to self-antigens is restricted by the host MHC (Matzinger, Zamoyska et al. 1984; Rammensee and Bevan 1984). Both groups show that in the T cell repertoire of normal animals, precursor CTL exist that have affinity for self-antigens (minor histocompatability antigens) when presented in the context of foreign MHC. The system of Rammensee and Bevan was quite elegant. Mixed lymphocyte cultures were set up in the classical way, however directly alloreactive T cells were caused to undergo apoptosis by labeling with bromodeoxyuridine and exposure to UV light. This protocol resulted in a population of CTL, from strain A, that could not kill strain B targets. The few remaining cells, following multiple rounds of selection by this protocol, were re-stimulated with irradiated A.B cells (from MHC congenic mice expressing the B MHC molecules). A population of CTL were expanded that could lyse A.B targets, but neither A targets nor B targets. Presumably these CTL recognized self- (A) minor histocompatability antigens in the context of B MHC. This presumption was shown to be true by the finding that these CTL lysed AxB F_1 cells. Thus, tolerance to self-antigens is achieved in an MHC-restricted fashion.

Kappler et al. were the first to directly demonstrate negative selection in the thymus (Kappler, Roehm et al. 1987). Thymocytes that express Vβ17a-encoded TCR segments, which imparts affinity for I-E to many of the TCRs, exist in a normal proportion in I-E deficient mice. However, in I-E expressing mice, the single positive thymocytes and peripheral T cells expressing Vβ17a are drastically reduced. Importantly, immature double positive thymocytes contain normal populations of $V\beta17a^+$ cells suggesting that negative selection occurs at the single positive stage. T cells that express TCRs that bind strongly to self-components are removed, or negatively selected in the thymus.

Additional evidence suggested that distinct areas of the thymus fulfill distinct functions in positive and negative selection. The epithelial cells in the thymic cortex are thought to be mainly responsible for positive selection. Restriction of I-A expression to the thymic cortex results in positive selection without corresponding negative selection, leading to I-A reactive cells accumulating in the periphery (Laufer, DeKoning et al. 1996). The epithelial cells of the thymic medulla express self-antigens promiscuously because they are under the control of non-specific promoters such as AutoImmune REgulator (AIRE) (Anderson, Venanzi et al. 2002). These cells were once thought to be the main mediators of negative selection (Petrie 2002). However, recent evidence suggests that thymic resident dendritic cells acquire antigens from medullary epithelial cells, and mediate deletion of T cells that react with self peptide-MHC complexes (Brocker, Riedinger et al. 1997; Gallegos and Bevan 2004). The major role of the thymic medullary epithelial cell appears to be in inducing regulatory T cells (Aschenbrenner, D'Cruz et al. 2007), as I will discuss further below. As maturing thymocytes pass through distinct areas of the thymus, they are subject to distinct selection stimuli.

Given the evidence above, it is clear that the thymus plays a major role in determining the repertoire of T cells. This is carried out by thymic selection for thymocytes that have some affinity for self-MHC molecules, but by facilitating the elimination of maturing thymocytes with high affinity for self-antigens. Even in the face of heavy negative selection in the thymus, autoreactive T cells do emigrate to the periphery, particularly when the antigens for which they are specific are not well expressed in the thymus (Pircher, Burki et al. 1989). Thus, a mechanism for inducing

tolerance in T cells in the periphery should exist to limit autoimmunity. Evidence for such a mechanism of peripheral inactivation is outlined below.

1.2.6 Peripheral Inactivation of CD4 T cells

The activation of T cells was found to require more than simply antigenic stimulation. While the nature of this second-signal or "co-stimulation" was unclear in the mid-1980's, several groups turned to investigating the minimal requirements for the activation of T lymphocytes. However, both the models of Bretscher and Cohn and of Lafferty and Cunningham suggested that, when a lymphocyte receives a signal 1 from binding antigen in the absence of a second signal, it will be inactivated. In contrast to Lederberg's idea that lymphocytes pass through a unique developmental stage, during which they are susceptible to deletion by self-antigen, two-signal models predict that inactivation, under the correct circumstances, may occur at any time. The shaping of the T cell repertoire in the thymus, and indeed the inactivation of self-reactive B cells in the bone marrow, are processes more reminiscent of the Lederberg model. A critical question for investigators was whether inactivation of lymphocytes, by signal 1 alone, can occur efficiently in the periphery. As will be discussed below, evidence supports the idea that tolerance to self- and experimental- antigens can be induced in mature peripheral lymphocytes.

The predictions of the two-signal models were investigated in both B and T lymphocytes concurrently, and definitive evidence for the role of signal 1, in inducing inactivation of these cells, was obtained at roughly the same time. Extensive similarity in the rules governing the activation and inactivation B and T lymphocytes exists, as originally proposed by Bretscher and Cohn, and so it is informative to review evidence from the investigations of both of these cell types.

Nossal and Pike were the first to directly demonstrate that the interaction of B cells with antigen, in the absence of appropriate "help", resulted in inactivation (Nossal and Pike 1975). When murine bone marrow was incubated in the presence of haptenated 2,4 dinitrophenyl- (DNP-) HGG or HGG alone, and subsequently adoptively transferred into lethally irradiated hosts. Upon challenge with DNP conjugated to a protein

component of flagellin, mice that received bone marrow that was previously exposed to DNP were specifically unresponsive, in terms of antibody production, to the hapten. Metcalf and Klinman showed that B cells, particularly those immature cells in the bone marrow, were susceptible to inactivation upon exposure to antigen (Metcalf and Klinman 1977). Thus, signal 1, received through interaction of the BCR with antigen inactivates B cells.

Transgenic mouse technology greatly aided investigations into B cell tolerance induction and led to experiments that confirmed that mature B cells can be inactivated by signal 1. In 1988 Christopher Goodnow, employing a dual transgenic mouse system in which a strain of mice that ectopically expressed hen egg lysozyme (HEL) were crossed with a strain of mice that expressed a transgene-encoded BCR (immunoglobulin; Ig), that had affinity for the HEL protein. The resulting offspring, though functionally tolerant of HEL, still contained B cells that expressed the transgenic Ig (Goodnow, Crosbie et al. 1988). The state of these B cells, not apoptotic but unresponsive to antigen stimulation, was called anergy. This initial study seemed to be at odds with the idea that inactivation of lymphocytes by signal 1 generally resulted in their death. In a subsequent study, Nemazee and Burki did not observe B cell anergy. They employed a similar transgenic system, wherein B cells expressed a transgene encoding a BCR with affinity for an MHCI molecule, of a given haplotype, not present in the host animal. When these transgenic mice were bred with mice of the H-2 haplotype, for which the transgene-encoded BCR had affinity, there was a severe defect in the generation of mature B cells, both in the bone marrow and periphery (Nemazee and Burki 1989). The difference between Goodnow's model and Nemazee's model was sorted out a few years later. When mice transgenic for a *membrane bound* form of HEL were employed in crosses with BCR transgenic mice, transgene-expressing B cell development was impaired (Hartley, Cooke et al. 1993). B cells with affinity for self-antigens, particularly those antigens that are readily accessible by virtue of existing on the cell surface, are deleted as they are generated after experiencing signal 1 alone.

Recently, Nemazee's group has shown that polyclonal B cells that bind self-antigens only in the *peripheral tissues* are subject to deletion. Employing an elegant experimental system that allowed them to avoid the complications that transgenic B cell

receptors may introduce, the authors show that an engineered superantigen, that binds kappa-encoded light chains of the BCR, expressed solely in the liver, induces deletion of kappa-bearing polyclonal B cells (Ota, Ota et al. year). These results, taken together, support the idea that B cells in the periphery, when exposed to antigen alone, signal 1, are removed via apoptosis, resulting in functional tolerance to these antigens. Thus the rules governing the activation and inactivation of B lymphocytes appear to be consistent with the original two-signal hypothesis.

The identification and characterization of both the TCR and MHC gene products greatly facilitated the study of T cell biology. Once the nature of the interaction of T cells and the antigens for which they have specificity was known, investigators were able to address, in more reductionist and clearly defined ways, the mechanisms governing the activation and inactivation of these lymphocytes.

Some of the first, and certainly some of the most influential studies, showing that T cells can be inactivated by the antigen-mediated signal 1 alone, came from Jenkins and Schwartz. These authors demonstrate that the pre-incubation of rested CD4 T cell clones with chemically "fixed" antigen presenting cells, from the spleen of normal mice, loaded with peptide antigen in culture, results in specific unresponsiveness of the CD4 T cell clones to further stimulation with the peptide. Similarly, unresponsiveness towards peptide antigens could also be achieved *in vivo* by the administration of fixed peptide-loaded APC (Jenkins and Schwartz 1987). Thus, signal 1, delivered by fixed splenocytes, resulted in the inactivation of CD4 T cells.

Jenkins also investigated the molecular mechanisms involved in the inactivation of T cells using his experimental protocol. During the process of CD4 T cell inactivation by interation with antigen alone the IL-2 receptor was upregulated, but the cells fail to produce IL-2. This suggested that the clones require second signals for IL-2 production and, thus the lack of IL-2 stimulation may explain the inactivation of these clones (Jenkins, Pardoll et al. 1987). Importantly, the signals delivered to T cells required extracellular $Ca^{2+,}$ since cultures containing EGTA, a chemical that strongly chelates Ca^{2+}, did not allow for antigen to inactivate the CD4 T cell clones. Moreover, the calcium ionophore, ionomycin, which artificially raises intracellular Ca^{2+} levels, could inactivate clones on its own. The authors also report a relative deficiency in protein kinase c (PKC)

activation and inositol triphosphate (IP3) accumulation in inactived clones compared to activated clones. They suggest that tolerance, mediated by the receipt of signal 1 alone, results in an imbalance between signals mediated by intracellular Ca^{2+} levels and those mediated by PKC.

In a third paper, the authors show that the inactivation of CD4 T cell clones, by fixed APC, can be prevented by supplementing the cultures with unfixed allogeneic spleen cells (Jenkins, Ashwell et al. 1988). Presumably, the cells were S+ in phenotype, providing critical second signals that rescued CD4 T cell clones from inactivation. The second signals could not be replaced by IL-1 in culture, but were partially replaced by the phorbol ester PMA, which aggregates many cell surface receptors. Thus, signaling through these surface receptors, and not through soluble cytokine, is critical in preventing inactivation. Moreover, the induction of tolerance by fixed APC could be prevented by provision of Cyclosporine A (CsA). CsA functions by inhibiting the action of the Ca^{2+} dependent phosphatase calcineurin, thus confirming that Ca^{2+} is an important mediator of signal one, and leading to the suggestion that its major effect is mediated through the activity of calcineurin.

Working in the same laboratory, Quill et al. demonstrated, in a more reductionist way, that signal one alone resulted in death of CD4 T cell clones. Employing a system, previously used to show that antigen presentation to CD4 T cells required only peptide MHC complexes (Watts, Brian et al. 1984), Quill stimulated CD4 T cell clones with I-E molecules, loaded with the antigenic peptide, supported on an artificial planar lipid membrane (Quill and Schwartz 1987). Upon ligation of antigen, in this system, the clones were initially activated, as demonstrated by an increase in cell size, but became unresponsive to normal activation by antigen-pulsed splenocytes within 16-24 hours. The provision of PMA partially rescued clones from tolerance induction, but in this system, provision of IL-2 did not. The authors also demonstrated that a down-regulation of the TCR is not responsible for the inability of inactivated clones to be stimulated by antigen, suggesting that intracellular mechanisms are likely to be involved in specific inactivation of CD4 T cell clones in this manner. On receipt of signal one alone, CD4 T cells undergo apoptosis.

Taken together, the finding of Jenkins, Quill, and Schwartz are striking. These findings brought two-signal hypotheses back into the consciousness of immunologists with regards to CD4 T cell activation. In clearly defined systems, and with insight into signaling events, the authors were the first to definitively demonstrate that the antigen-mediated signal 1 was tolerogenic in CD4 T cell clones. However, this laboratory was not the only one to publish evidence that CD4 T cells are inactivated upon receiving signal one alone. Lamb, employing cultures of human CD4 T cell clones, demonstrated that incubation with the peptide of influenza HA, for which these cells had affinity, resulted in unresponsiveness (Lamb and Feldmann 1984). Critically, the induction of unresponsiveness in this system required MHC, as a blocking antibody could prevent inactivation. Human CD4 T cells express MHCII, and so in this system, act as both the stimulated cell and the APC. This finding was confirmed and extended, showing that inactivation of the cells in these cultures was long-lived, as was the case in the Jenkins system (Lamb, Zanders et al. 1987). The finding of Burkly et al. provided evidence that the inactivation of CD4 T cells occurs exrathymically *in vivo* (Burkly, Lo et al. 1989). Employing a similar methodology to Kappler (Kappler, Roehm et al. 1987), she showed that when I-E expression is restricted to pancreatic β-cells, inactivation, but not immediate deletion of Vβ17a+ T cells occurs. Thus, T cells can be "paralyzed" by antigen alone, a finding that is reminiscent of the anergy induced in B cells in Goodnow's (Goodnow, Crosbie et al. 1988) BCR transgenic mice.

Although the findings of antigen-induced inactivation of CD4 T cell clones in culture were clear, questions about whether similar mechanisms could inactivate normal precursor T cells, *in vivo*, remained. As with the study of B cell tolerance, the study of the activation/inactivation decision of CD4 T cells was greatly aided by transgenic mouse technology. Development of a TCR-transgenic mouse, in which the T cells expressed the genes encoding rearranged α and β chains from the DO11.10 CD4+ T cell clone that recognizes a peptide of OVA spanning residues 323-339 ($OVA_{323-339}$), was pivotal. Murphy et al. demonstrated that the administration of large amounts of the $OVA_{323-339}$ peptide to DO11.10 TCR transgenic mice resulted in apoptosis of mature thymocytes expressing the TCR (Murphy, Heimberger et al. 1990). These transgenic T cells, it

appeared, are regulated in a similar manner to normal T cells; they are susceptible to negative selection in the thymus.

Are mature TCR transgenic CD4 T cells also susceptible to inactivation in the periphery? Kearney, working with Jenkins, addressed this question directly (Kearney, Pape et al. 1994). After finding that administration of 300μg of $OVA_{323-339}$ resulted in peptide-specific unresponsiveness in normal mice, she seeded mice with 2.5 x 10^6 DO11.10 TCR transgenic cells, so that they could be easily followed by flow-cytometry. Upon administration of 300μg $OVA_{323-339}$ intravenously or intraperitoneally, these cells were found to divide rapidly, implying that they were initially activated. DO11.10 TCR-bearing cells rapidly underwent apoptosis and disappeared from all lymphoid tissues examined. Liblau et al. reported confirmatory results, in a different TCR transgenic mouse system (Liblau, Tisch et al. 1996), and Aichele et al. showed that endogenous CD8 T cells can be inactivated in a similar manner (Aichele, Brduscha-Riem et al. 1995). These results, which closely resemble those of Quill (Quill, Carlson et al. 1987), indicate that mature, but naive, peripheral CD4 T cells are highly susceptible to inactivation, by the provision of peptide alone, in the periphery.

The finding that administration of soluble peptide, via systemic routes, results in inactivation of the corresponding CD4 T cells implies that the cells that present this peptide do so in a manner that is primarily tolerogenic. According to the two-signal model of Lafferty and Cunningham, it is likely that these cells do not possess the S+ phenotype, or express what became known as co-stimulatory activity. The major populations of MHCII-bearing cells that could possibly present injected peptide are B cells, macrophage and DCs. As I will describe in section 1.4, resting and antigen-presenting B cells are often found to be tolerogenic in culture with T cells (Chesnut, Endres et al. 1980; Kakiuchi, Chesnut et al. 1983; Croft, Joseph et al. 1997). Evidence also supports the idea that without activation by certain inflammatory stimuli, DC are tolerogenic (Hawiger, Inaba et al. 2001; Steinman, Hawiger et al. 2003). Thus, the results of Kearney, Liblau, and others imply that under normal conditions the majority of MHCII-bearing cells in the mouse are resting and unable to offer appropriate co-stimulation to CD4 T cells for their activation. In addition, some extrathymic, MHCII-bearing, stromal cells have recently been identified in the peripheral tissues. These cells

appear to function in a similar manner to the thymic medullary epithelial cells and express AIRE (Gardner, Devoss et al. 2008). It is possible that these cells also play a role in presenting peptides to induce CD4 T cell tolerance, particularly since these cells do not express the major co-stimulatory molecules. Systemic administration of peptide antigen leads to presentation by primarily resting APC that lack co-stimulatory activity. We now turn, briefly, to investigations concerning the nature of co-stimulation.

1.2.7 The Molecular Nature of Signal 2 for T cells

The activation of CD4 T cells in culture with "macrophages" and antigen is a classical system for the study of antigen-specific T cell biology. However, once the nature of antigen recognition by T cells became clear in the 1980s, some groups began to investigate the activation of T cells in more reductionist systems. Many people employed antibodies to the CD3 antigen, a component of the TCR signaling complex, to stimulate T cells (Geppert and Lipsky 1987). However, it rapidly became clear that signaling through the TCR alone was insufficient to activate naive T cells, resulting in acquisition of effector function. Another study employing different antigen presenting cell populations, illustrates this point. Upon culture of peptide-specific CD4 T cells with either IFNγ-stimulated fibroblasts (that express MHCII) or macrophages, pulsed with peptide, T cell proliferation was observed only in macrophage-containing cultures, indicating that presentation of antigen, through peptide-MHCII alone, is insufficient to result in proliferation of antigen-specific T cells (Geppert and Lipsky 1987). Clearly fibroblasts do not possess an essential co-stimulatory activity that "macrophages" do.

An antigen expressed almost universally on the surface of T cells was identified as having stimulatory activity complementary to TCR signaling. CD28, a member of the immunoglobulin superfamily of cell surface receptors, is found on virtually all peripheral T cells and stimulation via antibody crosslinking was shown to stabilize the messenger RNA for IL-2 (Lindstein, June et al. 1989). This finding was interesting in light of the earlier observations of Larsson (Larsson and Coutinho 1979) and Jenkins (Jenkins, Pardoll et al. 1987) that T cells, stimulated with antigen alone, fail to produce IL-2, preceding their inactivation, which can be prevented by provision of exogenous IL-2.

Thus it seemed that a regulatory loop had been closed: TCR stimulation results in up-regulation of the IL-2 receptor, but not IL-2 production, co-stimulation through CD28 leads to IL-2 production, and subsequent proliferation, and activation of the T cell. CD28 stimulation enhances Ca^{2+} mobilization but does not increase IP3 or phosporylated protein tyrosine kinase (PTK) levels, which are normally a result of TCR ligation, thus these two signals are distinct (Ledbetter, Imboden et al. 1990).

The natural ligand for CD28 was identified as the B cell activation-associated molecule, B7, which turned out to have at least two separate isoforms, B7.1 and B7.2 (CD80 and CD86). These molecules also bind very strongly to another T cell surface receptor CTLA-4, which has different downstream effects than CD28 ligation. Blocking studies, *in vitro*, demonstrated that B7 was essential for proliferation of highly purified T cells in response to allogeneic B cells (Koulova, Clark et al. 1991). Confirmation of this finding, in live animals, was reported by Linsley et al. Administration of a soluble, recombinant CTLA-4 fusion protein completely blocks T cell dependent immune responses (Linsley, Wallace et al. 1992). The rapidly emerging findings regarding the molecular nature of the co-stimulatory signal were summarized by Schwartz at the time (Schwartz 1992) in a tidy minireview. In a number of experimental systems, the blocking of CD28 co-stimulation, during antigenic stimulation, was shown to result in anergy induction in T cells. The molecular nature of the Lafferty and Cunningham S+ phenotype had finally been resolved. However, the above description is altogether too simplistic.

Only a few short years following the discovery of the B7 molecules as potent co-stimulators of T cells, Ding and Shevach described a B7/CD28 independent co-stimulatory activity of B cells (Ding and Shevach 1996). As it turns out, there are a great number of different molecules, expressed differentially on APC, that signal T cells in a co-stimulatory fashion. These fall broadly into two categories, the Ig superfamily and the tumor necrosis factor superfamily (TNFSF) (Peggs and Allison 2005). TNFSF members are generally homotrimeric surface-bound molecules with primary sequence similarity to the secreted cytokine TNF. These molecules, particularly OX40L and its corresponding receptor OX40, play an important role in inducing the effector function and survival of CD4 T cells.

There have been dozens of APC surface molecules identified that have some form of co-stimulatory activity in terms of the activation of T cells. The sheer numbers of molecules leads to the suggestion that some level of redundancy exists. It is generally believed that the regulation of these molecules stems from circumstances surrounding the acquisition of antigen by APC and thus, T cells receive distinct co-stimulation upon interaction with differentially activated APC (Manicassamy and Pulendran 2009). However, the details are not well understood at this time.

1.2.8 Control of Survival and Apoptosis by Signal 2

As has been outlined above, experimental evidence demonstrates that T cells that experience signal 1 alone undergo apoptosis. The simultaneous provision of signal 2, to these cells, results in activation and prevents apoptosis. The findings of Jenkins, Quill, and Schwartz led to the suggestion that activation of the calcium dependent phosphatase, calcineurin, through the influx of Ca^{2+} to the T cell cytoplasm, is a critical event in signal 1-mediated apoptosis. TCR signaling results in rapid Ca^{2+} influx, which eventually leads to cellular apoptosis if not prevented by factors generated when signal 2 is delivered to T cells. Molecular studies lend support to this basic two-signal tenet.

Apoptosis can regulate the growth of all cells. Unrestrained growth, or neoplasia, can result in cancer; cancers of immune cells are common. However, unrestrained growth and activation of autoreactive lymphocytes has another danger, the potential to induce autoimmunity. Therefore complex regulatory mechanisms have evolved to prevent autoimmunity by harnessing the normal apoptotic machinery present in all cells. The cellular mechanisms of this regulation are outlined above. The intracellular molecular signaling events that lead to apoptosis in lymphocytes that receive signal 1 alone are less well defined. However, over the past two decades, much about the molecular mechanism governing apoptosis has been learned. I will present a very oversimplified view of how the two-signal hypothesis for the activation of lymphocytes integrates with our current understanding of cellular death mechanisms.

All cells constitutively express proteins that are both pro- and anti-apoptotic. These proteins antagonize one another, resulting in a molecular stalemate, allowing

survival of the cell. Thus, tipping of the balance in either direction supports either enhanced survival or results in rapid apoptosis. The first enzymes that were shown to be associated with apoptosis, caspases, cleave one another in a cascade of reactions, resulting in degradation of the cellular DNA and eventually apoptosis. Lymphocytes are sensitive to death induced by this pathway. Kishimoto and Sprent demonstrate that TCR ligation in the absence of co-stimulation results in high-sensitivity to Fas-mediated apoptosis. Fas is a cell surface receptor with an ability to initiate caspase-dependent apoptosis. Thus, upon the receipt of signal 1 alone, T cells become very sensitive to ligation of Fas. The ligand for Fas, FasL, is expressed on the same activated T cells in this system. This leads to signal-1-activated T cells interacting with one another, inducing death amongst them - T cell fratricide (Kishimoto and Sprent 1999). Presumably, the provision of FasL by other cells would result in death of the TCR-stimulated T cells as well. The Fas pathway, it seems, is not as important, in the regulation of T cell survival, as a second pathway, the Fas-independent mechanism for apoptosis.

The Fas-independent apoptosis pathway consists of a highly complex network of signaling intermediates that integrate pro- and anti-apoptotic signals; the balance of these signals ultimately determines whether the T cell survives or undergoes apoptosis (Song, Ouyang et al. 2005). The signals that are integrated come from ligation of cell surface receptors like the TCR and CD28 as well as cytokine receptors, and other molecules (Salmond, Filby et al. 2009). Many of the ultimate effectors in this Fas-independent pathway share sequence homology to BCL-2, a protein originally isolated from B cell lymphomas. BCL-2 and its related family members exert potent anti-apoptotic activity. Current models hold that the function of these proteins is to sequester (and keep in an inactive form) other structurally related proteins, like Bax and Bak. Free (or appropriately activated) Bax and Bak molecules form oligomers in the mitochondrial membrane, which releases the mitochondrial stores of cytochrome C, initiating apoptosis. Bax and Bak associate with BCL-2 and other anti-apoptotic family members through their third BCL-2 homology (BH3) domain. Other proteins, homologous to BCL-2 in only the BH3 domain (BH3-only proteins), are thought to displace (and activate) Bax and Bak from BCL-2. Thus, apoptosis can be initiated by the binding of BH3-only proteins such as BAD to BCL-2 (Lomonosova and Chinnadurai 2008). The ability of BAD to bind BCL-2 is

regulated by two factors, Akt/PKB and calcineurin. Phosphorylated BAD does not bind BCL-2; Akt phosphorylates BAD (Zhou, Liu et al. 2000) and calcineurin dephosphorylates BAD (Wang, Pathan et al. 1999). Thus, an excess of calcineurin activation or a deficiency of Akt-pathway activation should result in apoptosis. In T cells, calcineurin activation is induced by TCR-mediated calcium flux, and Akt-pathway stimulation is associated with co-stimulation and growth-factor (like IL-2) receptor ligation (Koyasu 2003). It appears therefore, that BAD activation acts as a fulcrum in the seesaw that balances apoptosis in the Fas independent apoptosis pathway.

Given this highly simplified description, it is clear that signal 1 alone should normally result in the apoptosis of T cells. However, the pathways are more complex than outlined and there is considerable overlap in the induction and activation of the signal 1- and signal 2-associated pathways mentioned above. Moreover, other pathways may share intermediates, further complicating interpretation. Recent evidence suggests that both Fas-dependent and Fas-independent pathways are essential in maintenance of tolerance to self-antigens (Hughes, Belz et al. 2008). Induction of a state of anergy, in T cells, in the absence of apoptosis, is sufficient in some cases to prevent autoimmunity (Barron, Knoechel et al. 2008). Overall molecular studies appear to back up findings from cellular immunology regarding the roles of signal 1 and signal 2 in the activation/inactivation decision for lymphocytes.

1.2.9 New Two-Signal Models for the Activation of CD4 T Cells

Charles Janeway Jr. proposed a new model for the initiation of immune responses. First, at a Cold Spring Harbor symposium, and later in *Immunology Today*, taking into account emerging evidence that the expression of second-signal mediators appeared to be regulated, rather than constitutively present, he proposed that the mammalian immune system evolved from a primitive system that had the capacity to recognize conserved molecular patterns often associated with pathogens (Janeway 1992). Janeway hypothesized that, upon recognition of these pathogen-associated molecular patterns, or PAMPS, the innate APC, the macrophage and the DC, would be induced to express critical second signal molecules that would allow for the activation of T cells. Thus, the

decision criteria, for whether the host responds to an antigen or not, is whether or not this antigen is presented by APC that are activated in the presence of a PAMP (in addition to the availability of lymphocytes with antigen receptors that can recognize this antigen). Janeway then all but predicts the course of immunological study over the next decade by placing the importance of DC and germline encoded receptors as central controllers of immune responses. Many of Janeway's ideas proved to be true and his rational and well-reasoned arguments formed the basis for the next, and current paradigm for the initiation of the immune response.

Matzinger extended Janeway's idea in proposing that the activation of innate APC could be achieved by "danger signals" (Matzinger 1994). According to Matzinger, these danger signals could include conserved microbial products, but also cell stress molecules like heat-shock proteins, or interferons. This proposition was seen as an improvement, at the time, of Janeway's model, as it explains why harmless organisms, for example commensal bacteria, are not normally subject to immune clearance. That is, commensal bacteria do not initiate immunity because they do not damage tissues. Matzinger also makes a point to reiterate the classical idea that signal 1 alone should result in tolerance of a lymphocyte. Since experimental evidence for this abounds (see above), the proposition is far from novel. However, in the context of a Matzinger's model for the activation of lymphocytes, one would predict that inactivation of autoreactive cells would occur, one by one, as they are generated and interact with APC bearing self-antigens but not activated by the presence of danger signals.

A major conceptual problem arises from the models of Janeway and Matzinger (Bretscher 1999). These models, like that of Lafferty and Cunningham before, are principally concerned with the activation of T cells, and in particular, the CD4+ helper subset. Because these cells are seen as central to the induction of most antibody responses, and some CTL responses, help from CD4 T cells is often the limiting factor in the generation of immunity. Assuming that autoreactive CD4 T cells escape selection and emigrate to the periphery, and that APC present self-antigens, one can envisage how autoreactive CD4 T cells would readily be activated during the course of infection by a pathogen. Specifically, DCs bearing both pathogen-derived and self-antigens, but activated by PAMPS, would have the capacity to activate autoreactive T cells. If this

happens, the activated T cells, which depend less on signal two for their continued activation than naive cells (Dubey, Croft et al. 1996; Curtsinger, Lins et al. 1998), may initiate a cascade of autoimmune activation, leading to pathology. Thus, it would seem that the activation of autoreactive CD4 T cells should be stringently controlled, more stringently perhaps than is achievable under the models of Janeway and Matzinger.

Bretscher proposed that while activated innate APC may initiate priming and expansion of CD4 T cells, their full activation and acquisition of effector function requires an antigen-mediated collaboration between CD4 T cells and B cells (Bretscher 1999). Since both populations of lymphocytes are negatively selected for autoreactivity, the chance that autoreactive cells could achieve this type of collaboration was thought to be exceedingly rare. Thus, this model, called the "two-step, two-signal" model for the primary activation of CD4 T cells, takes into account observations concerning the role of inflammatory stimuli in activating innate APC, but remains consistent with the original two-signal model. As I will discuss later in this introduction, and demonstrate experimentally in this thesis, some of the implications of this model, particularly, the role of antigen-mediated CD4 T cell and B cell collaboration and CD4 T cell-CD4 T cell cooperation, may have important roles during the development of the immune response. However, experimental evidence does not appear to support the *requirement* for this type of lymphocyte interaction for the full activation of CD4 T cells as envisaged by Bretscher (1999).

Since the proposals of Janeway and Matzinger, much evidence has been reported that appears to support their hypotheses. The biology of DC, central to their theories, and shown to be an extremely potent APC in the induction of CD4 T cells was heavily investigated. DCs were shown to express B7.1 and B7.2 (CD80/86) which allows them to stimulate T cells in culture (Inaba, Witmer-Pack et al. 1994). The expression of CD86 was found to be inducible on DCs, by the innate cytokine GM-CSF, which also caused their "maturation" as APC (Larsen, Ritchie et al. 1994). These findings led to the idea that DCs can be induced to express B7 molecules in response to inflammatory stimuli.

The link between the maturation of DCs to express potent co-stimulatory molecules, and the detection of PAMPS was made by Medzhitov, working with Janeway.

The Toll protein was shown to be involved in resistance to fungal infection in *Drosophila* (Lemaitre, Nicolas et al. 1996). Toll was previously described as having sequence homology to the human IL-1 receptor suggesting a role in innate immune activity. Employing a homology search of the National Center for Biotechnology Information (NCBI) sequence bank, Medzhitov was able to identify and clone a human homologue of Toll. He expressed a constitutively active form of this human Toll in leukocyte cell lines and demonstrated that this leads to stimulation of the nuclear factor kappa-B (NF-κB) pathway and upregulation of IL-1, IL-8, IL-6 and B7.1-encoding mRNA (Medzhitov, Preston-Hurlburt et al. 1997). Subsequently, another protein homologous to Toll, encoded by the previously named Toll-like receptor gene, *Tlr4,* was shown to be critical in responses to bacterial lipopolysaccharide (LPS) (Poltorak, He et al. 1998). Toll-like receptor 4 (TLR-4) was identified as a receptor for a known PAMP, which induced the expression of co-stimulatory proteins, strongly supporting the hypotheses of Janeway. Serving to underscore the importance of these findings to the study of basic immunology is the fact that the 2011 Nobel Prize in physiology or medicine was awarded to the principle investigators of the work describing the discovery of the function of Toll in drosophila, Jules Hoffman, and the discovery of the function of TLR-4, Bruce Beutler. Ralph Steinman, for his discovery of DC, was also awarded the prize. Presumably, had he been alive at the time, Charles Janeway Jr. would have shared in this prize.

New members of the TLR family were identified, and subsequent studies identified ligands for these receptors (Pasare and Medzhitov 2004; O'Neill 2006; Manicassamy and Pulendran 2009). TLR ligands include hypomethylated CpG sequences of bacterial DNA, and double-stranded RNA products found in some viruses. Most TLR ligands are derived from microbial pathogens and some respond to endogenous "danger signals" like heat-shock proteins. These TLR adaptor proteins signal via a variety intermediates, eventually leading to the transcription and protein expression of various co-stimulatory molecules, inflammatory cytokines, and homing molecules in DC that allow trafficking of these cells from peripheral tissues, where they reside, to the lymph nodes, as reviewed in: (Moynagh 2005; O'Neill 2006; Manicassamy and Pulendran 2009). DC, it appears, are able to fully prime CD4 T cells, in an antigen and danger-signal-dependent manner, upon migration to the lymph nodes. Experimental evidence

also suggests that both surface molecules borne by TLR-activated DC and the innate cytokines that they produce are critical for T cell activation (Pasare and Medzhitov 2004).

Elizabeth Ingulli was one of the first to employ imaging to observe the interaction of DC with CD4 T cells *in situ* (Ingulli, Mondino et al. 1997). In the paracortical region of lymph nodes, draining areas where DCs were seeded, interactions between antigen-specific CD4 T cells and peptide-loaded DC were observed whereas unloaded DC did not form many T cell contacts. Interestingly, peptide-loaded DCs, but not un-loaded DCs, disappear from the lymph node at 48 hours after the initial injection, suggesting that loaded cells are actively removed by some antigen-dependent mechanism. Subsequent dynamic imaging studies demonstrated the interaction of CD4 T cells and DCs in great detail (Stoll, Delon et al. 2002; Miller, Safrina et al. 2004). Thus interactions between DC and CD4 T cells have been visualized, underscoring the role of this interaction in the generation of immunity.

Further evidence supports the idea that Toll-like receptor stimulation enables the selective formation of peptide-MHC complexes from ingested antigens (Inaba, Turley et al. 2000; Blander and Medzhitov 2006; Yarovinsky, Kanzler et al. 2006). DCs may preferentially avoid the presentation of self-antigens when exposed to inflammatory stimuli. This mechanism presumably serves to limit the activation of autoreactive T cells.

These findings, taken together, illustrate the major role of DCs, activated by inflammatory stimuli, in the primary induction of CD4 T cell effector function. Comprehensive reviews are available on this subject (Banchereau and Steinman 1998; Villadangos and Schnorrer 2007; Segura and Villadangos 2009). However, DCs are not necessary for the induction of immune responses, as B cells can be sufficient to prime CD4 T cells in some cases (Constant, Schweitzer et al. 1995), and DC deficient mice generate CD4 T cell responses to DNA vaccines (Castiglioni, Lu et al. 2003). Moreover, danger signals are not absolutely required for immunity to sterile xenogeneic red-blood cells or to allografts (Bingaman, Ha et al. 2000), and signaling by TLRs is not required for immune responses to many conventional adjuvants (Gavin, Hoebe et al. 2006), despite the continual re-assertion of Janeway's statement that the microbial components in adjuvants are immunologists' "dirty little secret". Overall, it appears that DCs facilitate CD4 T cell activation, often so greatly that it is seen as essential to developing effective

immunity, and this requires activation of the DCs by inflammatory stimuli. The role of DC in the primary activation of CD4 T cells is thus one of the most important factors in the generation of immunity. As it turns out, DCs are also efficient in aiding the maintenance of self-tolerance, the evidence for which will now be discussed.

1.2.10 Maintenance of Self-Tolerance by DC

Thymic DCs are able to negatively select mature thymocytes by presenting self-antigens. However, if the models for activation of CD4 T cells of Janeway and Matzinger are correct, then inactivation of autoreactive CD4 T cells, by DC presenting self-antigens in the periphery, should also occur. Naturally, Ralph Steinman's group was amongst the first to demonstrate that resting DCs can inactivate CD4 T cells in the periphery. Hawiger and colleagues devised an experimental system to target antigen uptake to immature DC. They constructed fusion protein antigens by joining a peptide derived from the primary sequence of hen-egg lysozyme (HEL) to an antibody molecule with affinity to DEC205 (αDEC-HEL), a lectin receptor constitutively expressed on the surface of DC. Mice that were given αDEC-HEL, systemically, over a number of days, became tolerant to the HEL peptide as assessed by their failure to mount a T cell response to this peptide upon subsequent challenge. Interestingly, the effect of tolerance induction by immature DC targeted with αDEC-HEL could by prevented by the co-administration of FGK45 antibody, an agonist to CD40, which systemically activates APC, including DC (Hawiger, Inaba et al. 2001). Thus, antigen presented by resting DC inactivates CD4 T cells.

It has been shown that under non-inflammatory conditions immature DCs sample self-antigens for presentation to T cells, leading to their inactivation. Normally, DCs continually sample antigen in the periphery through endocytosis, mediated by surface receptors, like DEC205 or DCSIGN. The antigens that are taken up, self-proteins, are processed and loaded onto MHC molecules. These DCs also normally leave the peripheral tissues and traffic to the lymph nodes where they are free to interact with T cells. Because these cells have not been activated by exposure to PAMPS or other inflammatory stimuli, most DCs in this state express relatively low levels of MHC and

co-stimulatory molecules (Mahnke, Schmitt et al. 2002). DCs continually sample antigens from the periphery, where naive T cells do not normally have access to them, and bring them to lymph nodes where they are presented in a fashion that inactivates, rather than activates, T cells. This phenomenon was directly demonstrated by Liu et al. They administered dying, chicken ovalbumin- (OVA-) expressing, but antigen-presentation incompetent, cells to normal mice. This protocol resulted in systemic unresponsiveness to OVA. However, if the OVA-expressing dying cells were given at the same time anti-CD40 agonistic antibody, in the fashion of Hawiger, OVA-specific T cell responses were observed (Liu, Iyoda et al. 2002). Thus, T cell tolerance to self-antigens is achieved, by immature DCs in the periphery, under what Ralph Steinman called the "steady state" (Steinman, Hawiger et al. 2003).

Raymond Steptoe's group has addressed the role of steady-state DCs in tolerance induction in a more technologically advanced way. They make use of a transgenic mouse model wherein the DCs express ovalbumin under the control of the DC-specific promoter for CD11c. Because, in this system, immature DCs constitutively express OVA and cross-present OVA peptides on MHCII molecules, it was hypothesized that these transgenic mice would become tolerant of OVA at the T cell level. In a series of papers the authors have shown that, by virtue of this expression under normal conditions, DC can induce OVA-specific tolerance in naive CD4 and CD8 T cells (Steptoe, Ritchie et al. 2007; Doan, McNally et al. 2009). The authors employ adoptive transfer experiments to ensure that inactivation occurred only in the periphery. Given this evidence, it is clear that steady state DCs play a major role in the induction of T cell tolerance in the periphery at many time points along their lifecycle.

The terms activated and inactivated DCs are somewhat inaccurate, as Caetano Reis e Sousa points out (Reis e Sousa 2006). This is because, in some cases, DCs that are activated and express higher levels of MHC and co-stimulatory molecules still lead to inactivation of T cells when they present antigen. For example, Perona-Wright et al. determined that DCs, activated by LPS in the presence of interleukin-10 (IL-10), though phenotypically indistinguishable in surface marker expression from LPS-alone-activated DCs, failed to elicit robust T cell responses in culture and *in vivo*. Mice that were treated with these IL-10-matured DCs became tolerant of peptides that the DCs were loaded

with. Whereas DCs matured with LPS alone generated robust T cell responses. The authors extended these observations to a model of experimental allergic encephalomyelitis (EAE) and found that IL-10 matured DCs pulsed with a myelin oligodendracyte glycoprotein (MOG) peptide could protect against EAE when given before MOG immunization (Perona-Wright, Anderton et al. 2007). Dendritic cells that reside in the lungs also, it appears, have a tendency to elicit tolerance in T cells. Akbari et al. found that lung DCs, by virtue of their production of IL-10, tolerized T cells when they presented antigen. However, lung DC from IL-10 knockout mice primed T cell responses (Akbari, DeKruyff et al. 2001). Thus, even some "activated" dendritic cells play a role in maintenance of T cell self-tolerance.

In general, it appears that DCs are highly efficient at inactivating T cells in both the thymus and the periphery. Findings, such as those outlined above support the Janeway/Matzinger/Steinman-type two-signal model for the activation/ inactivation of CD4 T cells. However, B cells are also highly efficient at inactivating CD4 T cells, under appropriate conditions, and the large numbers of resting B cells, that exist in the lymph nodes and spleen, lead one to suggest that B cells are more likely to interact with autoantigen-specific CD4 T cells, leading to inactivation, provided that these B cell express the self-antigen endogenously. Some experimental evidence supports this proposition (Dalai, Mirshahidi et al. 2008). Thus, DCs and B cells likely act in concert to maintain self-tolerance.

Regardless of what cell type is mainly concerned with deleting autoreactive T cells in the periphery, the fact that autoimmunity arises, suggests that the removal of autoreactive T cells, sometimes fails. Fortunately, as I will outline below, the immune system has evolved at least two additional mechanisms to deal with activated autoreactive T cells in the peripheral tissues.

1.2.11 Inactivation of Effector CD4 T cells

Two-signal models for the activation of T cells, are also concerned with the maintenance of self-tolerance. Signal 1 alone has been shown to result in the inactivation and death of T cells. It is clear that, under appropriate conditions, two signals, delivered

together, result in activation of CD4 T cells. The prevention of the activation of autoreactive cells was once thought to be critical for the maintenance of self-tolerance. This belief stemmed, in part, from the recognition that activated T cells appear less dependent on signal 2 for their continued activation than naive cells (Dubey, Croft et al. 1996; Curtsinger, Lins et al. 1998). Thus, it seemed critical that autoreactive T cells are never activated, lest they proliferate and cause pathology. However as Adler reviews, experimental evidence suggests that both *effector* and *memory* T cells are susceptible to inactivation in the periphery by the *same mechanisms* that inactivate naive T cells (Adler 2005). That is, signal 1 alone, presented by resting APC, inactivates T cells at virtually any stage in their life cycle.

Some of the first evidence that effector CD4 T cells can be inactivated came from studies in the EAE model of autoimmunity in mice and rats. Critchfield et al. demonstrated that administration of large quantities of a soluble MHCII-binding peptide, derived from the primary sequence of myelin oligodendracyte protein (MOG), to mice during the course of symptomatic EAE, resulted in attenuation of the normally self-limiting disease (Critchfield, Racke et al. 1994). This finding was independently confirmed (Brocke, Gijbels et al. 1996) and recently has been extended to a different murine model of multiple sclerosis (Verbeek, van der Mark et al. 2007). Our own observations bear on this issue, as discussed in Chapter 5 of this thesis. Encephalitogenic T cells appear to be inactivated upon the administration of high doses of soluble peptide for which they have affinity.

Does the administration of high doses of peptide reflect the natural biology of tolerance induction by resting APC? In order to test whether endogenous expression of experimental antigens in peripheral cells leads to tolerance in T cells, antigen-transgenic mouse models have been established. The results obtained in these systems support the idea that steady-state DCs inactivate naive T cells in an antigen-specific manner. Recently, the question of whether effector and memory T cells can also be inactivated by antigen-presenting cells expressing experimental antigens, but not activated by inflammatory stimuli, has been addressed. Influenza HA-specific effector CD4 T cells are inactivated within days of their adoptive transfer into HA-transgenic mice that express this antigen ubiquitously (Higgins, Mihalyo et al. 2002). Recent evidence suggests that

the majority of this inactivation is due to B cells presenting the experimental antigen (Dalai, Mirshahidi et al. 2008). However, when expression of the experimental "self" antigen is restricted to the CD11c-expressing cells (conventional DCs), similar results are obtained; effector and memory CD4 T cells are efficiently inactivated by steady-state DCs that bear antigens for which these T cells have affinity (Nasreen, Waldie et al. 2010). Similarly, memory CD8 T cells (Kreuwel, Aung et al. 2002; Kenna, Thomas et al. 2008) and even effector CTL (Kenna, Waldie et al. 2010) are readily inactivated in mice where APC express the antigen for which they are specific. The bulk of experimental evidence, therefore, supports the idea that signal 1 alone inactivates all types of conventional αβ TCR-bearing T cells, at virtually any stage during their development or during the effector phase. The results obtained employing antigen-transgenic mice are strikingly similar to those obtained upon injection of soluble peptide. Thus, it would appear that administration of high doses of soluble peptide is a valid protocol for the study of T cell tolerance.

These findings may be surprising to immunologists familiar with the original rationale behind the development of two-signal models. Based on these results, one can envisage how autoimmunity is avoided: In the event that a microbial pathogen subverts the primary barriers and innate defenses, it is picked up by a DC. The DC is activated to express co-stimulatory molecules, inflammatory cytokines, and lymph node homing molecules, by TLR signaling following binding of the PAMPs associated with this microbe. Many of these DCs migrate to the lymph nodes draining the infected area where they present both the pathogen-derived peptides and some self-peptides along with potent signal 2 mediators. The T cells that recognize these peptides are activated. While the majority of these T cells are specific for the pathogen, some may be autoreactive. Upon emigration from the lymph node where they are activated, these T cells circulate to the inflamed peripheral tissues and through other lymph nodes. While pathogen-specific T cells are continually activated by relatively rare DCs (and other APC) presenting pathogen-derived peptides, always in association with co-stimulation, the auto-peptide-specific effector cells are inactivated by the vast majority of APC that are resting, having never been activated by a PAMP. In cases where the majority of APC are activated, as may occur during disseminated infections, activation of autoreactive T cells may be

supported. However, if the individual recovers, clearing the pathogen, the majority of APC will return to a resting state and continue to inactivate the autoreactive cells. Thus, frank autoimmunity will only occur in special cases, which I will speculate upon later in this thesis.

With the evidence given above, one would expect that clinical applications for therapy of autoimmune disease would be achievable. Indeed, the use of synthetic peptides is currently being explored in human models of allergy (Campbell, Buckland et al. 2009). While it is generally thought that administration of peptides, to allergy patients, results in the induction of regulatory T cells (discussed below) the possibility that some allergen-specific T cells are inactivated and deleted remains (Larche and Wraith 2005). However, due to the great polymorphism in the human MHC, the peptides that are recognized by allergy mediating or autoreactive T cells are likely to be different in different individuals. To circumvent this problem, therapeutic strategies employing anti-CD3 (a signaling component of the TCR) have been developed. These therapies, currently in clinical trials for the treatment of various forms of autoimmunity, rely on the idea that anti-CD3 antibodies stimulate T cells in a manner analogous to signal 1 alone, thus causing their inactivation (Chatenoud and Bluestone 2007).

The finding that even activated T cells can be readily inactivated, under the appropriate conditions, has interesting implications for the mechanism by which autoimmunity arises. It would appear that, due to the continual inactivation of autoreactive T cells, outlined above, that autoimmunity would likely never occur. However, experientially, we know this is not the case. Even in the face of heavy selection against autoreactivity in T cells, at all stages of their development, autoimmunity does arise. The mammalian immune system has therefore evolved another mechanism that exerts dominant control over auto reactive cells. It turns out that this mechanism involves T cells that are able to actively suppress the activation of other T cells, the regulatory T cell subset.

1.2.12 Dominant Self-tolerance

The importance of thymus derived cells in the generation of immune responses has been known since the early 1960's (Miller 1961); but, by the end of that decade, the first clues that thymic emigrants were involved in the active suppression of autoimmunity were emerging in the literature. A curious study by Nishizuka and Sakakura, working with a murine models of cancer, demonstrated that thymectomy leads to autoimmune oophoritis and wasting in mice (Nishizuka and Sakakura 1969). The results of this experiment could not be interpreted at the time, given that the study of thymus-derived cells was still in its infancy - so these results were nearly forgotten. However, when he began working with a similar neonatal thymectomy protocol decades later, Sakaguchi observed the development of an equivalent wasting syndrome. This observation led to the identification of thymus dependent cells (T cells) that were essential suppressors of autoimmunity in mice (Sakaguchi, Takahashi et al. 1982; Sakaguchi, Fukuma et al. 1985). Thymectomy after day ten of life often did not result in serious autoimmunity, indicating that the suppressive cells emigrate from the thymus by day ten. The thymus facilitates the generation of T cells that are able to dominantly suppress autoimmunity.

By the early 1990s, observations regarding this suppressive T cell subset were accumulating. Powrie and Mason found that the subset of CD4+ T cells that express low levels of CD45RB, can regulate autoimmunity mediated by the CD45RB high-expressing population (Powrie and Mason 1990). Subsequently, this regulation was found to be a dominant process (Modigliani, Pereira et al. 1995), meaning that the suppression of autoimmunity, mediated by CD45RB low-expressers, was an active process that could be transferred to recipient mice through administration of T cells. CD4 T cells with suppressive capacity were shown to constitutively express the high-affinity IL-2 receptor α-chain, CD25 (Sakaguchi, Sakaguchi et al. 1995). In order to distinguish this suppressive subset from other types of suppressor cells, the term regulatory T cell or Treg, was coined. In 2003, a Treg lineage-specific transcription factor, foxp3, was identified (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003). The proportion of foxp3+ cells of CD25+ CD4 T cells was estimated at around 95%, and enforced, ectopic expression of foxp3 is sufficient to cause naive CD4 T cells to acquire a regulatory phenotype. Foxp3, also known as scurfin, was identified earlier as a gene that was

involved in autoimmunity. Scurfy mice, with a natural mutation of the scurfin protein, succumb to a fatal lymphoproliferative disorder (Godfrey, Wilkinson et al. 1991).

The TCRs of Tregs are very heterogeneous. It has been shown that in a naïve animal, variable (V) region usage in the TCRs of CD25- and CD25+ cells is not significantly different (Takahashi, Kuniyasu et al. 1998). This fact implies that Treg and naïve T cells are equally diverse. However, Hsieh *et al*. found the complementarity determining region 3 (CDR3) sequences (known to be a major specificity determining factor for the TCR) of CD25- and CD25+ CD4 T cells only overlap by around 25% in clones found frequently in either population (Hsieh, Liang et al. 2004). CD25+ derived TCRs appear to be dramatically more self-reactive than their CD25- derived counterparts. Following this finding, Hsieh et al. have shown that thymically derived Tregs are similar to their counterparts in the periphery, which may indicate that the Treg lineage is established mainly in the thymus and is stable upon emigration (Hsieh, Zheng et al. 2006). The heterogeneity of the TCRs of Treg cells indicates that they are randomly generated, as are conventional CD4 T cells, and may derive from the same population of thymocytes.

Pacholczyk et al. have demonstrated that in the periphery, the TCR diversity of the CD25+ population is in fact greater than that of the CD25- fraction (Pacholczyk, Ignatowicz et al. 2006). In contrast to the findings of Hsieh, this group finds that, in general, the specificity of Tregs and naïve T cells in the periphery is not significantly different (Pacholczyk, Kern et al. 2007). Seddon and Mason found that Tregs with a specificity for a peripheral autoantigen were only induced in mice where that antigen was present (Seddon and Mason 1999). Furthermore, it has been well established that antigen specific Tregs can be generated in the periphery (Belkaid, Piccirillo et al. 2002; Graca, Cobbold et al. 2002; Apostolou and von Boehmer 2004; Dahlberg, Schartner et al. 2007; Sun, Hall et al. 2007).

In 2003 Bluestone and Abbas proposed that a distinction be drawn between Tregs generated in the thymus and those generated in the periphery (Bluestone and Abbas 2003). This separation of regulatory T cells into two populations called natural Tregs (nTregs) or inducible (iTregs), is currently considered to be valid. As mentioned above, some have found differences in the repertoire between the subsets. Natural, thymus

derived Tregs, appear to be self-reactive. This finding is entirely consistent with the classical thymectomy experiments, which suggested that the thymic suppressors were responsible for regulating autoreactive cells. More recently, decisive experiments have demonstrated that the Tregs that are generated in the thymus and can be selected for by the presence of their cognate antigen there. By crossing TCR transgenic mice with transgenic mice expressing the antigen for which the cloned TCR is specific, Jordan et al. have shown that antigen-specific Tregs are produced in the thymus. The authors also show that the relative expression level of the antigen is critical for this induction. Only at intermediate levels of expression were significant numbers of Tregs generated. High expression resulted in deletion of TCR transgenic cells, while low expression levels were insufficient to cause Treg conversion of thymocytes (Jordan, Boesteanu et al. 2001). It is thought that Tregs undergo the normal selection processes experienced by other naïve T cells. Both positive and negative selection occur. After these, the process that is responsible for Treg conversion takes place (Takahama 2006). As Aschenbrenner et al. have elegantly shown, negative selection is mediated by haemopoetic cells (such as thymic DC), while the $AIRE^{+}$ medullary thymic epithelial cells seem to play a critical role in the induction of Treg function (Aschenbrenner, D'Cruz et al. 2007).

Inducible Tregs can be produced from thymic emigrants in the periphery under conditions known to be non-immunogenic, or *ex vivo* by the provision of exogenous cytokine. In antigen specific systems, these cells can be produced at a very high ratio. In TCR transgenic RAG knockout mice, this 'sub-immunogenic' condition is achieved by the administration of the antigenic peptide in saline (Apostolou and von Boehmer 2004; Dahlberg, Schartner et al. 2007). In TCR transgenic mice that normally do not develop Tregs, this protocol can induce around 50% of the antigen-specific cells to become foxp3. Antigen-specific T cells can also be induced to express foxp3 *in vivo* by the oral feeding of the cognate antigen (Sun, Hall et al. 2007). This conversion has been attributed to the microenvironment of the *lamina propria* (LP) created by the resident dendritic cells (DC). Retinoic acid (RA), a vitamin A derivative expressed by various cells in gut lymphoid tissues, seems to be key for the induction of Tregs there. Additionally, TGF-β has been identified as a factor that is essential for foxp3 induction both in vivo and in

vitro (Chen, Jin et al. 2003). With TGF-β stimulation, other cytokines may synergize to produce optimal signaling for foxp3 induction (Namba, Kitaichi et al. 2002).

Classically, CD4+CD25+ Tregs were known to be refractory to stimulation i*n vitro*. Though this was shown not to be the case *in vivo*, the question arose as to the nature of the APC that is able to expand Tregs. To address this question Sayuri Yamazaki et al. purified CD4+ CD25+ T cells from DO11.10 ($OVA_{323-339}$-specific) T cell receptor transgenic mice. They were able to show that bulk populations of splenocytes as APC were able to stimulate the division of Treg cells very poorly *in vitro*. However, it was found that conventional CD11c-expressing DCs purified from the spleen of these mice were able to expand DO11.10 Tregs very efficiently when they were loaded with the appropriate $OVA_{323-339}$ peptide (Yamazaki, Iyoda et al. 2003). Thus, it appears that DCs play a role in the expansion of Tregs.

A perhaps more important role for DCs in the promotion of Tregs has been described – their ability to generate, *de novo,* Tregs from naive precursors. Cheng-Ming Sun, working with Yasmine Belkaid, demonstrated that a certain population of DCs that reside in the LP of the small intestine of mice preferentially induce CD4 T cells to become Treg when presenting antigen. The authors find that in the presence of TGF-β, which is constitutively produced in the LP, resident DCs, but not spleen DCs, were able to generate high numbers of Tregs from naive precursors. Further fractionation revealed that the CD103+ LP DCs are responsible for this effect. Purified CD103+ LP DCs, without exogenously supplied TGF-β, are capable of inducing Tregs in culture due to their production of RA (Sun, Hall et al. 2007). The production of RA appears to be crucial for the *de novo* generation of Tregs by tolerogenic DCs. By employing a sophisticated cell-sorting scheme, Martin Guilliams et al. were able to identify RA-producing DCs in the dermis. Interestingly these cells do not express CD103, which implies that the presence of this surface marker does not exclusively identify tolerogenic DCs. These CD103-, RA producing DCs were able to generate Tregs in culture (Guilliams, Crozat et al. 2010). DCs are able to promote the generation of antigen-specific Tregs under certain circumstances.

It has become clear that *de novo* Treg generation is a common function of DCs. The microenvironment in which the DCs reside plays an extremely important role. As

mentioned above, mucosal tissues constitutively express factors that aid in Treg differentiation. It has become appreciated that part of the role of these factors is to condition the resident DCs. Aside from what we might call constitutively tolerogenic DCs (RA-producers mentioned above), tolerogenic DCs may also be induced from immature precursors. There is evidence that tolerogenic DCs can be induced by certain pathogens (D'Ambrosio, Colucci et al. 2008) and in the tumor microenvironment (Gabrilovich 2004) which leads to regulation of protective immune responses. Moreover, Tregs themselves may induce a tolerogenic phenotype in DCs, promoting further Treg generation, a phenomenon known as "infectious tolerance" (Andersson, Tran et al. 2008).

A key question in the field of Treg biology remains: What is the mechanism of the suppressor function of these cells? Though much effort has been expended in trying to answer this question, no general mechanism has been demonstrated for Tregs. This may suggest that under different conditions Tregs may function, or appear to function, in different ways. Tregs have been shown to exert their suppressive function by a number of mechanisms. The first in vitro assays used to detect suppressive function indicated that inhibition of IL-2 synthesis was a major mechanism of Treg suppression (Thornton and Shevach 1998). In this system, it was found that T cell-T cell contact was essential and that APC were not necessary to 'bridge' this interaction (Thornton and Shevach 2000). Subsequently, inhibitory roles for the cytokines IL-10 and TGF-β, produced by Tregs, have been defined *in vivo* (Nakamura, Kitani et al. 2001; Zhang, Koldzic et al. 2004). In addition, cell contact dependent mechanisms such as CD80/86 ligation by CTLA-4 on suppressive cells have been described (Paust, Lu et al. 2004). These contact dependent mechanisms may result in direct signaling from T cell to T cell or through antigen presenting cells (von Boehmer 2005). Tregs may exert their suppressive function through any or all of these mechanisms.

Whatever the specific molecules involved in this suppression are, the effects of Tregs appear to be exerted at both the inductive and effector stages of T cell responses. Klein et al. have demonstrated that *in vivo*, the once-thought-to-be anergic Treg cells are able not only to proliferate upon antigen stimulation but to suppress the proliferation of naïve antigen specific cells as well (Klein, Khazaie et al. 2003). Additionally, the development of effector function by naïve cells was drastically limited, as assessed by

cytokine secretion. In tumor models, the removal of CD25+ cells results in tumor regression (Onizuka, Tawara et al. 1999; Shimizu, Yamazaki et al. 1999; Yu, Lee et al. 2005). It is unclear whether the removal of these cells results in a switch to a protective class of immunity, or if Tregs (and therefore suppression) are specifically being removed. If the later is true, it is evidence that Tregs actively inhibit functional effector cells at a late effector stage. There is also evidence that Tregs specifically inhibit memory T cell responses but not primary responses (Kursar, Bonhagen et al. 2002). Taken together, this evidence suggests that Tregs function at virtually all stages of a T cell mediated response.

The presence of Tregs has been shown in many disease models of chronic infections and tumors (Belkaid, Piccirillo et al. 2002; MacDonald, Duffy et al. 2002; Chen, Zhou et al. 2007; Kakinuma, Nadiminti et al. 2007). These cells are often found to be specific to pathogen derived antigens (MacDonald, Duffy et al. 2002; Suffia, Reckling et al. 2006). This line of evidence suggests that these cells are derived from peripheral naïve cells sometimes even in inflammatory conditions. This suggestion is paradoxical in light of the current paradigms. However, it has been suggested that pathogen-specific Treg cells act to limit the complete clearance of pathogens, leading to enhanced memory (Pepper and Jenkins).

Taken together, the experimental evidence concerning the existence, functional properties, and importance in limiting autoimmunity, of Tregs, suggests that the inactivation of autoreactive T cells by the conventional provision of signal 1 alone is insufficient to prevent damaging autoimmunity. The existence of many, somewhat redundant, pathways for the avoidance of autoimmunity also suggests that autoimmunity was a major selective pressure in the evolution of the immune system. This suggestion serves to validate the concerns of Ehrlich, Burnet, Lederberg, Talmage and Pearlman, Bretscher and Cohn, Lafferty and Cunnigham, Janeway, and Matzinger.

1.2.13 Summary

Even at the very outset of the study of immunology it was recognized that the immune system had the potential to damage the host. As I have reviewed, several

mechanisms exist to limit autoimmunity; this underscores the potential that our immune system has to cause us harm.

The originators of the clonal selection theory for the generation of antibody attempted to integrate control mechanisms into their models for how lymphocytes could be activated. However, these models could not account for accumulating experimental evidence, and in the 1970s the two signal Models of Bretscher and Cohn, and subsequently Lafferty and Cunningham were put forward. Both models held that the signal mediated by antigen binding to the antigen receptor, signal 1, was tolerogenic, leading to the inactivation of a lymphocyte in the absence of an additional stimulus, signal 2. It took until the mid 1980s for experimental evidence for these models to accumulate to the point where many immunologists could begin to address predictions of them. The nature of the antigen receptor of T cells was determined and the MHC restriction of immune responses was integrated into the contemporary models for T cell activation.

The molecular nature of signal 2 was elucidated in the late 1980s and early 1990s and these discoveries led to new suggestions about how signal 2 could be regulated. Janeway proposed that the detection of infectious microbes, by innate antigen-presenting cells of the immune system would be critical to the induction of immunity. The dendritic cell, first identified by Steinman, emerged as a likely candidate for the initiation of T cell activation. The discovery of Toll-like receptors, that bind conserved microbial patterns, leading to enhanced presentation of antigen, particularly by dendritic cells, confirmed this suspicion. However, it is quite clear that DC can also efficiently maintain T cell self-tolerance.

Recently, it has become clear that T cells can be inactivated at many stages along their life cycle. However, the existence of a subset of CD4 T cells that actively suppress the generation of autoreactive T cells serves to prove that negative selection processes are insufficient to prevent autoimmunity.

1.3 CD4 T Cell Cooperation

1.3.1 Introduction

Cooperative interactions amongst cells of the adaptive immune system are well described. Indeed, cooperation between lymphocytes is essential for the induction of most immune responses. CD4 T cells provide help to B cells that present antigens in the context of MHCII molecules, endocytosed after binding the BCR (Claman, Chaperon et al. 1966; Miller and Mitchell 1968; Lanzavecchia 1985). CD8 T cells are aided in their transition to CTL effectors by CD4 T cell help (Keene and Forman 1982; Guerder and Matzinger 1992). In the case of B cell- T cell interaction, this cooperative interaction is achieved by direct cell-to-cell contact; B cells are both effector cells and antigen-presenting cells. However, in the case of CD4 T cell help for CTL generation, this help proceeds via an APC intermediate (Shevach and Rosenthal 1973; Ridge, Di Rosa et al. 1998; Schoenberger, Toes et al. 1998). Activation of DCs via the ligation of CD40, on their surface, by CD40L, present on the surface of activated CD4 T cells, serves to "license" these DCs to allow them to potently activate and maintain CTL responses (Shevach and Rosenthal 1973; Ridge, Di Rosa et al. 1998; Schoenberger, Toes et al. 1998; Bachmann, Hunziker et al. 2004). Experimental evidence also suggests that activation of B cells, via ligation of CD40, makes these lymphocytes, normally tolerogenic in the resting state, potent APC for generating CD4 T effector cells (Jaiswal and Croft 1997; Evans, Munks et al. 2000). Given that CD40L is normally provided by activated CD4 T cells, it would seem likely that B cells, following antigen-mediated interaction with an activated CD4 T cell would then be able to potently activate other CD4 T cells. This type of phenomenon has been called CD4 T cell cooperation (Bretscher 1999), and is reminiscent of the phenomenon of epitope spreading, as I will discuss below.

1.3.2 CD4 T Cell Cooperation

At the time of the first proposal of the two-signal hypothesis (Bretscher and Cohn 1968; Bretscher and Cohn 1970), Bretscher was aware of the quantitative implications of one of the most contentious parts of the model. The assertion that T cells require help from other T cells, for their induction, became a conceptual problem for other

immunologists. However, this "help-for-help" also allowed for a mechanism by which the class of immune response could be controlled.

A classical observation, made by Salvin, demonstrated that the dose of antigen, given in an immunization, affects the class of immunity generated (Salvin 1958). He observed that DTH generally precedes antibody production, and that the administration of very low doses of antigen tended to result in DTH that does not evolve into antibody production. Moreover, increased amounts of antigen result in a rapid transition from DTH to antibody production. Later, Pearson and Raffel linked a number of older observations together in proposing that the degree of foreignness of an antigen had a similar impact on the nature of the immune response generated upon immunization (Pearson and Raffel 1971). They summarize that minimally foreign antigens, either very small molecules, or minimally foreign proteins, are capable of inducing only DTH, upon injection. Taking these observations together, it appears that the fewer CD4 T cells that are stimulated (or the less well they are stimulated), the more prone these cells are to becoming mediators of DTH; enhanced foreignness, or higher doses of antigen, generally favor the induction of antibody. Considering this evidence, in light of the findings of Parish, that DTH and antibody responses are often exclusive, one suppressing the induction of the other (Parish 1971; Parish 1971; Parish and Liew 1972), it can be suggested that CD4 T cells play a major role in determining the class of immunity generated upon immunization.

Bretscher put forward a model of immune class regulation that was consistent with the two-signal hypothesis (Bretscher 1974). According to this hypothesis, the number of inductive cellular interactions that occur in a population of responding lymphocytes, during the course of the development of an immune response, determines whether this population will express DTH or antibody. Thus, the cellular interaction involved in the generation of immune responses, as originally envisaged, may also be responsible for the determination of the class of an immune response. This hypothesis, extended to its natural ends, would then predict that 1) the absence of cellular collaboration results in inactivation of lymphocytes (by signal 1 alone), 2) small amounts of cellular collaboration results in DTH, and 3) increased interactions between lymphocytes result in antibody responses. These basic principles have been tested, in various experimental models, over the last four decades, and appear to be generally true.

Initial experimental evidence that T cells provide help for the induction of DTH-mediating T cells was provided by the experiments of Tucker and Bretscher (Tucker and Bretscher 1982). In order to determine the cellular requirements for the generation of DTH-mediating cells, the authors developed a culture system in which burro erythrocyte (BRBC)-specific DTH-mediating cells could be induced. After establishing that culturing, with BRBC, of a specific density of spleen cells was required to produce DTH-mediating T cells, the number of spleen cells was reduced to a level where DTH-mediating T cells were no longer induced. Only antigen-specific T cells, supplemented back into these cultures, resulted in restoration of the induction of DTH-mediating T cells. Furthermore, T cells specific for fowl gamma globulin (FGG) could aid in the induction of BRBC-specific DTH if FGG was *physically linked* to BRBC in culture. These results suggest that the density of T cells in the tissue culture dish had a great influence on the generation of DTH-mediating T cells.

An interpretation of this finding is that density influences the number of antigen-mediated interactions that occur in the dish over time. Bretscher published further evidence supporting this interpretation, showing that while low density cultures of splenocytes, with antigen, did not induce antigen-specific responses, medium densities of cells yielded DTH-mediating T cells and high-density cultures led to the production of antibody (Bretscher 1983). Further observations showed that activated CD4 T cells were responsible for the "help" in inducing DTH-mediating T cells *in vitro* (Bretscher 1986), and that culturing splenocytes from humorally immune mice, in medium densities, with antigen, resulted in a reversion to DTH, suggesting that the phenotype of immune responses can be modulated over time by cooperative interactions between lymphocytes (LeClercq and Bretscher 1987). These results have been confirmed *in vivo*, by the adoptive transfer of different numbers of syngeneic lymphocytes (Bretscher 1983; Ismail and Bretscher 2001; Ismail, Basten et al. 2005), and *in vitro*, by culturing different numbers of antigen-specific CD4 T cells with constant numbers of T cell depleted APC (Rudulier, Kroeger et al. 2012). In each case, it appears that increased interactions amongst lymphocytes enable the evolution of DTH to antibody. In modern terms, infrequent interactions between antigen-specific CD4 T cells results in the Th1 cells, as

assessed by the production of IFNγ and frequent interactions between these CD4 T cells results in their evolution to IL-4-producing Th2-like cells.

Conceptually, this idea is very simple. However, the cellular and molecular mechanisms that enable the interaction of CD4 T cells were, and are, not well understood. A major part of this thesis deals with my investigations into the mechanisms governing CD4 T cell cooperation.

More recently, other groups have shown that cooperative interactions amongst CD4 T cells can serve to enhance their activation. Gerloni et al. demonstrated that mice immunized with a DNA vector encoding a polypeptide derived from the primary sequence of the tumor-associated antigen MUC.1 generate a greater CD4 T cell response if the vector also encodes an immunodominant peptide from a protozoan parasite (Gerloni, Xiong et al. 2000). Interestingly, the expression of proteins from this DNA vector was restricted to B cells, suggesting that B cells are sufficient to mediate the cooperative interaction between CD4 T cells. They subsequently showed that priming of CD4 T cells, following similar immunization, could also occur in mice made genetically deficient in the generation of DCs, indicating that DCs are not essential for cooperative responses in their system (Castiglioni, Lu et al. 2003). Taking their findings a step further, Gerloni demonstrated that cooperation between CD4 T cell populations, mediated by adoptively transferred, vector-transfected B cells, resulted in enhanced immunity to MUC.1 and tumor resistance in MUC.1 transgenic mice (Gerloni, Castiglioni et al. 2005). Creusot et al. have also reported that cooperation between two TCR-transgenic CD4 T cell populations can occur in response to in vivo vaccination with a DNA vector encoding proteins that give rise to peptides recognized by the transgenic CD4 T cells (Creusot, Thomsen et al. 2003). The cooperation observed was most efficient when two vectors, encoding each protein, were delivered on a single gold particle to the same cell, implying that cooperation occurs most efficiently by a linked mechanism.

Another recent publication, from our own laboratory, has demonstrated cooperation between endogenous CD4 T cells in the primary generation of protein-specific immune responses. Evidence was obtained in experiments in BALB/c mice made tolerant to $HEL_{105\text{-}120}$. Critically, these mice fail to respond to $HEL_{105\text{-}120}$ *and all other HEL peptides* upon immunization with HEL protein on ALUM. The generation of CD4

effector T cells specific for the other HEL peptides could be rescued by immunizing with HEL coupled to OVA, thus replacing the $HEL_{105-120}$-specific cooperating CD4 T cells with an OVA-specific CD4 T cell population (Peters, Kroeger et al. 2009). Thus, it appears that in some cases CD4 T cells are required for the optimal generation of effector CD4 T cells even in the face of stimulation with potent adjuvants.

1.3.3 The Role of the B Cell in CD4 T Cell Cooperation

The results of the studies, cited above, are supportive of a role for cooperation between CD4 T cells in their optimal generation of effector cells and in determining their Th1/Th2 phenotype. Some of these results also suggest, and directly demonstrate, that CD4 T cell cooperation can be mediated by B cells acting as APC. Theoretically, the B cell seems to be the only APC that could achieve CD4 T cell cooperation, in a manner that would be consistent with observations of the past. The antigen-specific collaboration between CD4 T cells has been shown to be achieved by a linked mechanism (Tucker and Bretscher 1982; Creusot, Thomsen et al. 2003; Ismail, Basten et al. 2005). That is, presentation of epitopes to both the helper CD4 T cell, and to the CD4 T cell being helped, must be accomplished by the same APC. Given the B cell's role as an antigen-specific APC, that samples and presents antigen by virtue of its binding to the BCR, the B cell is ideally suited to mediate "linked" cooperative effects. If DCs and other APC, that endocytose antigen via germline-encoded receptors, normally mediate CD4 T cell cooperation, then no requirement for linked recognition would be seen; unlinked antigens could be taken up and presented together. In fact, upon simultaneous immunization with two antigens, one at a dose that normally induces Th1 responses, and the other at a dose that normally induces Th2 cells, results in independent generation of the appropriate phenotype, even if these antigens are presented in the same lymphoid organ (Ismail and Bretscher 1999). Thus, based on this experimental evidence, one would propose that B cells must be the APC that normally mediates CD4 T cell cooperation, as Bretscher did in his two-step, two-signal hypothesis for the primary activation of CD4 T cells (Bretscher 1999).

1.3.4 Epitope Spreading

1.3.4.1 The Phenomenon of Epitope Spreading and Autoimmunity

Epitope spreading, in immunity, is a process whereby the number of B cell- and T cell-recognized antigenic determinants that are responded to accumulate over the course of the immune response. In other words, while initially antibody and T cell recognition of antigen may be restricted to one, or a few, epitopes, during the evolution of the immune response, more antigenic determinants are incorporated into the repertoire of activated lymphocytes. This phenomenon has been observed in response to infection (van der Most, Sette et al. 1996), and experimental immunization, but is most commonly discussed in terms of the development of autoimmunity. However, as Mamula points out in a 1998 review,

> It must first be understood that epitope spreading is a fundamental mechanism of the immune system that has evolved for the survival of organisms, not for the development of autoimmunity. It seems obvious that the most effective means by which to clear an infectious agent or tumor challenge is to attack as many target epitopes as possible. (Mamula 1998)

Thus, epitope spreading appears to be a common mechanism for potentiating efficient immunity.

In cases where autoimmunity arises, spreading in the autoantigenic determinants that are recognized by T cells may result in chronic disease. An example of epitope spreading, in the commonly employed mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), was reported by Lehmann et al. who showed that primary immunization with myelin oligodendracyte glycoprotein (MOG) results in generation of effector CD4 T cells that recognize only the peptide consisting of amino acid residues 1-11. However, as the course of clinical disease progresses, CD4 T cells that respond to other peptides of MOG can be found. Eventually, CD4 T cells that recognize at least four different epitopes of MOG are generated (Lehmann, Forsthuber et al. 1992). Thus, while initially, these additional MOG epitopes are not responded to, following immunization with MOG in CFA, they become part of the CD4 T cell

repertoire through epitope spreading. Others have confirmed (Jansson, Diener et al. 1995) and extended this work in EAE and have even suggested that administration of high-dose peptide, which inactivates CD4 T cells, may be effective clinical therapy for MS (Yu, Johnson et al. 1996). Similar findings have been reported in murine models of lupus (Bockenstedt, Gee et al. 1995) and autoimmune diabetes (Kaufman, Clare-Salzler et al. 1993). Epitope spreading appears to be a common mechanism by which the repertoire of lymphocytes is expanded during autoimmune responses, prolonging the clinical course of the disease.

Accepting that epitope spreading in CD4 T cells occurs, one is led to question the cellular and molecular mechanisms that underlie the phenomenon. Mamula (Mamula 1998) outlines a two stage cellular model for epitope spreading in the context of autoimmunity. In stage one, the initial priming of autoreactive CD4 T cells is achieved by the activation of these cells by DC. The activated CD4 effector T cells, specific for a single peptide of the autoantigen then break B cell tolerance by activating an autoreactive B cell that presents the autoantigen following uptake via the BCR. The second stage of epitope spreading involves the expansion of B cells that bind, take up, and present autoantigen to T cells. Diversification of the T cell repertoire is achieved by activation of CD4 T cells, specific for linked determinants, taken up in physical association with the initial autoantigen by the activated and expanded pool of autoreactive B cells. A cascade of such reactions ensues leading to further diversification of the B cell and T cell repertoire. This mechanism is strikingly similar to the two-step two-signal model for the activation of CD4 T cells (Bretscher 1999). As I will outline in section 1.4, B cells are efficient APC for CD4 T cells. The presence of un-inactivated autoreactive B cells, in the periphery, is sufficient to allow for the loss of CD4 T cell self-tolerance (Lin, Mamula et al. 1991). The evidence that B cells, as antigen presenting cells, play a major role in the development of many autoimmunities supports this hypothesis.

1.3.4.2 The B Cell as APC in the Induction of Autoimmunity

In order for autoreactive B cells to activate autoreactive CD4 T cells, leading to epitope spreading, they must avoid negative selection and persist in the periphery. Here, I

outline the evidence supporting the hypothesis that autoreactive B cells can survive in the peripheral tissues, and have the ability to initiate autoimmunity. Further evidence for the role of B cells in the activation and generation of CD4 effector T cells is discussed in section 1.4.

Two model systems, discussed in section 1.2.5 above, lent not only to our understanding of central tolerance in B cells, but to how some autoreactive B cells persist in host animals.

Some B cells with autoreactive BCRs may persist following dilution in avidity for self-antigens following a secondary immunoglobulin rearrangement. Nemazee noticed that a few B cells persisted in the offspring of BCR transgenic mice mated with mice transgenic for the antigen recognized by that BCR. They had somehow managed to alter their receptor immunoglobulin (Ig) to a form that was not self-reactive (Tiegs, Russell et al. 1993). This phenomenon became known as "receptor editing". It appears that B cells that rearrange an immunoglobulin light chain (IgL) that confers autoreactivity, may reinitiate recombination of the IgL genes. The B cell has a second chance to generate a functional Ig molecule that does not bind self-antigens. More often than not, these secondary rearrangements fail to rescue the B cell, and upon further stimulation, the cell is deleted after binding of the autoreactive BCR to antigens in the bone marrow stroma. However, a small proportion of non-autoreactive, re-rearranged IgL abrogate the self-affinity of the prior Ig molecule. In some cases, the new arrangement destroys the old IgL locus, but occasionally, a new locus is rearranged, resulting in B cells of dual specificity. Dilution of the autoreactive Ig with the new non-autoreactive Ig lowers the avidity of interaction with self-components and can allow these dual-specific B cells to escape deletion and emigrate to the periphery. This is a phenomenon that has been called allelic inclusion. Liu and colleagues demonstrated this phenomenon, in a system similar to Nemazee's, and found that persisting dual specific B cells could be stimulated to produce autoantibody under certain circumstances (Liu, Velez et al. 2005). In some cases, B cells with dual BCRs, one specific for self, may persist in the periphery.

Chris Goodnow's original dual transgenic system revealed another way that B cells may escape negative selection, anergy. In these mice HEL-specific B cells did emigrate to the peripheral tissues, though they remained unable to respond to Ig

stimulation. Even though anergic cells are found to be mostly unresponsive to antigenic stimulation, their reactivity can be recovered when antigen is removed, suggesting that anergic cells can recover their ability to respond to antigen (Goodnow, Crosbie et al. 1988). Thus, anergic B cells, specific for self-antigens may persist in the periphery, and these cells can be activated under certain circumstances.

A third way that autoreactive B cells can persist in the periphery is as normal follicular B cells ignorant of their self-reactive potential. This "clonal ignorance" was observed by Mark Shlomchik, working in Martin Weigert's lab, when he made mice transgenic for an Ig that had affinity for self-IgG molecules. In secreted form, these antibodies make up what is known as rheumatoid factor (RF). The B cells in these transgenic mice not only persist in the periphery, but enter the follicles and seem to be undisturbed by their reaction to self (Shlomchik, Zharhary et al. 1993).

How are autoreactive B cells activated under pathologic conditions to give rise to autoimmunity? A classical example of B cell mediated autoimmunity is rheumatic fever following group-A streptococcal infection. The highly immunogenic M polysaccharide antigen is thought to cross-react with protein components of the valves of the heart. Upon infection anti-M antibodies are induced which can subsequently bind the heart valves causing a type II hypersensitivity reaction in the heart which results in congestion and, in extreme cases, death (Mims, Nash et al. 2001). Clearly there are B cells specific for this cross-reacting antigen in the body prior to infection. Possibly these cells persist being ignorant of their affinity for the heart-valve tissue. The autoreactive B cells are activated via cognate interaction with CD4 T cells specific for peptides derived from streptococcal antigens. Thus, in some cases, activation of autoreactive B cells can occur via the conventional route.

In the case of rheumatic fever, there is an abundance of CD4 T cell help for the autoreactive B cell. However, normally both the B cell compartment and T cell compartment are heavily biased against self-reactivity making this interaction very unlikely. There are at least two other ways in which autoreactive B cells may be activated in the periphery.

Despite the variable nature of systemic lupus erythramatosis (SLE), the antibodies that are generated are towards a limited number of targets. These antigens are often

normally hidden from Ig interaction by virtue of their location within cells. Major examples are DNA and Smith's complex (Sm), a complex of RNA and associated proteins. It is easy to speculate that B cells that are specific for these antigens are often clonally ignorant and therefore need only to be induced. In special cases, B cells can produce antibody without cognate interaction with CD4 T cells. These cases involve strong stimulation through the interaction of the B cell with conserved microbial products. For example, the provision of LPS to anergic B cells can partially rescue antibody responses (Goodnow, Crosbie et al. 1988). A common thread between many lupus antigens is that they often provide such stimulation. Both DNA and RNA have the potential to stimulate toll-like receptors. The aggregation of Fc portions of antibodies causes complement deposition, which is highly inflammatory, and may be important in the generation of RF. Lupus prone mice that are deficient in toll-like receptor signaling have defects in the production of anti-DNA and anti-Sm autoantibodies (Shlomchik 2008). Moreover, the ability of immune complexes and complement components to cause antibody production by anergic B cells has been directly demonstrated (Nemazee 1985; Lyubchenko, Dal Porto et al. 2007). Certain antigens, it seems, are able to provide both signal 1 and a second signal that allows for antibody production.

Defects in the normal signaling pathways in B cells may result in the third potential way that auto-reactive B cells can be induced (Shlomchik 2008). In general, defects in BCR signaling components, for example, may result in altered tolerance induction. Moreover, defects in molecular mediators of apoptosis may allow autoreactive B cells to survive negative selection processes, increasing the likelihood that one of the two previously mentioned mechanisms will take over. The complex genetic component to B cell mediated autoimmunities, was revealed by back crossing BCR transgenic strains with mice of the MRL.Fas SLE prone strain. Thus, in some cases, genetic abnormalities may predispose individuals to autoimmunity by altering the normal tolerance pathways.

Autoreactive B cells can persist in the periphery, but how do they mediate autoimmunity? Firstly, and most obviously, is the production of autoantibody, which leads to tissue destruction and inflammation, as in the case of rheumatic fever above. However, there are many reports of the critical role that B cells play in supporting T cell mediated autoimmunities (Lin, Mamula et al. 1991; Lyons, San et al. 1999; Matsushita,

Yanaba et al. 2008), and even in supporting SLE beyond antibody production (Chan, Hannum et al. 1999; Chan, Madaio et al. 1999). Treatment of a broad variety of primarily T cell mediated autoimmunities, by systemic depletion of CD20+ bearing B cells, is effective in treating these diseases (Lund and Randall 2010). Moreover, given the capacity of B cells to stimulate T cells, resulting in epitope spreading of the T cell repertoire, B cells are likely to be critical to the development of many different autoimmune diseases. It is likely then, that the major role for B cells in T cell autoimmunity is to present antigen. The role of B cells as antigen-presenting cell for T cells will be further discussed in section 1.4.

1.3.5 Summary

Cooperative interactions amongst lymphocytes are essential for the generation of immunity. Experimental evidence, obtained in our laboratory and others, over the past three decades, has led to the conclusion that CD4 T cells cooperate with one another in the generation of optimal CD4 T cell responses. Moreover, it appears that the degree of the cooperative interactions involving CD4 T cells can have a role in determining the Th1/Th2 nature of the immune response generated. Due to the observation that CD4 T cell cooperation requires physical linkage of the T cell epitopes, both in culture and *in vivo*, B cells are thought to be the APC mediators of this interaction.

Another well-described phenomenon, from the literature regarding autoimmunity, is epitope spreading. As an immune response develops, the number of epitope-specific clones that react with the antigen increases. In normal immune responses, this phenomenon appears to ensure efficient clearance of the pathogen and the development of robust memory. In autoimmune disease, epitope spreading serves to prolong the course and to leads to immunopathogy. In some cases B cells, acting as APC, have been shown to be critical for the induction of T cell autoimmunity. Moreover, it appears that B cells mediate epitope spreading.

Because of the high degree of similarity between the phenomena of CD4 T cell cooperation and epitope spreading, it can be suggested that these processes are manifestations of the same basic mechanism. As I will outline in the next section, much

evidence supports the hypothesis that B cells acting as APC can potentiate and mediate the evolution of Th1 to Th2 responses during the course of responses to experimental antigens and to infections. This evidence serves to further underscore the importance of CD4 T cell cooperation in the development of immunity.

1.4. The Role of B cells as Antigen Presenting Cells in CD4 T Cell Activation

1.4.1 Introduction

While T cells fulfill their effector function in eliminating infection by provision of cytokine, of by directly killing infected self-cells, B cells differentiate to produce antibody molecules, which bind antigen and harness a variety of potent secondary effector mechanisms to clear invading organisms. During infection, the fates of antigen-specific B and T cells are intimately linked. B cell differentiation into antibody producing plasma cells depends on help from activated CD4 T cells. Whether this interaction is also essential for the optimal generation of effector CD4 T cells is the subject of a debate that has been ongoing for four decades.

In Chapter 4, I present evidence that cooperative interactions between CD4 T cells can be mediated by B cells. In light of this finding, the role of B cells as APC for CD4 T cells becomes more important to the study of their biology. Here, I attempt to explore, in an unbiased way, the literature surrounding the APC function of B cells. In bringing together diverse, and sometimes contradictory, observations on the role of B cells in the activation of CD4 T cells, I hope to reconcile these into a comprehensive model for B cell involvement in CD4 T cell responses.

1.4.2 Do B Cells Present Antigen to T cells?

Classical studies clearly defined a role for T cells in the activation of B cells leading to antibody production (Claman, Chaperon et al. 1966; Miller and Mitchell 1968; Mitchell and Miller 1968; Nossal, Cunningham et al. 1968). From this early perspective, the major role of T cells, in particular the $CD4^+$ subset, is to provide “help” to the antigen

receptor-bearing B cells to allow for optimal antibody production. Though this interaction is often envisaged as a "one-way street", with signals coming mainly from CD4 T cells and delivered to the B cell, a large amount of literature describes a role for the B cell in CD4 T cell activation. Many early studies were aimed at determining whether B cells were even capable of presenting antigen to CD4 T cells. Findings that B cells are capable of antigen presentation naturally led to the question of the functional significance of this interaction, beyond the induction of antibody production, a question to which we will return later.

By the early 1980s, technological advances led to the development of assays that allowed detection of immune responses *ex vivo*. The tritiated-thymidine incorporation assay, that allowed T cell-specific proliferation to be measured, was developed. Detection of the incorporation of radiolabeled nucleotides into the nascent DNA of dividing T cells, enabled the study of antigen presentation in a dish. These experiments involved mixing different combinations of antigen-primed T cells with different antigen-presenting cell populations and different antigens and then assessing the proliferation of the T cells. To ensure that the proliferation measured was due only to T cell incorporation of tritiated thymidine, the APC populations were most often heavily irradiated. Employing purified leukocyte populations allowed for direct assessment of APC capability. This basic assay, together with careful experimental design, yielded significant information regarding the role of B cells in presenting antigen to CD4 T cells.

Chesnut and Grey demonstrated that splenic B cells, thoroughly depleted of macrophage-like cells, could induce antigen-specific proliferation of T cells. This presentation could occur provided that the B cells took up this antigen by way of interaction with the BCR and that the B cells and T cells were MHC compatible (Chesnut, Endres et al. 1980). Other groups extended these observations and demonstrated that B cell lymphoma cells could also present antigen to CD4 T cells (McKean, Infante et al. 1981; Glimcher, Hamano et al. 1982; Glimcher, Kim et al. 1982). Presentation occurred at a range of antigen concentrations similar to those that allowed presentation by macrophages. B cells, it appears, are efficient APC for CD4 T cells.

Since B cells are subject to activation by interaction with antigen or with CD4 T cells, it was of interest to determine whether the activation status of the B cell had any

effect on antigen presentation to T cells. Following-up their earlier work, Grey and colleagues showed that small, dense, resting B cells, are inefficient APC for CD4 T cells, whereas less dense, presumably activated B cells, are highly efficient APC. Activation of resting B cells by culturing with LPS, or through stimulation with rat anti-mouse IgG, which aggregates the BCR providing an analog of antigen binding, resulted in efficient antigen-presentation to T cells (Kakiuchi, Chesnut et al. 1983). Making use of the A20 B cell lymphoma line, they also demonstrated that antigen processing, a phenomenon originally observed in presentation of antigen to T cells by macrophages, was required for antigen presentation by B cells (Shimonkevitz, Kappler et al. 1983). Malynn and Wortis demonstrated that antigen-specific B cells from the lymph nodes of immunized mice could present antigen to T cells *in vitro* provided that both the B cell and T cell epitopes were present and physically linked (Malynn and Wortis 1984), confirming long-recognized rules for B cell-T cell interaction *in vivo*. Moreover, antigen-specific presentation by B cells is highly efficient, requiring only very low antigen concentrations. Ashwell et al. re-visited the question of whether the activation status of B cells was important in their antigen-presenting role. They found resting B cells could productively present antigen to T cells provided this presentation led to T cell-dependent B cell activation and proliferation. (Ashwell, DeFranco et al. 1984). Thus the presentation function of resting B cells was radiosensitive. This finding explained why, in previous T cell proliferation type experiments, irradiated resting B cells were found to be inefficient APC. Therefore, B cells can present antigen to CD4 T cells if 1) they were activated in some way, often by way of BCR ligation, 2) they took up the polypeptide that gave rise to the T cell epitope and 3) the B and T cells were MHC compatible.

The work of Antonio Lanzavecchia tied together these observations leading to a model of how B cells present antigen to CD4 T cells (Lanzavecchia 1985). He observed that presentation by antigen-specific B cells required a 10^4 to 10^5 fold lower antigen concentration than presentation of Ag by polyclonal B cells. This indicated that B cells specifically acquired antigens for presentation via the BCR. In his experimental system, B cells bound, internalized, and proteolytically digested antigen to enable presentation to CD4 T cells. The B cells and T cells had to be MHC compatible for presentation to occur. Given these observations, it was suggested that B cells take up antigen by way of the

BCR, which is then processed and presented as peptides in the context of MHC molecules to T cells (Howard 1985).

This model accounted for most observations, regarding the B cell-T cell interaction, made up until this point, and resolved the long-standing issue of how MHC-restriction played into the generation of antibody responses. However, even though it was clear that B cells could present antigen to T cells, it remained unclear whether this presentation was solely involved in the activation of B cells or whether there was a reciprocal effect on T cell activation. To address this question, very many studies were undertaken, and many addressed the priming of T cells in B cell deficient mice. These studies have yielded a great deal of insight into the role of B cells as APC.

I will review the literature concerning B cell deficient animals and some evidence from therapeutically B cell depleted humans later. First, I will focus on studies where positive observations of a role for B cells in the activation of CD4 T cells were made.

1.4.3 Positive Evidence That B Cells Are Involved in CD4 T Cell Priming

The presence of autoreactive B cells in the periphery often leads to autoreactive CD4 T cell activation. Lin et al. demonstrated this phenomenon when they employed cross-reacting human cytochrome c (hCC) to break B cell tolerance to endogenous mouse cytochrome c (mCC) (Lin, Mamula et al. 1991). Despite the production of autoreactive anti-mCC antibodies, following immunization with hCC in complete Freund's adjuvant (CFA), mice remain tolerant to mCC at the T cell level. This maintained T cell tolerance possibly reflected the scarcity of mCC protein which limited its ability to be presented by autoreactive B cells. However, upon immunization with a mixture of hCC and mCC, tolerance at both the antibody level and the T cell level was broken. Furthermore, purified B cells from mice immunized with hCC alone, when transferred into naive recipients allowed efficient priming of mCC specific T cells upon immunization with mCC alone. Thus, the presence of autoreactive B cells, and sufficient amounts of antigen, allow for priming of autoreactive T cells. These findings were extended by de Vos et al. who showed that mice with BCR-transgenic B cells, specific for HEL, that were crossed with mice expressing HEL under the α-crystalin promoter, leading to expression in the lens of

the eye, supported the accumulation of autoreactive CD4 T cells (de Vos, Fukushima et al. 2000). Autoreactive B cells, in this case, remained at least partially ignorant of the experimental autoantigen. Thus, autoreactive B cells that are not eliminated by self-antigens in the periphery, support the survival and activation of autoreactive T cells that would normally be eliminated themselves. Taken together, these studies support the idea that B cells can play a positive role in antigen presentation, leading to the activation, of autoreactive T cells.

Buhlmann *et al*. demonstrated that the ability of B cells to prime alloreactive T cells in vivo required CD40-CD40L interactions (Buhlmann, Foy et al. 1995). Adoptively transferred, MHC disparate B cells elicited potent alloreactive T cell responses. However, under conditions where B cells could not receive CD40L signals from T cells, tolerance was induced. This finding confirmed, with a description of the signaling molecules involved, the work of Ashwell (Ashwell, DeFranco et al. 1984). However, in this system, where no peptides from exogenous antigen are presented, the role of activation via the BCR could not be addressed. B cells that present antigen without having previously been activated by T cells via CD40-CD40L interactions are tolerogenic, whereas antigens presented by B cells that have interacted with T cells in this way are potently immunogenic.

It appears that activation of B cells via the BCR is tied to their ability to activate CD4 T cells. Employing HEL-specific antigen-receptor transgenic B and T cells, Ho et al. re examined the role of BCR ligation in B cell antigen presentation (Ho, Cooke et al. 1994). In cultures containing only purified B cells and T cells from these transgenic mice, HEL protein could elicit substantial T cell activation whereas the MHCII-binding HEL peptide did not. This was not due to a defect in presentation alone, but rather a deficiency in the co-stimulatory activity of resting and anergic B cells, as agonist antibodies to CD28 restored T cell activation in peptide-containing cultures. Croft et al. subsequently demonstrated that, although resting B cells do present antigen to naive CD4 T cells, leading to initial proliferation, this activation is not sustained, and the T cells eventually become anergic (Croft, Joseph et al. 1997; Jaiswal and Croft 1997). A difference, in these cultures, between T cells, primed by resting B cells, and those primed by activated B cells, is their expression of CD40L. CD4 T cells stimulated by resting B cells, that do not

express high levels of B7 co-stimulatory molecules, fail to express CD40L, which in turn leads to their inability to reciprocally activate the antigen-presenting B cell. B cells that have been activated by binding antigen efficiently prime CD4 T cells.

It became accepted that while B cells can, and do under some circumstances, prime T cells, this priming is likely secondary to initial activation of the T cell by another APC (likely a DC). This line of reasoning depended on two main points. Firstly, resting B cells are tolerogenic when presenting peptide to naive T cells in the absence of CD40L stimulation. Secondly, T cells cannot provide CD40L if not previously activated. Thus a chicken-and-egg problem about the initial priming steps of T cell activation made it unlikely that a naive B cell could ever activate a naive T cell. If another cell, not dependent on CD40L stimulation for its activation, could initiate T cell priming, the problem is avoided. The large amount of accumulating evidence that DCs are extremely potent APC for CD4 T cells reinforced this notion. This viewpoint became pervasive and is widely held to this day. However, it would appear that at least under some circumstances resting B cells can be activated sufficiently to initiate the cascade of B cell-T cell interactions mediated by peptide-MHC-TCR and CD40-CD40L interactions (Kakiuchi, Chesnut et al. 1983; Ashwell, DeFranco et al. 1984), (Ho, Cooke et al. 1994).

According to Constant (Constant 1999), initial priming by B cells is possible *in vivo* under some circumstances. Transfer of TCR transgenic CD4 T cells specific for a peptide of moth cytochrome c (MCC) into either WT or MD4 HEL-specific BCR transgenic mice provided an opportunity to examine the presentation of peptide by antigen-specific B cells. MCC-specific T cell activation was not observed when limiting amounts of MCC alone was injected into WT or MD4 mice, nor was it observed when a conjugate of MCC-HEL was given to WT hosts. Conversely, MD4 mice were able to support activation of seeded MCC-specific CD4 T cells when MCC-HEL was given. The HEL-specific B cells in these mice became activated to express CD86 and downregulated peptide-MHC complexes indicating that they had presented antigen to T cells. Therefore, at antigen concentrations below what are required to allow activation by DC, B cells can mediate the initial activation of CD4 T cells.

More recent evidence supports a role for B cells in CD4 T cell responses indirectly. In a mouse model where the development of DC is severely impaired, the

presence of B cells is sufficient to allow T cell activation (Castiglioni, Lu et al. 2003). In addition, by employing two-photon microscopy, Gunzer et al. have observed similar patterns of T cell interaction, with activated DC and activated B cells in collagen matrices (Gunzer, Weishaupt et al. 2004). Activated B cells and CD4 T cells were visualized forming multiple productive immune synapses in an antigen-dependent manner. The B cell-T cell interactions have similar characteristics, in terms of duration and functional priming, as DC-T cell interactions. Thus, B cells appear to play an active role as APC for T cells in certain experimental settings.

Overall, experimental evidence employing B cells directly as APC supports the idea that B cells can directly activate T cells. However, the natural circumstances that would lead to exclusive antigen presentation by B cells are unclear. It would seem that activation of naive T cells, by B cells would be rare. Most B cell-T cell interactions will take place after initial T cell priming by DC. Therefore while B cells are capable of priming CD4 T cells directly, it appears that their role as APC is primarily accomplished during ongoing immune responses.

1.4.4 Evidence that B cells are involved in T cell priming from studies in B Cell Deficient Animal Models

1.4.4.1 Anti-μ Treatment

Given the findings discussed above, it is clear that B cells do not normally initiate CD4 T cell activation. However, B cell deficient animal models have pointed to a role for B cells in the generation of optimal immune responses and in achieving appropriate immune responses for clearance of pathogens.

In early experiments, *in vivo* depletion of B cells was employed to confirm their role in antibody production. Manning and Jutila undertook such experiments *in vivo* (Manning and Jutila 1972). They employed rabbit anti mouse IgM (anti-μ), given continuously to mice from the day of birth, to remove IgM-bearing cells (B cells). Not unexpectedly, the results of this study showed greatly decreased antibody production when anti-μ treated mice were challenged with sheep erythrocytes (SRBC). However, in

a subsequent study, the authors found that anti-μ treated mice were able to reject allografts equivalently to their untreated counterparts (Manning and Jutila 1972). Thus, it appeared that while B cell functions were removed by anti-μ treatment, the T cell functions remained intact.

Over the next decade, a number of researchers sought to harness this technique to study T cell activation in the absence of B cells. It became clear that in addition to removing B cells, anti-μ had an effect on the generation of effector T cells that could not be explained simply by the administration of the antibody.

Janeway et al. demonstrated that T cells from anti-μ treated mice were poor helpers for Ig production when co-transferred primed B cells (Janeway, Murgita et al. 1977). This finding was confirmed by Bottomly (Bottomly, Janeway et al. 1980) employing a different hapten-carrier system, and by Rosenberg who showed that SRBC-primed T cells from anti-μ treated mice were poor helpers for inducing class-switching in SRBC primed B cells (Rosenberg and Asofsky 1981). These findings led to the idea that in anti-μ treated mice there may also be some defect in T cells.

Jacov Ron observed that T cells from anti-μ treated and normal mice behaved similarly *in vitro*, indicating that the defect in T cell priming in anti-μ treated mice was not T cell intrinsic (Ron, De Baetselier et al. 1981; Ron, De Baetselier et al. 1983). Purified T cells from these mice responded equally well to ConA or PHA stimulation. However, upon challenge with OVA or KLH in CFA, the proliferative response of T cells was poor in anti-μ treated mice. This poor response could not be attributed to innate APC as the macrophages and DCs from normal and anti-μ treated mice were found to be very similar in their APC function. Infusion of immune serum into anti-μ treated mice did not restore T cell priming indicating that a lack of opsonized antigen was also not responsible for the T cell priming defect. Hayglass et al. described similar deficiencies in T cell priming in anti-μ treated mice which were attributed to a lack of antigen-presentation by B cells and not a defect in other APC (Hayglass, Naides et al. 1986). Taken together, these findings suggested that the defect in priming of T cells in anti-μ treated mice was due to a lack of antigen-presentation by B cells, not a problem with T cells.

Evidence for a defect in antigen presentation in B cell deficient mice continued to accumulate. T cell priming in the lymph-nodes of anti-μ treated mice was severely

impaired but could be reconstituted by providing live polyclonal B cells which seeded the lymph nodes (Ron and Sprent 1987). Kurt-Jones et al. showed that OVA-specfic T cell responses to haptenated OVA could only be reconstituted by the provision of hapten-specific B cells with compatible MHC (Kurt-Jones, Liano et al. 1988). Thus, antigen-specific B cells take up the antigen by way of recognition of the hapten, process the OVA into peptides and present these peptides to OVA-specific CD4 T cells, leading to mutual activation of hapten-specific B cells and optimal generation of OVA-specific T cells.

Overwhelmingly, the results of experiments in anti-μ mice seemed to support the idea that B cells had a functional role as APC in the generation of optimal T cell responses. It was clear that T cell priming in anti-μ mice was not completely absent, and in some cases the priming in the spleen to protein antigens was found to be equivalent in the presence or absence of B cells (Sunshine, Jimmo et al. 1991). Differences in T cell priming by way of route (systemic versus local) suggested that B cells were most critical in T cell priming when antigen did not have access to the potent non-B cell APC in the spleen.

1.4.4.2 Genetically B cell Deficient Models

Around the mid 1990s, with the advances in genetic manipulation of the mouse, the technology for addressing the role of B cells in T cell priming shifted. Two types of mouse model, made deficient in B cells by way of genetic mutation, were developed. Mice lacking the ability to generate the membrane form of IgM (μMT), and mice that could not functionally re-combine the Ig heavy-chain (JHD), came into widespread use. Though the technology and systems for assessing T cell activation were much improved by this time, many of the results obtain in μMT and JHD mice reflected those obtained in anti-μ treated mice.

Confirming earlier findings that T cell priming in the lymph nodes of B cell deficient mice is often impaired, Liu et al. found that upon immunization with OVA or KLH in CFA, very little T cell priming occurred in the lymph nodes of JHD mice (Liu, Wu et al. 1995). Again, spleen resident DC and macrophages in JHD and WT mice were equivalent in *ex vivo* antigen presentation ability. Similarly, employing the μMT model,

Constant et al. reported defects in T cell priming in response to pigeon cytochrome c (PCC) proteins, given in CFA, but not when the immunodominant PCC peptide was given in CFA (Constant, Sant'Angelo et al. 1995; Constant, Schweitzer et al. 1995). By assessing the *in vivo* loading of antigen to different APC populations, Constant was able to suggest that in situations where peptide was given, DC were much more efficient than B cells at presentation; conversely, when protein was given, B cells become loaded with peptide more efficiently and up-regulate B7 co-stimulatory molecules presumably after having taken up antigen by way of the BCR. Macaulay showed that CD4 T cells from JHD mice, primed with protein in CFA, did not provide help for B cells to class-switch to IgG1. These T cells were deficient in production of IL-4 (Macaulay, DeKruyff et al. 1998). In a more recent study, Crawford et al. demonstrated that B cells bearing class II MHC molecules were critical for the primary expansion, cytokine secretion, and memory cell transition of CD4 T cells following immunization with protein on ALUM (Crawford, Macleod et al. 2006). B cell deficiency in this system greatly limited the production of IL-4 by T cells while leaving IFNγ production relatively intact. Thus, the results of the majority of experiments undertaken in mice made genetically deficient in B cells supported the notion that B cells are critical APC, at least under certain circumstances, for optimal CD4 T cell priming.

At the same time there were a number of reports showing no difference in T cell priming between WT and μMT mice. In a comprehensive set of experiments Epstein directly addressed this issue. She found no difference in CD4 T cell proliferation, cytokine production, or CTL generation, between WT and μMT strains, in various experimental systems, such as allograft rejection, protein-in-CFA immunization, and immunization with schistosome eggs (Epstein, Di Rosa et al. 1995). The responses in control, WT mice in these experiments were somewhat lower than responses normally obtained by other groups, employing similar immunizations, which led some to question the relevance of Epstein's findings (Rivera, Chen et al. 2001).

There appears to be no difference between μMT and WT mice in T cell tolerance induction. Baird and Parker backcrossed μMT mice to a HEL-transgenic background and found that both strains of mice were tolerant to HEL (Baird and Parker 1996). Interestingly, T cells from μMT HEL tolerant mice, challenged with CFA and OVA,

produced about 2-fold more IFNγ than their WT counterparts. Similarly, Phillips demonstrated that both μMT and WT mice could be made tolerant to HGG (Phillips, Romball et al. 1996). T cells from CFA HGG challenged μMT mice generated 3-fold higher levels of IFNγ than T cells from WT C57Bl/6 mice. While these studies seemed to demonstrate a bias in the Th1/Th2 nature of immune responses in μMT mice, peripheral tolerance for T cells, once thought to proceed through an initial B cell-dependent priming step, appeared to be intact in B cell deficient mice.

Clearly, in different laboratories the level of CD4 T cell activation in B cell deficient mice could be variable. This variability was a source of confusion for those seeking to describe the role of B cells in CD4 T cell activation. Addressing this issue, Rivera et al. pointed out that most reports, in which activation of CD4 T cells was found to be similar between WT and B cell deficient animals, employed mice of the C57Bl/6 genetic background. After backcrossing the μMT mutation to BALB/c and SJL strains, the authors were able to offer an explanation for variable results obtained by different groups (Rivera, Chen et al. 2001). In μMT C57Bl/6 CD4 T cell priming was found to be decreased relative to WT mice, but highly variable from mouse to mouse, and at times, reached 30-40% of WT levels. In μMT BALB/c or SJL mice the results were much cleaner, i.e. CD4 T cell priming was severely impaired compared to WT controls. The demonstration of strain-specific differences in μMT mice has been widely ignored as most subsequent studies also employed C57Bl/6 μMT mice.

The findings of Baird (Baird and Parker 1996) and Phillips (Phillips, Romball et al. 1996), where IFNγ production was actually higher in response to immunization in μMT mice compared to WT mice, together with the findings of Crawford (Crawford, Macleod et al. 2006) and Macaulay (Macaulay, DeKruyff et al. 1998), wherein B cell deficient mice produced far fewer IL-4-secreting CD4 effector cells upon immunization, suggest that B cells are particularly suited to foster Th2 responses. This possibility is in line with physiological considerations of the role of B cells as APC, and the interplay between Th2 cells and B cell effector function (Harris, Goodrich et al. 2005).

A pair of interesting studies, undertaken in μMT mice, help reinforce a notion that B cell antigen-processing leads to increased Th2 responses. Bradley et al. showed that μMT mice, challenged with KLH in CFA, produced effector CD4 T cells that secreted

substantially less of the Th2-associated cytokines IL-4, IL-5 and IL-13 than did their WT counterparts (Bradley, Harbertson et al. 2002). Interestingly, the amount of IFNγ production was virtually identical in both the WT and μMT mice, and this was shown to be due primarily to IL-12 production by the remaining APC. Adoptively transferred B cells or large numbers of DC could restore Th2 development in μMT mice. Curiously, whether excess DC also increased the production of Th1 cytokines was not reported. In a follow-up study the specific effector molecules provided by the B cells that can re-constitute Th2 responses in μMT mice was examined. While reconstitution did not require B cell derived IL-4, IL-6, or IL-10, the expression of OX40L by B cells was required. These findings suggest that B cells allow for Th2 differentiation by way of APC function that depends on the OX40-OX40L interaction.

1.4.5 Confounding Problems in Mice Made Genetically Deficient in B cells

Concurrent with the investigation of the influence of B cells on T cell activation, a number of publications emerged that exposed potentially confounding factors in studies employing B cell-deficient mice. B cell derived lymphotoxin supports the generation of follicular DC clusters (Fu, Huang et al. 1998), the generation of germinal centers and overall lymph node organization (Endres, Alimzhanov et al. 1999), and supports the survival and expansion of tissue resident DC populations (Kabashima, Banks et al. 2005). In addition, μMT and JHD mice are deficient in Peyer's patch development, M cell generation (Golovkina, Shlomchik et al. 1999) and spleen stromal network development and have lower numbers of spleen resident DC (Ngo, Cornall et al. 2001). Clearly, the overall structure of lymphoid organs and the cell populations within are dramatically altered in B cell deficient mice. Therefore, when considering findings that are obtained regarding the priming of T cells in B cell deficient animals, one must bear in mind that DC presentation to T cells may also be impaired. However, given reports that B cells from WT animals, adoptively transferred into B cell deficient mice, largely restore T cell responses to WT levels, it is unclear, whether differences in lymphoid architecture truly affect the generation of T cell responses in B cell deficient mice.

1.4.6 Evidence that B cells are involved in CD4 T cell activation from Models of Autoimmunity

It was initially believed that T cell functions remained largely intact in animals made B cell deficient. Some of the first evidence that that was not the case came from studies in anti-μ treated rats. Willenborg et al. found that while normal Lewis rats were highly susceptible to the induction of EAE, their B cell deficient anti-μ treated littermates were not (Willenborg and Prowse 1983). None of the rats, in this study, that remained B cell deficient following EAE induction, developed symptoms. The very few anti-μ treated rats that developed antibody to myelin basic protein, indicating they were not truly B cell deficient, developed mild disease. Myers et al. reported that EAE could not be induced in anti-μ treated mice (Myers, Sprent et al. 1992). However, it was clear that adoptive transfer of encephalitogenic T cells from normal mice experiencing EAE, could not on their own, fully induce EAE in B cell deficient mice. The authors describe a role for autoantibody in the opsonization of myelin antigens. In parallel with the findings of Constant (Constant, Sant'Angelo et al. 1995; Constant, Schweitzer et al. 1995; Constant 1999) Lyons et al. described an inability to induce EAE, in μMT mice, when myelin oligodendrocyte protein was given in CFA. However, when the immunodominant peptide, MOG_{35-55}, was injected with CFA, both μMT and WT mice developed disease (Lyons, San et al. 1999). Taken together, findings in EAE, a model considered to reflect in some respects human multiple sclerosis, are consistent with basic findings in intact and B cell deficient mice. B cells are required as APC for the induction of autoreactive T cells particularly when protein antigens are given. Presumably this reflects in part the highly efficient antigen loading of B cells when protein concentrations are limiting.

Janeway et al. reported that mice, of the autoimmune prone strain MRL^{lpr}, failed to show signs of the fatal lymphoproliferative disease, common in untreated counterparts, when treated with anti-μ from birth (Janeway, Ron et al. 1987). Chan, working with Shlomchik, bred the MRL^{lpr} mutation into JHD knockouts and confirmed Janeway's findings (Chan, Madaio et al. 1999). Disease in these mice is associated with the presence, in lymphoid organs, of high numbers of CD4 and CD8 T cells with an activated and memory phenotype. In $MRL^{lpr./lpr}$ JHD mice, no evidence of increased levels of such

activated, pathogenic T cells was found. Chan extended these findings by developing a novel strain of mice that are capable of generating mature B cells, but whose B cells lack the ability to secrete antibody. These membrane IgM (mIgM) mice have become a valuable tool for researchers wishing to separate the APC and antibody producing roles of B cells, especially in models of autoimmunity. Employing the mIgM strain, crossed with MRL^{lpr} mice, it was found that the characteristic T cell activation and lymphoproliferation was similar, compared to mice of the MRL^{lpr} parental strain. A difference in immunopathology was found only in the kidneys, where Ig-complex deposition normally leads to tissue destruction. Thus, antigen presentation by B cells, and not autoantibody production, is key to the development of the characteristic autoimmunity found in MRL^{lpr} mice.

Although many factors have been found to influence the generation of insulin dependent diabetes mellitus (IDDM) in the non-obese diabetic (NOD) strain of mice, B cells appear to be absolutely essential for development of this T cell-mediated disease. NOD mice develop a form of IDDM that is the result of autoimmune reaction that destroys β-islet cell function. Serreze et al. report that after back crossing μMT to NOD mice, heterozygotes develop disease at frequencies expected of the parental NOD strain (Serreze, Chapman et al. 1996). Conversely, NOD mice homozygous for the μMT mutation are completely protected. Thus, B cells likely play a role in the genesis of β-islet reactive T cells. Whether this role is direct, involving antigen presentation to T cells, or indirect, in supporting lymphoid architecture, is not known.

Another common model of autoimmunity, induced autoimmune arthritis, which resembles rheumatoid arthritis in humans, has been found to be B cell dependent. O'Neill et al., in two recent studies have found that B cells are essential for priming the T cells that lead to severe disease in this model. Comparing JHD mice to mIgM mice, they were able to determine that antigen-specific B cell APCs supported the activation of autoreactive T cells which in turn lead to further B cell activation (O'Neill, Shlomchik et al. 2005). Because these studies were done in mIgM mice, the effects were found to be antibody independent. Extending these findings, the authors report that the presence of CD80 and CD86 on B cells is required for severe arthritis. While the level of autoantibody produced is similar between induced WT and mice where CD80/86 have

been removed specifically on B cells, the level of autoreactive T cell activation is severely limited (O'Neill, Cao et al. 2007).

Taken together, the majority of reports regarding B cell deficient mice and their susceptibility to experimental autoimmunity support the notion that B cells are essential APC for the induction of many autoreactive T cell responses. Because the pathology in the models described above is mainly due to tissue infiltrating effector T cells, it is clear that B cells, at least in these cases, do present autoantigen leading to the activation of autoreactive T cells.

1.4.7 Evidence that B cells are involved in CD4 T cell priming from models of infection

Infection models have led to great insights into how immunity is generated and maintained. Researchers have found that B cells play a role, independent of their production of antibody, in models of autoimmunity. However, it is more difficult to dissect the contributions of different cell types in infection models where effective immunity often involves multifaceted attack. Certain pathogens are susceptible to clearance by antibody, while others are cleared by CTL most efficiently, or by other effector mechanisms. Therefore, in some infection models, it appears that B cells play little role in immunity, whereas in others, B cells have been found to be critical.

Findings from basic work suggest that B cells are more critical for the generation of Th2 cells (Macaulay, DeKruyff et al. 1998; Bradley, Harbertson et al. 2002; Linton, Bautista et al. 2003; Crawford, Macleod et al. 2006), and thus lead to polarized antibody-mediated responses whereas a number of reports indicate that Th1-like IFNγ responses are similar in WT and μMT mice (Baird and Parker 1996; Phillips, Romball et al. 1996). Therefore, one may expect that B cell deficient mice infected with pathogens that are susceptible to Th1-mediated attack or CTL would not show deficiencies in clearance. Conversely, when antibody or Th2 cells were required for pathogen clearance, these B cell deficient mice may show enhanced susceptibility.

Viruses are most often susceptible to clearance by effector mechanisms of the Th1 axis. Although antibody can prevent infection of cells, most viral infections are

associated with development of CTL that destroy infected cells. Topham *et al*. found that after infection with a mouse adapted influenza virus, both WT and μMT mice cleared the pathogen and recovered in a similar fashion (Topham, Tripp et al. 1996). Mice of both strains contained similar numbers of IL-2-producing CD4 T cells, and μMT mice actually contained more IFNγ producing cells, at the height of immunity, than did WT mice. Similarly, Mozdzanowska and Gerhard reported that, after infection of μMT and WT mice with the PR8 strain of influenza, CD4 T cells were equivalently activated (Mozdzanowska, Furchner et al. 1997). However, CD4 T cells alone were insufficient for viral clearance, which required either B cells or CD8 T cells. Homann et al. reported similar results in μMT and WT mice infected with LCMV (Homann, Tishon et al. 1998). During primary acute or chronic infection of mice with different strains of LCMV, the number of effector CTLs generated were the same in μMT and WT mice. Upon transfer of CD4 T cells from acutely infected WT mice to chronically infected WT mice, the infection is resolved. Interestingly, when CD4 T cells from acutely infected μMT mice were transferred into chronically infected WT mice, the infection was not resolved. This finding seems to indicate that while primary CTL responses to virus are generated in μMT mice as well as WT mice, there is a defect in the helper function of CD4 T cells in these mice.

Not all viral infections are cleared equally well in μMT mice. Bergmann reported that μMT mice fail to clear CNS infection of the neurotropic mouse hepatitis virus, JMHV, while WT mice clear this infection (Bergmann, Ramakrishna et al. 2001). In this case, both virus specific CD8 and CD4 T cell responses were reduced in μMT mice. Iijima et al. employing a unique system where B cells and DC could be separately removed by administration of diphtheria toxin, showed that in infection with herpes simplex virus, both B cells and DC play critical roles in generation of sufficient Th1 responses to clear the virus (Iijima, Linehan et al. 2008). Thus, in some cases, it appears that optimal Th1 responses require B cells.

Some pathogens other than viruses are particularly susceptible to attack by Th1-associated effector mechanisms. Two commonly employed models are infections with intracellular parasites, the gram-positive bacterium *Listeria monocytogenes*, and flagellar protozoans of the genus *Leishmania*. Experimental infection of B cell deficient mice has

been reported, by many groups, employing these two pathogens. Matsuzaki et al. demonstrated that both μMT and WT mice clear *Listeria* infections well, but that μMT mice generate many fewer Th1 and CTL cells during clearance (Matsuzaki, Vordermeier et al. 1999). Similarly Shen et al. show efficient clearance of *Listeria* during primary infection of μMT and WT mice (Shen, Whitmire et al. 2003). However, in the absence of B cells post-contraction survival, or memory cell survival, was severely impaired. This finding is reminiscent of similar experimental infections in CD4 T cell deficient mice (Shedlock and Shen 2003) and supports the conclusion that CD4 T cell helper function is impaired in B cell deficient mice.

Leishmania major infection in mice results in either effective Th1 immunity and pathogen clearance or in ineffective Th2-associated immunity leading to progressive disease. While C57Bl/6 mice normally resist challenge with 10^6 parasites, BALB/c mice succumb due to Th2 immune deviation. BALB/c mice can be made resistant by immunization with low doses of parasite (Bretscher, Wei et al. 1992) or by blocking IL-4 (Uzonna and Bretscher 2001). David Sacks reported that partial depletion of mice with anti-μ resulted in Th1 development in BALB/c mice (Sacks, Scott et al. 1984). More recently, Ronet et al. showed that μMT BALB/c mice were relatively resistant to a particular strain of *L. major* (Ronet, Voigt et al. 2008). Susceptibility could be restored by transfer of polyclonal WT B cells but not by MD4 (anti-HEL BCR transgenic) B cells. These polyclonal B cells presented an immunodominant peptide of the pathogen. The authors conclude that B cells, as APCs, are essential for Th2 development in BALB/c mice infected with *L. major*.

The above-cited studies strongly support the hypothesis that B cell APCs are associated with Th2 development, whereas Th1 development is often independent of B cells. However, some infections, normally cleared or controlled by Th1-associated effector mechanisms, become progressive in B cell deficient mice. B cell deficient mice have been found to be more susceptible to infection with *Salmonella enterica* (Mastroeni, Simmons et al. 2000; Ugrinovic, Menager et al. 2003) *Chlamydia trachomatis* (Yang and Brunham 1998), and *Mycobacterium tuberculosis* (Vordermeier, Venkataprasad et al. 1996; Maglione, Xu et al. 2007). Taking these, and the above findings into consideration, it would appear likely that, while B cells are not generally required to generate Th1

responses, in certain cases, optimal and protective Th1 responses require B cells. This requirement may depend primarily on the antigen presenting function of B cells, leading to expansion of Th1 cells in cases where Th1 is predominant.

Infections that generally require Th2-associated effector mechanisms for clearance also appear to require B cells. For example, malaria parasites are cleared primarily by antibody. Comparing T cell responses in *Plasmodium* infected μMT and WT mice, Langhorne et al. showed that while μMT mice failed to clear infection, both strains generated large numbers of *Plasmodium*-specific CD4 T cells (Langhorne, Cross et al. 1998). The CD4 T cells from μMT mice failed to differentiate into Th2 cells, and were poor helpers for antibody production upon adoptive transfer. Significantly, adding back B cells to infected mice restored their ability to clear the infection and allowed the evolution of Th2 from "stalled" Th1 cells. Similarly, B cells have been shown to influence the number of Th1 and Th2 cells generated in murine infections of *Trypanasoma cruzi* (Cardillo, Postol et al. 2007). Thus, B cells are essential for the acquisition of the Th2 phenotype of CD4 T cells in some cases.

Nippostogulis braziliensis and *Heligmosomoides polygyrus* infections in mice result in potent Th2 immunity. Recently, two studies were undertaken to elucidate the role of B cells in immunity to these parasitic worms in murine models.

Again, antigen presentation by B cells appears to be critical in the development of appropriate immunity to parasitic worms. Liu et al. infected JHD mice with *N. braziliensis*. The mice were not able to resist this infection (Liu, Liu et al. 2007). A major deficit in Th2 generation was observed without a corresponding increase in Th1 cells. Imaging of the immune cells from draining lymph nodes of WT infected mice showed that production of IL-4 by T cells occurs as early as day 2 post-infection and is produced by T cells at the B cell-T cell border in draining lymph nodes. Moreover, JHD mice could be reconstituted in their Th2 responses by giving back WT or IL-4 knockout B cells, but not B7 knockout B cells, indicating that antigen presentation is the main role for B cells in the development of Th2 immunity in this model.

A comprehensive study by Wojciechowski examined the role of B cells in *H. polygyrus* infected mice (Wojciechowski, Harris et al. 2009). It was found that protective immunity to this worm depends on B cells; both μMT and JHD mice cannot become

immune to this infection. Antibody is necessary but not sufficient for immunity since secretory IgM deficient (similar to mIgM) mice are highly susceptible to infection, but immune serum alone from WT mice cannot protect B cell deficient animals. B cells that can present antigen associated with MHCII molecules are required for optimal generation of Th2 effector and memory cells in this model. Moreover, IL-4 secretion by B cells is dispensable for Th2 generation while IL-2 and TNFα production by B cells positively influenced, but was not absolutely required, for Th2 generation leading to effective immunity in parasite infected mice.

The results from B cell deficient mice, in general, appear to support the hypothesis that B cells are involved, and often are critical as APC, during infection. It is clear that in some cases B cells can support both Th1 and Th2 development. Following infection, some effector B cells differentiate into cytokine secreting cells (Harris, Haynes et al. 2000). Under Th1-inducing conditions, B cells by virtue of expression of the IFNγ receptor differentiate into IFNγ-secreting Be1 cells (Harris, Goodrich et al. 2005). Conversely, B cells exposed to IL-4, produced by Th2 cells, differentiate into Be2 cells that secrete IL-4 (Harris, Goodrich et al. 2005). Both B effector cell types likely potentiate their corresponding T cell responses by provision of paracrine lineage-specific cytokines. Thus, in some cases, B cells may be involved in the maintenance of specific Th phenotype and the propagation of effector T cells. Whether B cells are essential for clearance of a given pathogen, would depend heavily on the biology of the pathogenesis of infection. Differences here may explain why B cells are found to be essential for the clearance of some Th1-inducing pathogens but not all. Presumably, in some cases, Th1 development and sustenance occurs independently of B cell antigen presentation.

1.4.8 Evidence that B cells Are Involved in Activation of CD4 T cells From Therapeutic B cell-Depletion in Humans

Given the issues, highlighted in section 1.4.5 above, concerning the validity of observations employing B cell deficient animal models to assess whether B cells are involved in T cell activation, this important question has fallen somewhat out of the consciousness of many immunologists. Moreover, the widely held view that B cell

activation requires CD40L stimulation by activated T cells suggests the B cell-T cell interaction is secondary to the DC-T cell interaction. However, some recently emerging evidence from human studies employing B cell depleting monoclonal antibodies as therapeutic agents in treating cell-mediated autoimmunity has led some to suggest that B cell involvement, particularly in sustaining the activation of T cell responses, should be reconsidered.

Rituximab is a chimeric mouse/human anti-CD20 antibody that has been used clinically to selectively deplete B cells in vivo (Reff, Carner et al. 1994; Pescovitz 2006). Immunotherapy of this type has been effective in clinical treatment of B cell malignancies (Maloney, Grillo-Lopez et al. 1997) and autoimmune diseases such as lupus nephritis (Ramos-Casals, Diaz-Lagares et al. 2011). However, evidence is mounting that patients with many autoimmune diseases, where pathology is at least partially associated with T cells, also respond to treatment with rituximab. Moreover, studies of the treatment of lupus with rituximab has yielded evidence that, even though pathology is primarily mediated by antibody, autoreactive T cells are inhibited upon B cell depletion (Sfikakis, Boletis et al. 2005; Vallerskog, Gunnarsson et al. 2007). These findings have led to the suggestion that B cells, functioning as APC, are primary targets for rituximab in SLE (Liossis and Sfikakis 2008).

Despite the relative ineffectiveness of rituximab therapy in SLE (reviewed in (Ramos-Casals, Diaz-Lagares et al. 2011)), a number of clinical trials in patients with other autoimmune diseases have recently been undertaken (Dorner, Isenberg et al. 2009; Levesque 2009).

Rituximab treatment of rheumatoid arthritis patients has led to significant improvement of clinical symptoms (Edwards, Szczepanski et al. 2004; Cohen, Emery et al. 2006). Rheumatoid arthritis is characterized by destruction of joint tissue through a variety of immunopathological mechanisms. Classically it has been known that CD4 T cells play a central role in the pathogenesis of this disease, primarily due to joint tissue infiltration, and activation, of B cells to produce autoantibody (Harris 1990). While the pathogenesis of the disease is not fully understood, recent evidence suggests that CD4 T cells that produce IL-17 are particularly potent in the pathogenesis of the widely studied animal model induced by priming with collagen in CFA (Nakahama, Kimura et al. ;

Hirota, Hashimoto et al. 2007). Though direct effects on populations of autoreactive T cells have not been reported, the finding that B cell depletion ameliorates disease suggests that B cells play an important role in sustaining autoreactive T cells in rheumatoid arthritis.

Treatement of multiple sclerosis (MS) patients with rituximab has also been shown to be effective. The pathogenesis of multiple sclerosis (MS) involves formation of infiltrative central nervous system (CNS) lesions. The infiltrates are made up of both T cells and B cells. Therapeutic B cell depletion, after a single course of rituximab therapy, improves the clinical outcome of MS patients whose disease is refractory to standard therapies (Cross, Stark et al. 2006; Hauser, Waubant et al. 2008). Interestingly, Cross et al found that while autoantibody titers to a dominant antigen were not affected of B cell depletion, both B cells (presumably due to removal by rituximab) and importantly, T cells, were found in lower number in the CNS of treated patients. These findings are in line with findings in EAE models, outlined above, and support the hypothesis that antigen presentation by B cells is important in the pathology of MS.

In addition to MS and rheumatoid arthritis, rituximab therapy has been found to be effective in treatment of *pemphigus vulgaris* (Eming, Nagel et al. 2008), autoimmune diabetes (Pescovitz, Greenbaum et al. 2009), and Sjogren's syndrome (Abdulahad, Meijer et al. ; Meijer, Meiners et al. 2010). While the effects on the T cell compartment by B cell depletion in patients with autoimmune diabetes and Sjogren's syndrome can only be assumed, given the prominent role of T cells in the pathogenesis of these diseases, it was directly addressed in patients with *pemphigus vulgaris*. Eming et al. reported that while overall levels of CD4 and CD8 T cells remained constant, the number of desmoglein-specific T cells, the T cells primarily responsible for the pathogenesis of the disease, were dramatically decreased in patients depleted of B cells following rituximab treatment. Again, these findings suggest that B cells play a role in antigen-presentation to T cells in the pathogenesis of human autoimmune disease.

Taken together, the results of clinical trials where B cells are therapeutically depleted by the administration of the anti-CD20 monoclonal antibody, rituximab, support the hypothesis that B cells are somehow involved in autoreactive T cell responses in humans. This role is likely through APC function. However a subtle difference in these

findings, from human trials, and others, from animal models, is that in patients with autoimmunity, T cell responses are ongoing. If autoreactive T cells are affected by B cell depletion in these models, it is after their initial priming. Therefore the data from these trials leads to the hypothesis that, rather than being involved, solely and necessarily, in the initial priming of autoreactive T cells, antigen presentation by B cells *sustains* T cell responses. This conclusion is in line with our recent findings, as will become apparent later in this thesis. However, data bearing directly on the effect of B cell depletion on the generation of autoreactive T cells remains sparse, and thus, further investigation is warranted.

1.4.9 Conclusions

The history of the study of B cell involvement in CD4 T cell responses dates back almost as far as the identification of the two cell types. Both classical studies, and current evidence, suggest that B cells are not simply passive recipients of antigen-specific CD4 T cell help. By virtue of their ability to take up, process, and present antigen, in association with MHCII molecules, B cells are *antigen-specific* APC. This specificity makes B cells unique. B cells capture antigen through the BCR and, therefore, are not only highly efficient, picking up antigen at extremely low concentrations, but are also selective in the CD4 T cells with which they can interact. Only those CD4 T cells with affinity for peptides derived from the antigen for which the BCR has affinity, or antigens that are physically linked to this antigen, can be stimulated. Therefore, theoretically, the B cell is the ideal APC type to mediate cooperative interactions between CD4 T cells. As reviewed above, B cells appear to be critical in maintaining and enhancing the activation of CD4 T cells, under at least some circumstances. Furthermore, B cells are capable of directly activating CD4 T cells. B cells are intricately involved in the generation of effector CD4 T cells during immune responses.

Another commonality that emerges, from surveying the literature on experimental B cell deficiency, is that B cells may be involved in determining the Th1/Th2 phenotype of the activated CD4 T cells. In general B cell deficient mice are not more susceptible to pathogens, like viruses, that are normally cleared by Th1 effector mechanisms. However,

it is clear that in many cases B cells are required to generate Th2 cells. This role is not solely through the production of antibody, but is fulfilled by antigen-presentation to T cells. These findings are also consistent with the hypothesis that cooperative interactions between CD4 T cells, and mediated by B cells, can determine the Th1/Th2 phenotype of a particular immune response.

Findings, made in humans, therapeutically depleted of B cells to treat autoimmunity, add further weight to the idea that B cells mediate cooperation between CD4 T cells. As I have discussed in section 1.3, epitope spreading is a common mechanism by which autoimmunity progresses. If, as has been hypothesized, spreading in the repertoire of autoimmune CD4 T cells is a reflection of CD4 T cell cooperation, then one would expect B cell depletion to prevent this spreading. Moreover, if continued activation of CD4 T cells via cooperative events, mediated by B cells, sustains their activation, one would also expect B cell depletion to disrupt this cycle. It appears that current clinical evidence regarding the therapeutic efficacy of B cell depletion in various autoimmune diseases supports this view.

Given the evidence above, and previous findings in our, and other, laboratories, we conclude that the B cell-CD4 T cell interaction serves, at least under some circumstances, to both enhance the generation and activation of effector CD4 T cells and to enable the evolution of Th2 cells. Thus, it appears that the majority of findings, in forty years of literature on B cells as APC for the activation of CD4 T cells, are consistent with the hypothesis that CD4 T cell cooperation is mediated by B cells.

1.5 OX40 and its ligand

1.5.1 Introduction

According to Lafferty and Cunningham-type two-signal models of T cell activation, the interaction of an antigen-specific receptor on the surface of a T cell with antigen presented on an APC is insufficient for activation of the T cell. Activation requires a second signal, delivered by S^+ cells via cell-to-cell contact. When formulating this model, the authors were particularly concerned with addressing allogeneic stimulation of T cells by other cell types. The second signal that is required is thought to

be mediated by further interaction by a species-specific surface molecule that is expressed on the APC's surface (Lafferty and Cunningham 1975). The second signal that is normally required for T cell activation became known as the co-stimulation signal.

The first molecules to be identified as co-stimulators were the B7 molecules, which engage CD28 on the surface of T cells as discussed in section 1.2.6 and reviewed by Wang and Chen (Wang and Chen 2004). The effect of CD28 binding B7 has been thoroughly described and its role in the initiation of T cell activation is quite clear. However, in the early 1990's it became clear that there were other surface molecules, found on APC, that have an additional co-stimulatory role in the activation of CD4 T cells (Ding and Shevach 1996). One subsequently identified family of co-stimulatory molecules was the tumor necrosis factor superfamily (TNFSF) and their corresponding receptors (TNFR). These molecules have since become known for their role in T cell activation and modulation of effector T cell phenotype. There are a number of receptor and ligand pairs in the TNFSF with known effects on CD4 T cells (as reviewed by Croft (Croft 2003)). I will omit a comprehensive discussion of these molecules and focus on the receptor, OX40, and its ligand, OX40L, which have been shown, by others, and myself (see chapters 3, 4, and 5) to be involved in the generation and maintenance of CD4 T effector cells.

1.5.2 What are OX40 and OX40L and How Do They Function?

OX40 was first identified as a 50kDa surface protein on CD4 positive T cell blasts, and was highly specific to this cell type. The first paper on the antigen, recognized by the Oxford MRC monoclonal antibody -40 (OX-40 Ig), also demonstrated a potential functional role for OX40. Cross-linking of, OX-40, by OX-40 antibody, resulted in stimulation and enhanced proliferation of the T cell blasts (Paterson, Jefferies et al. 1987). Mouse OX40 was cloned a few years later and identified as a molecule that could potentially mediate T cell-B cell interactions. Calderhead and colleagues expressed the cloned sequence of mouse OX40 as a fusion protein with human IgG1. They subsequently employed this fusion to identify OX40L-bearing cells (Calderhead, Buhlmann et al. 1993). The ligand that bound OX40, OX40L, was identified the next

year and was found to have costimulatory activity. Wayne Godfrey and colleagues identified OX40L from a cDNA library made from transcripts found in phorbol myristate acetate (PMA)/ionomycin-stimulated B cells. Upon sequencing, OX40L was found to have homology to TNF. Fibroblasts engineered to express OX40L had potent co-stimulatory activity on T cells that were stimulated with analogs of signal one *in vitro* (Godfrey, Fagnoni et al. 1994). The year after that, the same group that had originally cloned OX40 reported that OX40L aggregation on the surface of B cells enhanced their proliferation and their secretion of IgG. They also demonstrated that the expression of OX40L was optimally induced by cross-linking the B cell receptor, while engaging CD40 (Stuber, Neurath et al. 1995). It appears that this ligand and receptor pair is involved in co-stimulation of T cell responses, especially CD4 T cell responses. Both the ligand and receptor are inducible, following antigen-receptor stimulation, and both enhance proliferation following cross-linking.

It appeared, based on expression patterns, that this pair of molecules is involved mainly in B cell-T cell interactions. Subsequently OX40L expression has been shown on dendritic cells, and that this expression is important for conventional T cell activation as well (Murata, Ishii et al. 2000). However, the notion that OX40L-OX40 interactions are quite important in B cell-T cell cooperation was further supported by evidence that ligation of CD40 on B cells (CD40L is expressed mainly by activated T cells) enhanced OX40L expression.

Irene Gramaglia demonstrated that stimulation of OX40 enhances the proliferation and survival of CD4 T cells. Employing T cell receptor transgenic CD4 T cells *in vitro*, she showed that the enhancement was downstream of B7 signaling, in that it could not replace the B7-CD28 interaction. However, OX40 signals acted potently to enhance the survival of activated CD4 T cells after antigen stimulation in culture, with the maximal difference being seen at 4-6 days of culture. Enhanced survival corresponded with the expression of OX40 on activated T cells, which was absent until 24-hours, and peaked at 48-96 hours. Taking these observations together, the authors suggested that the major role of OX40 was to sustain CD4 T cell responses by enhancing their survival (Gramaglia, Weinberg et al. 1998).

Stimulation of OX40 inhibits apoptosis in CD4 T cells. Paul Rogers, working in Croft's lab, demonstrated that OX40 stimulation directly enhanced the expression of the anti-apoptotic proteins Bcl-2 and Bcl-Xl. The upregulation of OX40 and OX40L was found to depend on CD28, which explained the group's previous findings that B7-CD28 interactions were up-stream in effect. Moreover OX40 deficient CD4 T cells had substantial defects in their ability to survive after antigen stimulation (Rogers, Song et al. 2001). The mechanism by which OX40 stimulates survival and proliferation, in T cells, was recently further elucidated by Jianxun Song. It was found that OX40 signaling mediated by the adaptor protein TNF-receptor associated factor- (TRAF-) 2 enhanced nuclear factor kappa-B1 (NFκB; RelA/p65 and p50) translocation to the nucleus and enhanced the expression of various pro-survival genes (Song, So et al. 2008). These findings explained the findings of Gramaglia at a molecular level; stimulation of OX40 leads to direct enhancement of activation pathways as well as anti-apoptotic molecular mechanisms.

1.5.3 Intracellular signaling by OX40

The major function of OX40, in CD4 T cells, appears to be to enhance cell survival, proliferation, and differentiation. As Croft outlines in comprehensive reviews (Croft, So et al. 2009; Croft 2010), OX40 signals through three main pathways. The first pathway stems from stimulation of TRAF-2 as described above. Two additional intracellular pathways, stimulated by the ligation of OX40, are outlined below.

It appears that OX40 stimulation enhances TCR-associated signaling intermediates. The cytoplasmic domain of OX40 directly associates with phosphoinositide 3-kinase (PI3K) which increases the levels of phosphoinositide phosphate$_{2/3}$ (PIP2/3; doubly or triply phosphorylated) in the inner leaflet of the plasma membrane. The enhanced levels of PIP2/3 attract proteins with the conserved pleckstrin homology domain to the membrane. One protein that is recruited is Akt, also known as protein kinase B (PKB), which is phosphorylated and leads to downstream effects such as enhanced survival, expansion and differentiation in T cells (Song, Ouyang et al. 2005). The precise molecular events, that control this interaction, were recently elucidated by So

et al. It appears that OX40 directly enhances Akt phosphorylation initiated by TCR signaling (So, Choi et al. 2011). Therefore OX40 functions via this pathway to augment TCR signaling.

The third way that OX40 has been found to signal is through synergy with the TCR in augmenting Ca^{2+} flux from the mitochondria leading to Th2 skewing in activated CD4 T cells. Though this mechanism is not completely understood, it appears that augmented Ca^{2+} flux following OX40 stimulation, allows a calcineurin dependent transcription factor to translocate from the cytoplasm to the nucleus, thus affecting gene transcription. So et al. demonstrated that, without OX40 stimulation, the calcineurin-dependent nuclear factor of activated T cells (NFATc1) fails to translocate to the nucleus. This failure resulted in decreased transcription and translation of the IL-4 gene. This finding highlights the role of OX40 in the generation of IL-4 producing CD4 T cells, which is consistent with the often-discussed function of OX40 in Th2 skewing of T cell responses, and the results reported in Chapters 3 and 4.

From the above-cited studies it can be seen that OX40-OX40L interactions play a major role in the CD4 T cell response. The role of OX40 signaling in CD8 T cells is less well defined. It seems that OX40-OX40L interactions are particularly suited to modulate CD4 T cells responses. As I will outline below, they have been implicated in many models of infection, cancer, and autoimmunity, which serves to underscore their importance to CD4 T cell biology.

1.5.4 OX40 and OX40L in the context of CD4 T cell cooperation

Integrating a role for OX40-OX40L interactions into a cooperative model for the activation of CD4 T cells places emphasis on the CD4 T cell-B cell interaction step. It is possible that, after the initial T cell-DC priming stage, a second round of activation serves to amplify the CD4 T cells numbers and to cause their further differentiation. B cells that bear OX40L could mediate this subsequent round of proliferation.

It is possible that OX40-OX40L interactions could also be involved in CD4 T cell-T cell interactions. Soroosh et al. reported that both OX40 and OX40L expression on T cells is required for their optimal activation and survival *in vitro* and *in vivo* (Soroosh,

Ine et al. 2006). It appears that T cell-T cell contact is involved in this activation, suggesting that in some cases, APC may act as a "dock" around which T cells interact by membrane-membrane contact.

Another study bears directly on the role of B cells, and their expression of OX40L, in the activation of CD4 T cells. By using a simple in-vivo model, Phyllis-Jean Linton showed that antigen presentation by B cells was essential for optimal expansion, and Th2 cytokine secretion by CD4 T cells. Moreover, the author also demonstrated that OX40L is essential for this interaction to occur (Linton, Bautista et al. 2003). The system made use of μMT mice (which are essentially devoid of B cells) and adoptively transferred ovalbumin (OVA) peptide-specific TCR transgenic CD4 T cells. B cell deficient mice seeded with 5 x 10^6 transgenic T cells were immunized with OVA on ALUM and generated poor T cell responses compared to similarly immunized WT mice. It was found that the defect in proliferation and differentiation of T cells, that was observed in μMT mice, could be overcome by supplementing these mice with *in vitro*-stimulated WT B cells. When various cytokine knockout mice were employed as a source of B cells, there was no detectable deficiency in their ability to aid CD4 T cell responses. However when $OX40L^{-/-}$ mice were employed as a source of B cells, their effect was *completely* lost. Thus, at least in one case, immunization with protein antigen in a conventional adjuvant preparation, B cells that express OX40L are necessary to allow full priming of CD4 T cells

Taken together, these studies suggest possible roles for OX40-OX40L interaction in CD4 T cell cooperation. It is possible that this cooperation occurs via both CD4 T cell-B cell interaction and through T cell-T cell interaction. Though the details remain unclear, both evidence from our work, demonstrated in Chapters 3, 4 and 5, and the findings outlined above, support the hypothesis that the OX40-OX40L interaction is involve in cooperation between CD4 T cells.

1.5.5 OX40 and OX40L in infection and cancer models

The role of OX40 in infection immunity is still controversial, however, it is likely that OX40 is not absolutely required for immunity, but rather it aids in the survival of effector cells and the transition to memory. Gramaglia et al. demonstrated that mice, immunized with KLH, mounted increased CD4 T cell responses when given an agonist antibody to OX40 at the same time. WT mice and $OX40^{-/-}$ T cells could secrete similar levels of cytokine during priming phases. However, OX40 deficiency resulted in impaired memory cell generation (Gramaglia, Jember et al. 2000). OX40 has also been implicated in the generation of Th2 immunity in *Leishmania major* infections in mice. Thus, OX40 signaling aids in survival and memory cell transition in CD4 T cells.

Much emphasis has been placed on determining whether OX40 stimulation can increase the generation of anti-cancer immune responses, which are notoriously weak. As Croft reviews (Croft, So et al. 2009), employing OX40 agonistic antibodies, as an adjuvant for increasing cancer immunity, has been extensively explored. Agonistic antibodies to OX40 have been shown to be effective at increasing anti-tumor CD4 and CD8 T cells, and in reducing the suppression of anti-tumor immunity by Tregs. Weinberg and colleagues demonstrated that either an agonistic antibody to OX40 or OX40L-Ig, administered to mice given a lethal dose of tumor, increased anti-tumor immunity and drastically increased survival in mice (Weinberg, Rivera et al. 2000). Interestingly the tumors employed in this study were MHCII and OX40 deficient suggesting that the agonist did not affect the tumor directly, but increased the generation of CTL by promoting CD4 T cell responses.

Silvia Piconese demonstrated a role for OX40 signaling in reducing the negative regulation, by Tregs, of tumor immunity in mice bearing a variety of aggressive syngeneic tumors (Piconese, Valzasina et al. 2008). She showed that intraperitoneal or intratumor injection of an OX40 agonistic antibody resulted in tumor rejection in at least 70% of mice. OX40 stimulation appears to antagonize the suppression of immunity by Tregs by directly inhibiting them.

OX40L and OX40 knockout mice have been infected with a variety of agents in order to directly test the relevance of these molecules to immunity. The results of these studies have been variable and so making general claims about the critical role of OX40 signaling is difficult. It is possible that in some cases OX40 normally plays a role in

immune class regulation (i.e. toward the Th2 pole) in which case knocking out either the receptor or the ligand would result in skewed immune class regulation. This would be beneficial in some cases and detrimental in others. Alternatively, in situations where OX40 would normally mediate effects, compensatory interactions between other molecules (maybe other TNFSF or TNFR family members) could take over, masking the effect of the OX40 or OX40L deficiency.

1.5.6 OX40 and autoimmunity

Because OX40 ligation is a very potent means of inducing effector function in T cells it is easy to imagine how aberrant stimulation of this receptor, particularly in autoreactive T cells, might lead to activation of autoimmunity.

OX40 and OX40L expression is regulated in immune cells and other tissues. As I have outlined above, both the ligand and receptor typically require activation via antigen stimulation to be expressed on immune cells. It can be intuitively understood that the regulation of the expression of these potent molecules should be tightly controlled to avoid inappropriate activation of immune cells, particularly T cells, because this could lead to immunopathology. Thus, aberrant expression of OX40 or OX40L may lead to inappropriate stimulation of autoreactive T cells.

How might inappropriate expression of these molecules occur? Though the specific endogenous transcriptional activators of *ox40* and *ox40L* genes are unknown, it appears that at least two factors are involved. Rudiger Pankow, and colleagues, showed that the 5 prime flanking sequences of both *ox40* and *ox40L* contain binding sites for the p65/P50 isoform of NF-κB (Pankow, Durkop et al. 2000). This finding built on previous work from another group that indicated the Tax protein of human T-cell leukemia virus (HTLV-1) induced expression of both OX40 and OX40L on murine T cells (Higashimura, Takasawa et al. 1996). Pankow demonstrated that the binding of Tax to the flanking sequences required NF-κB. Thus, it appears that both factors act in concert to drive expression of OX40 and OX40L. This also leads to an explanation of why cells often express both OX40 and OX40L. Because the upstream effectors of NF-κB activation may be varied, it is difficult to predict what specific types of abnormal events

might lead to the inappropriate expression of OX40 on immune cells, that could affect the development of autoimmunity. However, OX40 ligation is known to prevent inactivation of CD4 T cells. In this case, chronic inflammation or polymorphisms may lead to inappropriate expression of OX40L which could lead to inappropriate activation of autoreactive T cells by preventing normal T cell tolerance mechanisms. Whole genome association studies have shown a link between OX40L polymorphism and SLE (Manku, Graham et al. 2009).

It has been reported that ligation, usually by exogenous agonists, of OX40 can break T cell tolerance in at least two main ways. Firstly, employing a similar model to Elizabeth Kearney (Kearney, Pape et al. 1994), Pratima Bansal-Pakala et al. demonstrated that an agonist antibody to OX40 could reverse the tolerogenic effect of administration of high-doses of peptide to mice. It was shown that this agonist could prevent the induction of T cell anergy if given at the appropriate time (Bansal-Pakala, Jember et al. 2001). Another group extended this finding. Lathrop et al. demonstrated that stimulation through OX40 can break the unresponsiveness of already anergic T cells and allow them to acquire effector function (Lathrop, Huddleston et al. 2004). A second way that OX40 signals can break T cell tolerance is by interfering with the function of Tregs. Many groups have reported defects in Treg function upon OX40 ligation. In another groundbreaking paper, Takanori So and Michael Croft demonstrated that OX40 signals actively suppress the generation of Tregs from naive cells in vitro. This effect was due to antagonism of foxp3 induction (So and Croft 2007). Thus aberrant OX40 stimulation could lead to breakage of CD4 T cell tolerance.

Taken together these findings support the notion that OX40 signals mediate induction, to effector stage, of T cells, even in the presence of normally tolerogenic stimulation. Likely as a consequence of their potent activating effects, deficiencies in OX40 and OX40L have been tied to amelioration in inflammatory disorder models in rodents such as asthma and colitis and atherosclerosis. Moreover, deficiency in either the receptor or the ligand results in inability to generate experimental autoimmune encephalomyelitis and completely prevents diabetes in NOD mice, as reviewed in (Croft, So et al. 2009). By broad consensus, OX40 and OX40L have a role in autoimmunity and aberrant inflammation. However, the question remains as to whether abnormal expression

of OX40 and OX40L are at the root of the inappropriate response or whether they are simply a result of it.

1.5.7 Summary

It is clear that these molecules play a major role in the activation and survival of CD4 T cells. Functional roles for OX40 ligation, following experimental immunization, and in the development of autoimmune diseases, have been described. It appears that the patterns of expression of both OX40 and OX40L, combined with compelling evidence that expression of OX40L by B cells is necessary for the optimal generation of CD4 T effector cells, support the hypothesis that the OX40-OX40L interaction is somehow involved in cooperative CD4 T cell responses. The biology of OX40 and OX40L remain the topic of current research.

1.6 Trying to Tie it All Together

In sum, the evidence cited above leads to the belief that CD4 T cells, the cells that are centrally involved in orchestrating virtually all immune responses, are susceptible to regulation, both positive and negative, by other CD4 T cells. Many different researchers have indirectly observed CD4 T cell cooperation. Moreover, it appears that epitope spreading, a well-described phenomenon in models of autoimmunity, could be a reflection of CD4 T cell cooperation. Cooperative interactions amongst CD4 T cells have been shown to be involved in the increased generation of effector cells and Th2 differentiation; ligation of co-stimulatory molecules, like OX40, on the surface of CD4 T cells, leads to similar outcomes. Therefore it is possible that CD4 T cell cooperation involves the induction of expression of certain co-stimulatory ligands, by cells stimulated by CD4 T cells, which then further stimulate other CD4 T cells.

Given that the cellular and molecular mechanisms involved in CD4 T cell cooperation remain unclear, the central objective of the research, detailed in the following chapters, was to investigate these mechanisms. We hypothesized that cooperation between endogenous populations of CD4 T cells could be induced by the provision of synthetic peptide antigens to which these cells are known to respond. If such cooperation

could be observed, we hoped that by employing such a reductionist approach, we could attempt to answer a number of fundamental questions surrounding CD4 cell cooperation: 1) What APC are involved in mediating cooperative interactions amongst CD4 T cells? 2) Do these APC mediate cooperation in a "linked" fashion? 3) What types of co-stimulatory interactions are involved? 4) What are the functional consequences of CD4 T cell cooperation?

2.0 CHAPTER 2 – MATERIALS AND METHODS

2.1 Mice

BALB/c mice, aged between 6-10 weeks, were obtained from the College of Medicine animal facility at the University of Saskatchewan or from Charles River Canada (Sherbrooke, Canada). Parental TCR transgenic DO11.10 mice (H-2^d) were obtained from The Jackson Laboratory (Bar Harbour, Maine, USA) and were bred and housed in the Health Sciences Building Animal Quarters, College of Medicine, University of Saskatchewan (Saskatoon, Saskatchewan). In individual experiments, all mice were matched for sex and age. All mice were between six and ten weeks of age at the beginning of the experiment. All experiments were conducted under a protocol approved by the University of Saskatchewan's Animal Research Ethics Board and that adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.2 Media and Solutions

2.2.1 Carbonate/Bicarbonate Buffer for Coating ELISpot Plates

Carbonate/bicarbonate buffer (pH 9.5) for coating nitrocellulose-bottom wells in 96-well ELISPOT trays was prepared by combining 16mL 0.2M Na_2CO_3 with 34 mL 0.2M $NaHCO_3$ and 150mL ddH_20.

2.2.2 Leibovitz Media

Powdered Leibovitz medium (GIBCO Laboratories, Grand Island, NY) was prepared according to the manufacturers directions. After adjusting for pH, the prepared media was sterilized by filtration through 0.2μm nitrocellulose filter. Sterility of freshly prepared media is confirmed by culture at 37^O C for 24 hours.

2.2.3 NBT/BCIP Developing Solution

ELISPOT plates were developed by applying a solution of NBT/BCIP substrate diluted 1:100 in an alkaline (pH 9.5) substrate buffer consisting of 0.1M Tris-HCl, 0.1M NaCl and 0.05M $MgCl_2$

2.2.4 Phosphate Buffered Saline (PBS)

Phosphate buffered saline was prepared from a 10x stock solution containing 80g/L NaCl, 4g/L KCl, 11.5g/L Na_2PO_4, and 4g/L KH_2PO_4 in ddH_2O adjusted to pH 7.2. Working solutions are prepared by diluting the 10x stock with ddH_2O. Sterility was achieved by autoclaving working solutions.

2.2.5 PBS – Tween 20 (PBST)

PBS-Tween was used to wash ELISPOT plates after culture and after addition of secondary antibody. PBS-Tween was prepared by adding 0.05% v/v Tween 20 to a working solution of PBS.

2.2.5.1 MACS Buffer

MACS Buffer consisted of PBS supplemented with 2mM NaEDTA.

2.2.6 RPMI Media

Powdered RPMI medium, supplemented with L-glutamine (GIBCO Laboratories, Grand Island, NY) was prepared according to the manufacturers directions. After adjusting to pH 7.4 the prepared media is sterilized by filtration through 0.2μm nitrocellulose filter, resulting in a solution with pH 7.2. Sterility of freshly prepared media was confirmed culture at 37^{O} C for 24 hours.

2.2.6.1 RPMI Culture Media

For tissue culture and ELISpot blocking, RPMI media was supplemented with 10% fetal bovine serum (FBS; Hyclone; Canadian Origin), 0.1% 0.5M 2-mercaptoethanol, 100U/mL penicillin, 100 U/mL streptomycin and 0.8% 100mM sodium pyruvate.

2.2.6.2 ELISpot and pulsing Media

For overnight culture in the ELISPOT assay, and for overnight peptide pulsing cultures, RPMI media is supplemented with 0.1% 0.5M 2-mercaptoethanol, 100U/mL penicillin, 100 U/mL streptomycin and 0.8% 100mM sodium pyruvate without FBS.

2.3 Antigens

2.3.1 Synthetic Peptides

The peptides of hen-egg lysozyme, HEL_{11-25} (AMKRHGLDNYRGYSL), HEL_{48-63} (DGSTDYGILQINSRWW), HEL_{74-96} (NLCNIPCSALLSSDITASVNCAK), and $HEL_{105-120}$ (MNAWVAWRNRCKGTDV), as well as chicken ovalbumin peptide, $OVA_{323-339}$ (ISQAVHAAHAEINEAGR), and *Leishmania* analog of activated C kinase peptide, $LACK_{156-173}$ (ICFSPSLEHPIVVSGSWD) were synthesized by the Alberta Peptide Institute (Edmonton, Canada) or by GenScript (Piscataway, NJ USA), purified by HPLC, to >95% purity, as assessed by mass sprectroscopy. All peptide antigens were dissolved in PBS, and stored at -80C until needed.

2.3.2 Protein Antigens

Hen-egg lysozyme (HEL) (grade VI; Sigma, St. Louis, MO USA) was dissolved in PBS and sterilized by filtration through a 0.2μM nitrocellulose filter. In some cases heat-aggregated HEL (ha-HEL) was required. This procedure was previously described (Peters, Hamilton et al. 2005). 40mg/mL HEL solutions were incubated in a 100ºC water bath until visible aggregates formed. The aggregated protein was then thoroughly disrupted and pulverized using a sterile mortar and pestle. The concentration of ha-HEL was determined mathematically taking into account the amount of soluble HEL that remained in the supernatant as determined by the absorbance of this solution of light of λ=280nm.

2.4 Eliciting Immune Responses *in vivo*

2.4.1 ALUM Adjuvant Immunizations

In order to generate HEL-specific immune responses, mice were injected intraperitoneally with 200μL of a 1:1 mixture of heat-aggregated HEL protein in phosphate buffered saline with Alhydrogel(R) 2% aluminum hydroxide gel adjuvant (ALUM; Superfos Biosector a/s, Vedbaek, Denmark).

2.4.2 CFA and IFA immunizations

Immunizations with IFA and CFA were prepared by making a 1:1 mixture of the appropriate peptide(s) or protein, in phosphate buffered saline, with incomplete Freund's adjuvant (Sigma, Saint Louis, MO USA). Emulsification was achieved by passing the mixture rapidly between two syringes joined tip-to-tip. The syringes were kept cold by periodically resting them on ice. The emulsification process was continued until a consistency was reached where the colloid did not dissociate when dropped in water. In some cases, IFA was supplemented with 5μg/mouse of E. Coli serotype O111:B4 LPS (Sigma, Saint Louis, MO USA). Mice were immunized with 100μL of the IFA or CFA emulsion subcutaneously at the base of the tail or 50μL in the left hind footpad.

2.4.3 Adoptive Transfers of peptide pulsed APC

2.4.3.1 Pulsing

Spleens of euthanized naive mice were harvested into 10mL ice-cold Leibovitz media and disrupted by pressing through a fine stainless steel mesh. The resulting single-cell suspension was washed and centrifuged. The cell pellet was suspended to a final volume of 3mL. In most cases, LPS from E. Coli serotype O111:B4 LPS (Sigma, Saint Louis, MO USA), was added to a final concentration of 1μg/mL. Soluble peptides were added to a final concentration of 50μM to ensure loading. These splenocytes were then cultured for the indicated time, at 37ºC and 5% CO_2, in 60mm tissue culture-coated petri dishes (BD Falcon).

2.4.3.2 Washing and Injection

Following culture, peptide pulsed splenocytes were harvested by forceful pipetting of cold Leibovitz media. In some cases, certain cell populations were isolated by MACS, as described below. If cells were not MACS isolated they were washed, centrifuged, and re-suspended three times to remove excess peptide and LPS. The remaining viable leukocytes were enumerated by trypan-blue exclusion counting. Cells were suspended to an appropriate volume, in Leibovitz media, so that 50μL contained the desired number of cells. This 50μL injection was given into the left hind footpad and lower leg of Balb/c mice.

2.5 Antibodies

The CD40 agonist antibody FGK45, the corresponding isotype-matched control antibody 2A3, the OX40 (CD134) agonist OX86, and its corresponding isotype-matched control antibody HRPN, were obtained from Bio-X-Cell (West Lebanon, NH USA). The OX40L (CD252) blocking antibody RM134L, and the corresponding rat IgG2b control antibody were obtained, in functional grade, from eBioscience (San Diego, CA USA) or from Bio-X-Cell.

2.6 Tolerance induction *in vitro*

Activation of DO11.10 T cells was achieved by three-day cultures in 24-well culture trays (BD Falcon, Mississauga, ON, Canada). 10^5 CD4+ DO11.10 splenocytes were cultured with 3 x 10^6 T cell depleted Balb/c in the presence of 0.3μM $OVA_{323-339}$. After this culture some of these activated DO11.10 were subjected to secondary culture. Activated CD4+ T cells from primary cultures were isolated by MACS negative selection. These were then plated in 96-well V-bottom trays with 3 x 10^4 fresh T cell depleted spleen cells as APC with or without 0.3μM $OVA_{323-339}$. CTLA4-Ig (BD) at 10μg/mL was employed to block B7-CD28 interactions

2.7 Assessment of Immune Responses by ELISPOT Assay

2.7.1 ELISpot Plate Preparation

Ninety-six-well nitrocellulose bottom plates (Unifilter 350, Whatman-Unifilter, Clifton, NJ, USA) were coated with 100μL 1.25μg/mL purified monoclonal anti-interferon gamma (IFNγ), interleukin two (IL-2), or interleukin four (IL-4) antibodies (BD Pharmingen, San Diego, California, USA) at 37^{O}C for four hours in ELISpot coating buffer. The plates are then rinsed with RPMI ELISpot media and blocked with RPMI culture media for at least one hour at 37^{O}C.

2.7.2 Spleen and lymph node Cell Preparation

Spleens of immunized and normal mice were harvested aseptically following euthanasia via cervical dislocation. Individual spleens were placed immediately into ice-cold Leibovitz media and quickly passed though a stainless steel mesh to achieve a rough cell suspension. This suspension was mixed thoroughly and rested for two minutes on ice to remove remaining cell clumps. The top 5mL of the de-clumped suspension was harvested and kept in a separate tube on ice. The number of leukocytes present in this suspension was determined by counting non-red blood cells at a 1:5 dilution of on a haemocytometer after a further dilution of 1:2 in trypan blue. Only leukocytes that exclude the trypan blue stain were counted. The suspension was then centrifuged for ten minutes at 250g. The supernatant is removed and the cell pellet is suspended to 10^7 leukocytes/mL in RPMI ELISpot media.

2.7.3 Plating of cells and Internal Controls

The resuspended spleen cells from normal mice were plated at 50μL (5 x 10^5 leukocytes)/well. The number of leukocytes per well that reliably generates linear data has been determined to be 1.5 x 10^6 as previously reported (Peters, Hamilton et al. 2005). Accordingly, spleen cells from immunized mice were plated in triplicate wells for each

condition at 100μL (10^6 leukocytes)/well. In addition triplicate wells with 1.5 x 10^6 leukocytes from unimmunized animals were plated for each assay condition. To elicit antigen-dependent cytokine release, peptide antigens were diluted to 140nmol/mL in RPMI ELISpot media and 50μL of this solution was added to the appropriate wells. Media without antigen was also plated as an antigen-negative control for all spleen samples.

2.7.4 Developing and Counting

ELISpot plates were cultured for 16-20 hours at 37^{O}C and 5% CO_2. Following culture, the media was removed, and the remaining cells were lysed by washing the plate twice with ddH_2O. Plates are then further washed with PBS-Tween four times. Secondary, biotinylated, anti- IFNγ, IL-2, and IL-4 (BD Pharmingen, San Diego, California, USA) was diluted to 1.25μg/mL in PBS and 100μL is added to the appropriate wells. Plates were then incubated for at least 1.5 hours at room temperture. After incubation, excess secondary antibody is removed by washing five times with PBS-Tween and then two times with ddH_2O. Streptavidin conjugated alkaline phosphatase enzyme was then diluted to 0.2μg/mL and 100μL of this solution is added to every well. The plates were once again incubated at room temperture for 45 minutes. Plates were then washed extensively in dH_20 by submersion and 100μL of NBT/BCIP 1:100 developing solution is added to each well. Plates were incubated at 37^{O}C for about 10 minutes or until visible spots form. The developing reaction was stopped by washing twice in dH_2O. Plates were left to dry overnight. Antigen dependent cytokine producing (ELISpot forming) cells were enumerated by counting cytokine spots in antigen positive wells and subtracting the number of spots in antigen negative wells. Unimmunized control samples serve to verify the antigen specificity of the assay; in all reported experiments the number of Ag-dependent CSCs in unimmunized samples was less than twice the number of spots in antigen negative wells.

2.8 Characterization of Cellular Markers by Flow-cytometry

Flow-cytometry was employed for identification of cells bearing certain surface markers. Generally, spleen or lymph node cells were suspended at 10^7/mL in RPMI culture media. To this suspension an appropriate volume (as determined by prior experimentation) of fluor-conjugated antibody was added. The samples were incubated for 1h on ice and washed twice with ice-cold media. Cells were spun for two minutes at 850g between washes. For analysis cells are suspended to about 10^6 mL and analyzed immediately on an EPIC XL cytometer (Beckman-Coulter, Mississauga, Ontario).

2.9 Cell Isolations

2.9.1 MACS

Magnetic labeling and sorting were done according to manufacturer's recommendations except the media (PBS+ 2mM EDTA) contained no FBS. CD4 T cells were negatively selected with a T cell selection kit and spleen cells were depleted of T cells with CD90 labeled magnetic beads (Miltenyi Biotech, Auburn, CA USA). B cells were negatively selected with a B cell negative selection kit. DCs were isolated by positive selection with pan-DC microbeads. All samples were passed over magnetically charged LS columns (Miltenyi Biotech, Auburn, CA USA).

2.9.2 FACS

Fluorescence activated cell sorting was employed to partially purify CFSE-labeled cells from suspensions of lymph node cells. This was achieved with the assistance of Mark Boyd at the Cancer Research Unit of the Saskatchewan Cancer Agency with a Coulter EPICS ELITE ESP cell sorter running Expo32 acquisition software.

2.10 Determination of mRNA expression

2.10.1 RNA isolation

Following isolation by MACS negative selection, B cell mRNA was isolated by passage over RNA affinity columns. B cells were first disrupted using the QIAshredder spin columns (QIAGEN Canada, Toronto, ON) as per the manufacturer's instructions. The mRNA was isolated from the remaining solution with the RNAeasy kit (QIAGEN) as per the manufacturers instructions. The resulting purified mRNA was quantified by assessing the final concentration by Nanovue spectrophotometer (GE Healthcare).

2.10.2 cDNA preparation

Copy DNA was prepared with the iScript kit (BioRad, Mississauga, ON) as per the manufacturers instructions.

2.10.3 Q-RT-PCR

Quantitative real-time polymerase chain reaction was employed to assess the levels of mRNA transcripts from our cultured B cells. This was accomplished employing the cDNA prepared as above as a template. 30nM primers (Sequences: OX40L forward 5′-GGATGCTTCTGTGCTTCATCT; reverse 5′-GTTCTGCACCTCCATAGTTTGA; β-actin forward 5′-CCAGCCTTCCTTCCTGGGTA-3′; reverse 5′-CTAGAAGCA-TTTGCGGTGCA-3) were added to appropriate wells as well as the SsoFast EvaGreen supermix (BioRad). Reactions conditions were as per the manufacturer's instruction. Themocycling, data acquisition, and data analysis were accomplished using the CFX96 QRTPCR system (BioRad) and associated software in the laboratory of Dr. Joyce Wilson, department of Microbiology and Immunology, University of Saskatchewan. Confirmation that the sequences amplified were specific was accomplished by melt-curve analysis and by visualization of the PCR products by ethidium bromide agarose gel electrophoresis.

3.0 CHAPTER 3 - OBSERVATIONS AND MECHANISMS OF CD4 T CELL COOPERATION IN VIVO: I ANALYSIS EMPLOYING IMMUNOLOGICALLY INTACT MICE

3.1 Introduction

Put simply, cooperation between CD4 T cell populations is thought to increase CD4 T cell function. When multiple CD4 T cells, with specificity for different peptides in association with host MHCII molecules, interact with antigen presenting cells at the same time, the overall level of activation of the T cells is increased. Measuring relative numbers of cytokine producing cells, particularly those that produce IFNγ and IL-4, is a surrogate for measuring CD4 T cell function, since activated CD4 T cells mediate their effector function primarily by the secretion of cytokines and by providing stimulatory ligands through cell-cell contact, The cytokine ELISpot assay is therefore a most appropriate tool for measuring relative levels of CD4 T cell activation; it allows for ready enumeration of antigen-specific cytokine producing cells in a given population. By employing peptides as recall antigen in the ELISpot assay, we are able to detect T cells that recognize the most basic immunogenic unit of an antigen that can be recognized by a T cell and cytokine secreting cells that lead to the formation of ELISpots upon development, ELISpot forming cells, are enumerated. MHCII expressing CD4 T cells can be selectively stimulated by using synthetic peptide antigens of a length greater than about 10-12 amino acids since, for steric reasons, these peptides bind the groove of MHCII but do not bind well to MHCI molecules.

Given these considerations we sought to develop experimental protocols that would allow observation of cooperative interactions between subpopulations of peptide-specific CD4 T cells. Subsequent to observing CD4 T cell cooperation, we aimed to

explore the underlying mechanisms involved. I outline experiments undertaken with these aims in mind in this chapter.

3.2 Observations of CD4 T Cell Cooperation During Secondary Immune Responses

We wished to develop an experimental system, in intact mice, wherein the effects of CD4 T cell cooperation could be directly observed. This required establishment of an immunization protocol that resulted in sub-optimal generation of CD4 effector cells which, under appropriate conditions, could be enhanced. The administration of soluble synthetic peptides, known to bind MHCII molecules, in IFA, was found to result in such sub-optimal activation of CD4 T cells; the same peptides given in CFA induce much more robust effector cell generation (not shown).

CFA contains the remains of heat-killed *Mycobacterium tuberculosis* whereas IFA consists simply of mineral oil and an emuslifier without dead bacteria. Given the plethora of different substances associated with CFA, many of which have known immunostimulatory properties (Freund and Bonanto 1946; Freund, Stern et al. 1947; Pasare and Medzhitov 2004; O'Neill 2006; Manicassamy and Pulendran 2009), it is difficult to dissect their various roles in the enhancement of immunity when this adjuvant is employed directly in intact mice. However, we believe that at least some of the effect of CFA could be due to cooperative interactions between CD4 T cells that are specific for mycobacterial antigens and those CD4 T cells that recognized the incorporated peptide.

In order to test this possibility directly we developed a simple experimental system in which we assessed the involvement of cooperation on the potency of secondary immune responses. Secondary immune responses are almost always of a greater magnitude and generate increased numbers of effector CD4 T cells than primary responses. We hypothesized that enrichment of CD4 T cells specific for mycobacterial peptides by prior exposure to CFA would allow for greater cooperative interaction between these CD4 T cells and $HEL_{105\text{-}120}$-specific CD4 T cells when CFA containing $HEL_{105\text{-}120}$ was given subsequently. As multiple immunizations with CFA are discouraged for ethical reasons, we measured secondary immune repsonses naive mice in which we had adoptively transferred nylon wool purified (Julius, Simpson et al. 1973) T cells from

normal or CFA primed mice. The recipients were then challenged with CFA containing 10μg of $HEL_{105-120}$. The results of an experiment of this type are shown in Figure 3.2.1. There is a clear increase in the number of $HEL_{105-120}$-specific cytokine producing cells generated in mice that received CFA primed T cells compared to those that received normal T cells. Correspondingly, we observed greater numbers of mycobacteria-specific cytokine secreting cells in mice that received T cells from CFA-primed mice. This clear effect, seen upon transferring T cells, made it plausible that antigen-specific T cells are responsible for, by some unknown mechanism, enhancing the activation of CD4 T cells specific for $HEL_{105+120}$. We interpreted this experimental result to mean that cooperative interactions may occur between CD4 T cells specific for two antigens incorporated into the same adjuvant emulsion. However, because CFA is a general immune stimulator and its components are complex and uncharacterized we could not omit the possibility that the effects were not directly due to cooperation between CD4 T cell populations.

Thus, we designed a similar system in which CFA was not used for the second immunization. In these experiments the second challenge contained HEL_{11-25} and $HEL_{105-120}$ together in IFA to test whether two peptide-specific populations of CD4 T cells, activated by administering two peptides together in IFA, could cooperate to enhance the activation of CD4 T cells specific for at least one of two peptides. We undertook the experiment, the results of which are shown in Figure 3.2.2. Mice were were primed with CFA alone or with CFA with an incorporated MHCII-binding peptide, HEL_{11-25}, and then injected with HEL_{11-25} and $HEL_{105-120}$ together in IFA Mice that were primed with CFA containing HEL_{11-25}, in comparison to those primed with CFA alone, supported the generation of significantly more $HEL_{105-120}$-specific CD4 T cells upon injection with HEL_{11-25} and $HEL_{105-120}$ together in IFA. These observations support our hypothesis that CD4 T cells can cooperate *in-vivo* to enhance their own effector function

The findings made in these systems are complicated by their dependence on previous priming by CFA. It was likely that CFA mediated effects that were residual and not directly attributable to the activation of CD4 T cells. We therefore sought to eliminate this complication in our further experiments.

Figure 3.2.1 *T cells from CFA-primed mice, upon adoptive transfer to normal mice, enhance the generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells following challenge with $HEL_{105-120}$ in CFA*. Normal BALB/c mice were immunized with CFA and saline or were left unimmunized. Fourteen days later, splenocytes were isolated from both groups of mice and passed over nylon wool. One million of the non-adherent T cells were then adoptively transferred into naive BALB/c mice. On the day of transfer, all mice were challenged, subcutaneously at the tail base, with CFA containing 10μg of $HEL_{105-120}$. The CFA preparation contains heat-killed *mycobacterium tuberculosis*. Ten days following challenge, the $HEL_{105-120}$ and the related *mycobacterium*, BCG-, specific cytokine producing cells in the spleen were enumerated by ELISpot assay. Each symbol represents the number of antigen-specific ELISpot-forming cells found in the spleen of an individual mouse. This is the result of a single experiment with three mice per group (n = 3).

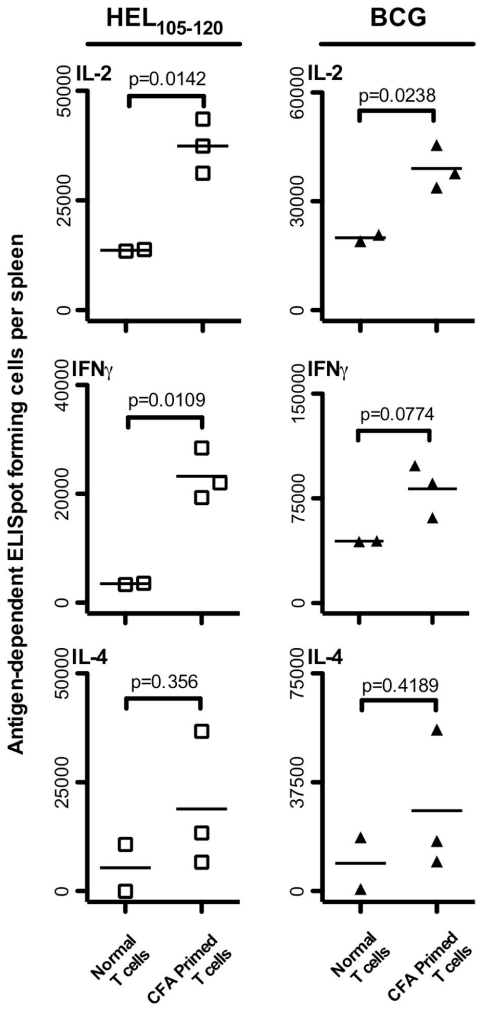
HEL105-120
BCG
IL-2
p=0.0142
IL-2
p=0.0238
IFNγ
p=0.0109
IFNγ
p=0.0774
IL-4
p=0.356
IL-4
p=0.4189
Antigen-dependent ELISpot forming cells per spleen
Normal T cells
CFA Primed T cells
Normal T cells
CFA Primed T cells

Figure 3.2.1

Figure 3.2.2 *Mice previously immunized with HEL_{11-25} in CFA support enhanced generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells following challenge with $HEL_{105-120}$ and HEL_{11-25} in IFA*. Normal BALB/c mice were immunized with CFA alone (CFA primed) or with CFA containing 10μg HEL_{11-25} (CFA/HEL_{11-25} primed). Fourteen days later, all mice were challenged with IFA containing 10μg each of HEL_{11-25} and $HEL_{105-120}$. Ten days following challenge, the $HEL_{105-120}$ and HEL_{11-25}-specific cytokine producing cells in the spleen were enumerated by ELISpot assay. Each symbol represents the number of antigen-specific ELISpot-forming cells found in the spleen of an individual mouse. This is the result of a single experiment with three mice per group (n = 3).

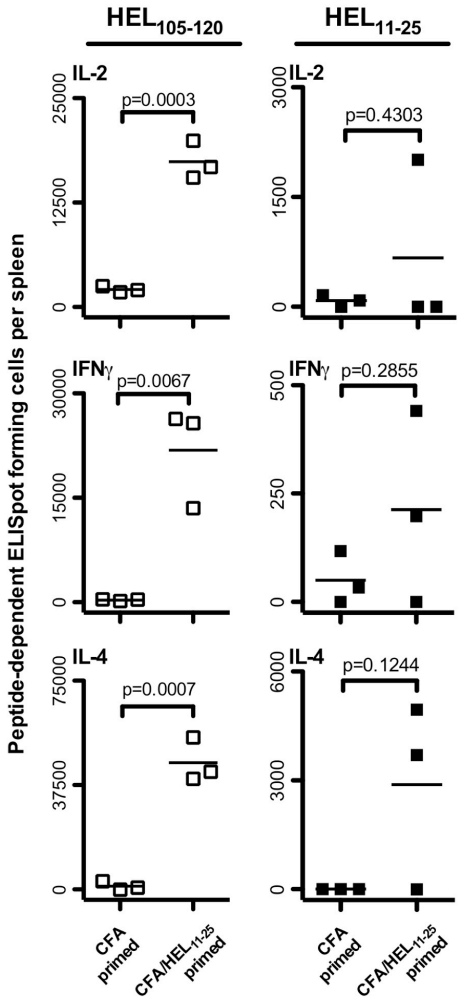
HEL105-120
HEL11-25
IL-2
IL-2
p=0.0003
p=0.4303
IFNγ
IFNγ
p=0.0067
p=0.2855
IL-4
IL-4
p=0.0007
p=0.1244
Peptide-dependent ELISpot forming cells per spleen
25000
12500
0
3000
1500
0
30000
15000
0
500
250
0
75000
37500
0
6000
3000
0
CFA primed
CFA/HEL11-25 primed
CFA primed
CFA/HEL11-25 primed

Figure 3.2.2

3.3 Direct Observation of Cooperation between CD4 T cells During Primary Immune Responses

Upon administration of protein antigens, they are endocytosed by APC, processed by proteolytic digestion into peptides, and loaded onto MHC molecules for presentation to T cells. The repertoire of peptides that are presented depends on the amino acid sequence of the protein digested (Weaver, Lazarski et al. 2008), the peptides generated by lysosomal proteases (Honey and Rudensky 2003), and the affinity of these peptides for host MHC molecules (Buus, Sette et al. 1987). Further on in the generation of T cell immunity towards this antigen, the repertoire of CD4 T cells that are generated will depend on not only which peptides are available, but also on whether T cells, bearing TCRs with affinity for these peptides in the context of MHCII, are present. As the repertoire of naive TCRs, due to positive and negative selection in the thymus, depends mostly on a combination of host MHC and self-peptides, genetically identical mice likely have similar precursor T cell repertoires (Moon, Chu et al. 2007). Similarly, the peptides generated and presented in genetically identical mice, immunized with the same protein, should be the similar. Therefore, one would predict that the repertoire of CD4 T cells, generated upon immunization with a given protein antigen, would be similar between individuals of the same mouse strain. This is, in fact, found to be true (Peters, Hamilton et al. 2005).

Because we were very familiar with the peptides to which an immune response is generated in BALB/c mice immunized with hen egg lysozyme (HEL), we saw these as ideal peptides to employ in assessing cooperation between CD4 T cell populations. Moreover, we had previously published evidence that cooperation between HEL-peptide-specific CD4 T cell populations facilitated the generation of optimal numbers of effector CD4 T cells towards this antigen (Peters, Kroeger et al. 2009). BALB/c mice immunized with HEL generate cytokine producing CD4 T cells with specificity for the peptides HEL_{11-25} and $HEL_{105-120}$ consistently, and effector CD4 T cells specific for HEL_{48-63} and HEL_{74-96} are often detected, but vary between mice (Figure 3.3.1).

Figure 3.3.1 *The repertoire of CD4 T cells generated upon immunization with HEL protein is predictable and is made up of four main peptide-specific populations*. Three normal BALB/c mice were immunized, intraperitoneally, with 100μg of heat-aggregated HEL on ALUM. Ten days following this immunization, mice were sacrificed and their splenocytes were harvested, and either left untreated or were depleted of CD4 T cells by MACS. These splenocyte populations were then plated into ELISpot wells to enumerate the HEL-specific cytokine producing T cells therein. Individual HEL-peptide-dependent ELISpot-forming cells in the spleen of a given mouse are shown in stacked columns. Total numbers of HEL-specific ELISpots, produced when HEL protein was added to the ELISpot well are shown beside for comparison. This is the result of a single experiment.

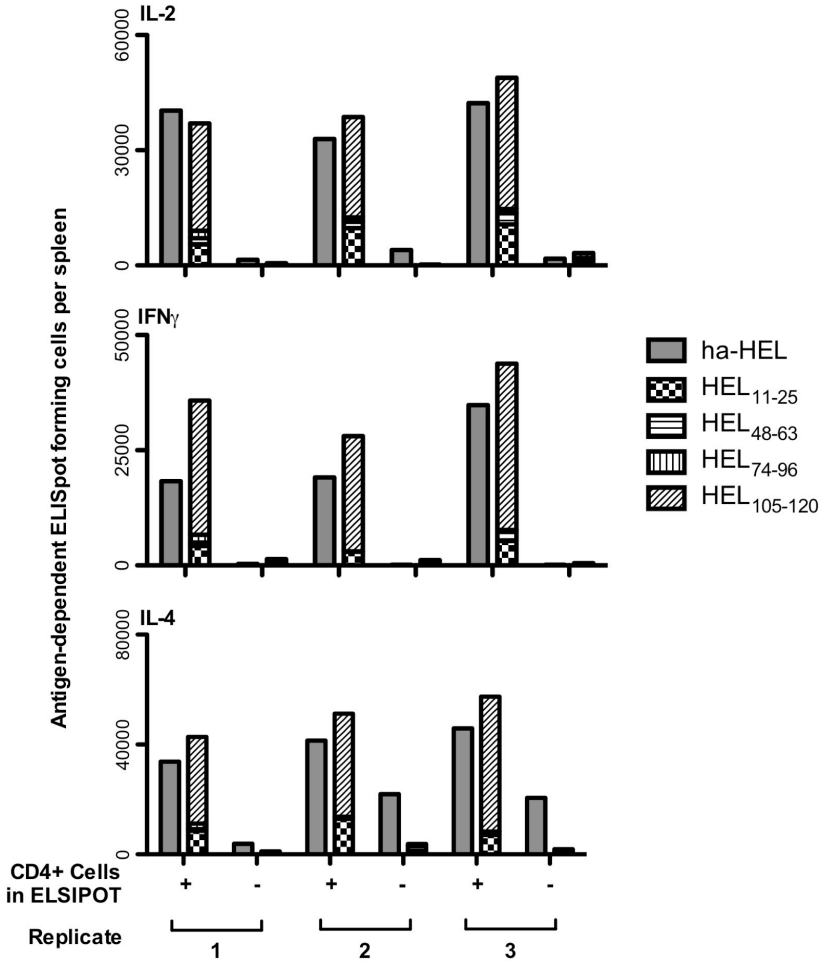
IL-2
60000
30000
0
IFNγ
50000
25000
0
IL-4
80000
40000
0
Antigen-dependent ELISpot forming cells per spleen
ha-HEL
HEL11-25
HEL48-63
HEL74-96
HEL105-120
CD4+ Cells in ELSIPOT
+ - + - + -
Replicate
1 2 3

Figure 3.3.1

We hypothesized that if cooperation between HEL derived peptide-specific CD4 T cells occurs when the protein antigen is injected into BALB/c mice, then cooperation may also occur when the HEL peptides are given together in IFA. We had already observed an increased generation of CD4 T cells specific for a target peptide when this target peptide and another peptide, against which the mouse had been primed were given together in IFA (Figure 3.2.2). Because $HEL_{105-120}$-specific cytokine producing CD4 T cells are generated sub-optimally by the injection of this peptide alone in IFA, we saw these cells as ideal targets for assessment of cooperation mediated enhancement of the generation of effector function.

We tested the possibility that administration of the four MHCII-restricted HEL peptides, normally responded to by BALB/c mice, could elicit cooperation between CD4 T cells specific for these peptides. Mice were given either $HEL_{105-120}$ alone, or $HEL_{105-120}$ plus equal amounts of each of the other three HEL peptides together in IFA. As can be seen in Figure 3.3.2, mice immunized with a combination of the four HEL peptides generated significantly more $HEL_{105-120}$-specific effector CD4 T cells than did their counterparts immunized with $HEL_{105-120}$ alone. We also observed that activation of HEL_{11-25}-specific CD4 T cells occurred when the four HEL peptides were given (Figure 3.3.3). This observation is the first, to our knowledge, to show cooperation between endogenous populations of CD4 T cells in response to primary immunization. However, we were unable to detect cytokine producing CD4 T cells specific for HEL_{48-63} or HEL_{74-96} suggesting that cooperation does not occur for every linked peptide in the mixture.

Initially, we sought to confirm that the cooperative effect in the generation of $HEL_{105-120}$-specific effector CD4 T cells, that we observed upon administration of four HEL peptides, compared to when $HEL_{105-120}$ was given alone in IFA, was not merely a transient difference between immune responses developing differently over time. Thus, we enumerated the $HEL_{105-120}$-specific cytokine producing CD4 T cells in the spleen of mice treated with these two different immunizations on different days post-immunization. As can be seen in Figure 3.3.4, the generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells when four peptides are given, roughly parallels what is observed when protein antigens are administered; the peak of this response occurs between 7 and 10 days post-immunization and wanes by fourteen days.

Figure 3.3.2 *Other HEL peptides co-administered with $HEL_{105-120}$ increase the generation of $HEL_{105-120}$-specific effector CD4 T cells*. BALB/c mice were immunized with either 10μg of $HEL_{105-120}$ alone or 10μg of each of HEL_{11-25}, HEL_{48-63}, HEL_{74-96}, and $HEL_{105-120}$ ($HEL_{105-120}$ + 3 HEL peptides), subcutaneously in IFA. $HEL_{105-120}$-specific cytokine producing CD4 T cells in the spleen were enumerated ten days post-immunization. Individual $HEL_{105-120}$-dependent ELISpot-forming cells in the spleen are shown. Each symbol represents the number of antigen-specific ELISpot-forming cells found in the spleen of an individual mouse. Three independent experiments, and pooled results from six independent experiments, with three mice per group (n = 18) are shown. P-values indicate the probability that the means of the indicated samples are not significantly different as assessed by unpaired, two-tailed, T tests.

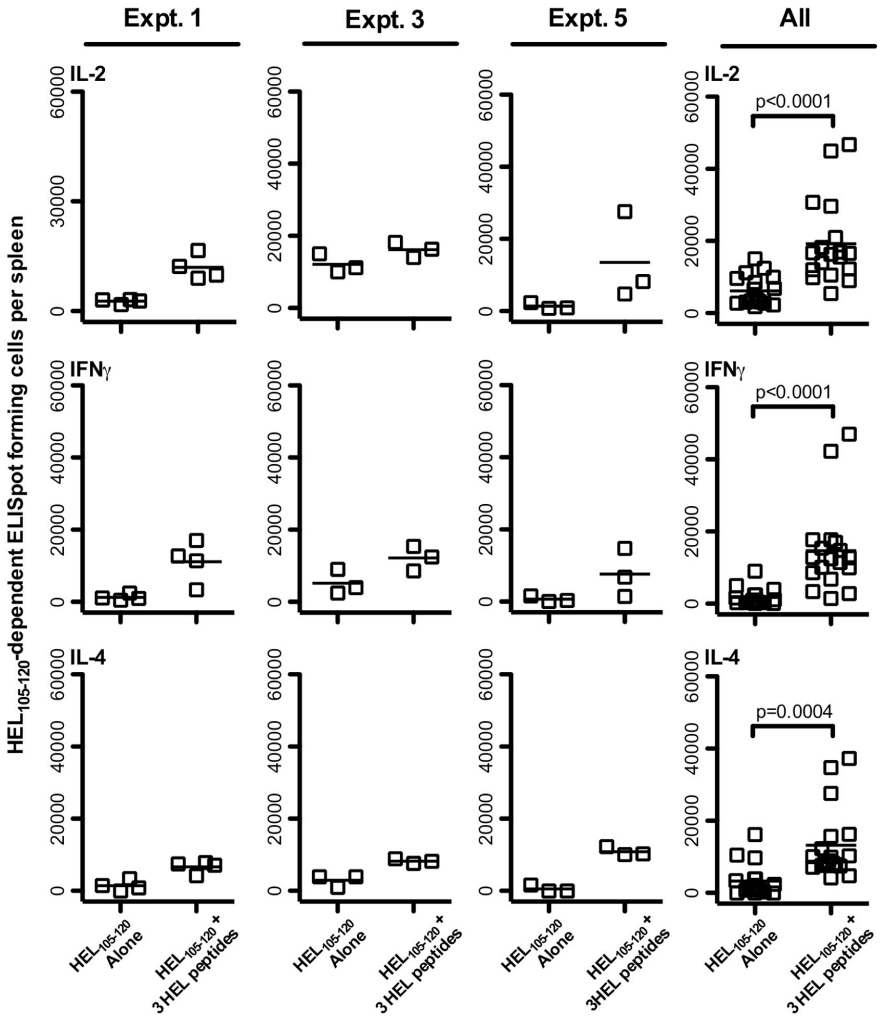
Expt. 1
Expt. 3
Expt. 5
All
IL-2
IFNγ
IL-4
p<0.0001
p<0.0001
p=0.0004
HEL105-120-dependent ELISpot forming cells per spleen
0
20000
40000
60000
30000
HEL105-120 Alone
HEL105-120 + 3 HEL peptides

Figure 3.3.2

Figure 3.3.3 *Minor HEL peptide-specific CD4 effector T cells are generated upon immunization with four HEL peptides in IFA*. Mice were immunized with four HEL peptides together in IFA as in Figure 4. HEL_{11-25}, HEL_{48-63}, and HEL_{74-96}-specific cytokine producing CD4 T cells in the spleen were enumerated ten days post-immunization. Each symbol represents the number of antigen-specific ELISpot-forming cells found in the spleen of an individual mouse. Pooled results from two to three independent experiments with three mice per group are shown.

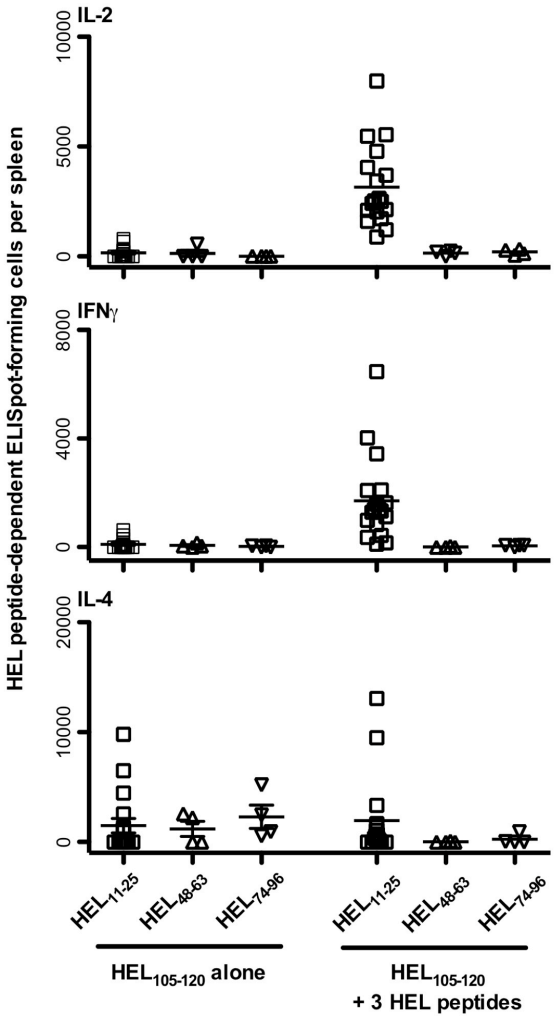
IL-2
IFNγ
IL-4
HEL peptide-dependent ELISpot-forming cells per spleen
0
5000
10000
4000
8000
20000
HEL11-25
HEL48-63
HEL74-96
HEL105-120 alone
HEL105-120
+ 3 HEL peptides

Figure 3.3.3

In contrast, mice immunized with $HEL_{105-120}$ alone in IFA displayed minimal generation of $HEL_{105-120}$-specific cytokine producing cells at all time points investigated. Thus we conclude that cooperation between CD4 T cells responding to multiple peptides, administered together in IFA, results in genuine increases in levels of effector CD4 T cells and not simply increased kinetics of the response to $HEL_{105-120}$.

We subsequently began to inquire as to whether our observations could be generalized to other peptide combinations. We reasoned that if we could employ combinations of peptides known to activate CD4 T cells this would facilitate further analysis of the underlying mechanisms of cooperation between peptides. Much work has identified peptides that are consistently responded to, and generate large numbers of CD4 effector T cells. These so-called "immunodominant" peptides have long off-constants when bound to the groove of MHCII molecules (Lazarski, Chaves et al. 2005; Weaver, Lazarski et al. 2008) and thus serve to stimulate CD4 T cells particularly well over time. Two such peptides that bind well to $I\text{-}A^d$ are chicken $ovalbumin_{323-339}$ ($OVA_{323-339}$) and *Leishmania* analog of activated $C\text{-}kinase_{158-173}$ ($LACK_{158-173}$). We hypothesized that administration of these peptides with $HEL_{105-120}$ in IFA would result in activation of CD4 T cells specific for them and that these activated T cells could affect the generation of $HEL_{105-120}$ specific CD4 T cells. We administered each of these peptides alone, or all three together, in IFA, and assessed the generation of CD4 cytokine-producing T cells ten days post-immunization. The results of this experiment were somewhat surprising in that it appeared that both $OVA_{323-339}$ and $LACK_{158-173}$ specific cytokine producing CD4 T cells were generated fairly efficiently when the peptides were given alone (Figure 3.3.5). Furthermore, it appeared that $LACK_{158-173}$ might have competed with $OVA_{323-339}$ for binding to $I\text{-}A^d$ as the numbers of $OVA_{323-339}$-specific cytokine producing cells were significantly lower in mice given all three peptides together compared with mice given $OVA_{323-339}$ alone. Competition was not observed between $HEL_{105-120}$ and the other peptides; it is known that $HEL_{105-120}$ binds $I\text{-}E^d$ (Weaver, Lazarski et al. 2008). Although there was a trend towards increased generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells in mice given these three peptides together, the competition between $OVA_{323-339}$ and $LACK_{158-173}$ complicated these experiments and thus made them less than ideal for further analysis.

Figure 3.3.4 *Kinetics of the $HEL_{105-120}$-specific Effector CD4 T cell population following immunization with $HEL_{105-120}$ alone or $HEL_{105-120}$ together with 3 HEL peptides*. BALB/c mice were immunized with either 10μg of $HEL_{105-120}$ alone or 10μg of each of HEL_{11-25}, HEL_{48-63}, HEL_{74-96}, and $HEL_{105-120}$ ($HEL_{105-120}$ + 3 HEL peptides), subcutaneously in IFA. $HEL_{105-120}$-specific cytokine producing CD4 T cells in the spleen were enumerated before injection (day 0) and seven, ten, and fourteen days post-immunization. Pooled responses from three individual mice are displayed +/- SEM. This is the result of a single representative experiment of three with three mice per group per time point.

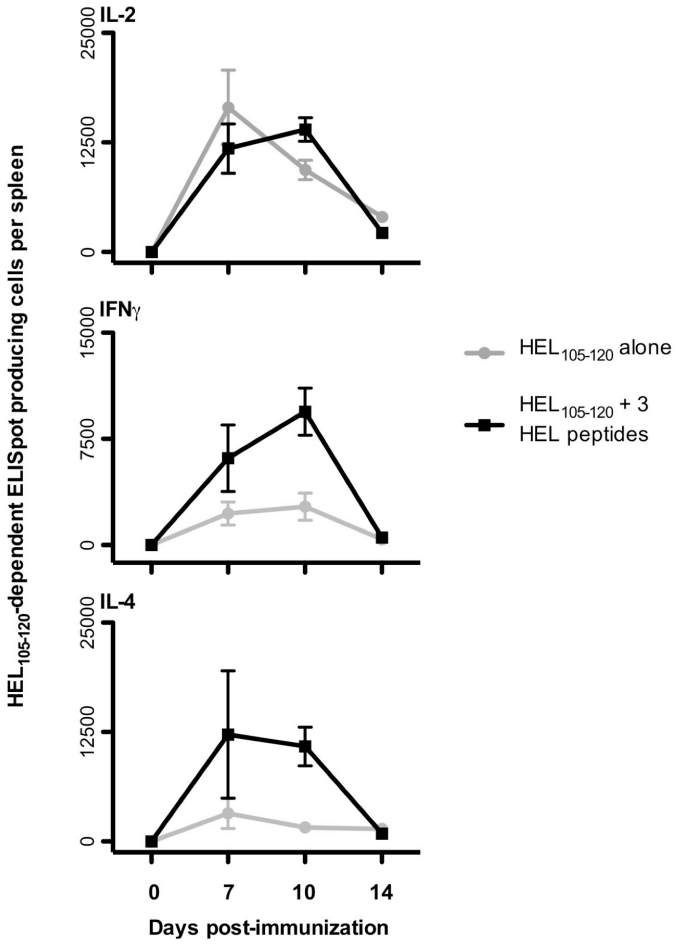
IL-2
IFNγ
IL-4
25000
12500
0
15000
7500
0
0
7
10
14
Days post-immunization
HEL105-120-dependent ELISpot producing cells per spleen
HEL105-120 alone
HEL105-120 + 3 HEL peptides

Figure 3.3.4

Figure 3.3.5 *Inferred Competition between I-A^d-binding peptides, when co-administered in IFA in the generation of cytokine-producing CD4 T cells specific for these peptides*. BALB/c mice were immunized with either $HEL_{105-120}$ alone, $OVA_{323-339}$ alone, $LACK_{158-173}$ alone, or all three peptides together, subcutaneously in IFA. Peptide-specific ELISpot forming cells in the spleen were enumerated on day ten post-immunization. Each symbol represents the number of ELISpot forming cell detected in the spleen of an individual mouse. Pooled results from two independent experiments with three mice per group are shown (n = 3-6). P-values indicate the probability that the means of the indicated samples are not significantly different as assessed by unpaired, two-tailed, T tests.

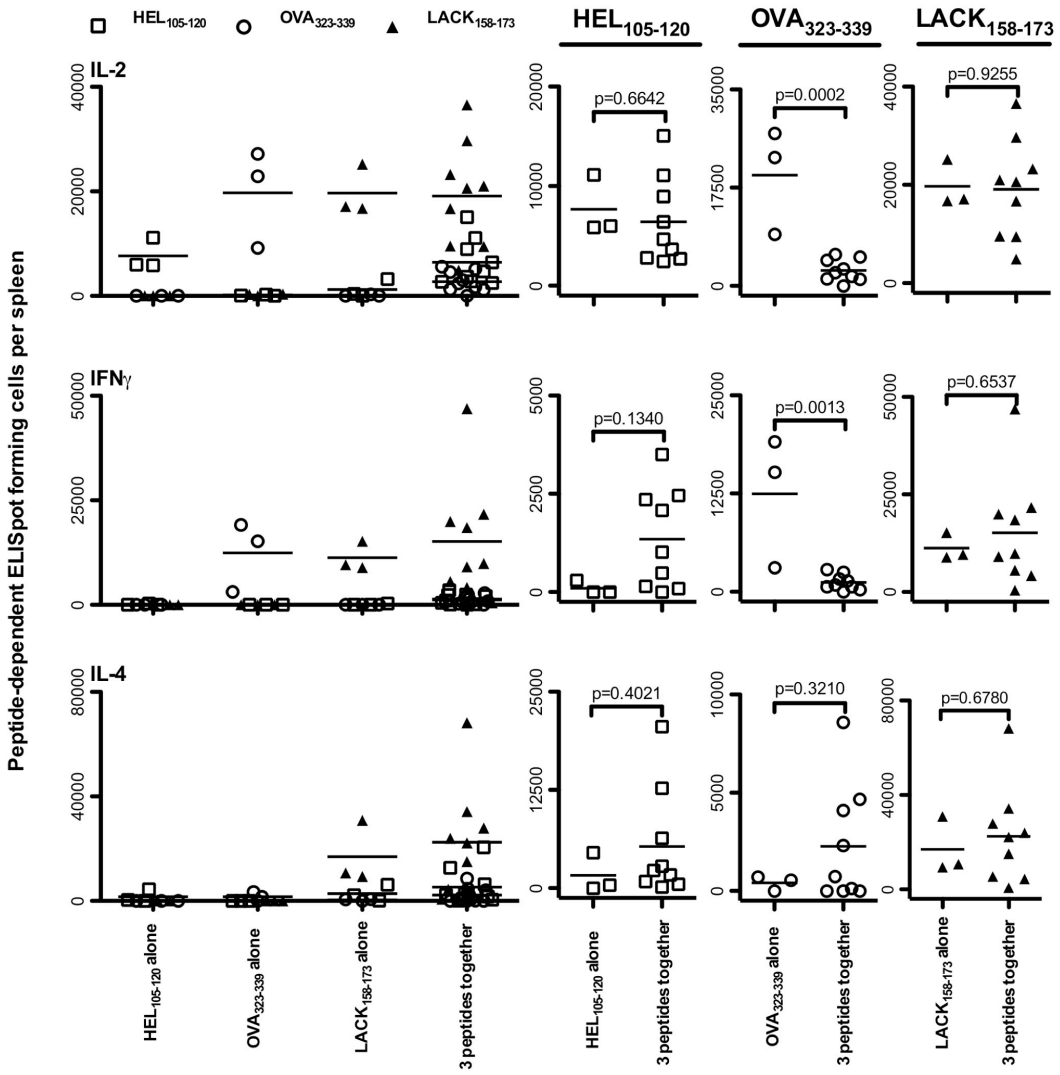
HEL105-120
OVA323-339
LACK158-173
HEL105-120
OVA323-339
LACK158-173
IL-2
IFNγ
IL-4
p=0.6642
p=0.0002
p=0.9255
p=0.1340
p=0.0013
p=0.6537
p=0.4021
p=0.3210
p=0.6780
Peptide-dependent ELISpot forming cells per spleen
HEL105-120 alone
OVA323-339 alone
LACK158-173 alone
3 peptides together
HEL105-120 alone
3 peptides together
OVA323-339 alone
3 peptides together
LACK158-173 alone
3 peptides together

Figure 3.3.5

However, this experiment caused me to become acutely aware of the possibility of competition between peptides for MHCII binding, and thus I sought to avoid it in my further experiments.

Drawing on our findings from the previous experiment, I reasoned that the administration of only two MHCII-binding peptides in IFA would result in the simplest situation where endogenous populations of CD4 T cells could facilitate the activation of one another when bound to the same APC. If the two peptides were chosen to bind separate MHC molecules, competition could be avoided. We therefore undertook an experiment to determine whether the I-E^d-binding peptide $HEL_{105-120}$ and the I-A^d-binding peptide $OVA_{323-339}$, when co-administered in IFA, could elicit cooperative interactions between endogenous CD4 T cells. Substantial and significant increases in the generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells were observed when $HEL_{105-120}$ was given with $OVA_{323-339}$, together in IFA to BALB/c mice, compared to when $HEL_{105-120}$ was given alone (Figure 3.3.6). As previously observed, mice immunized with $OVA_{323-339}$ alone in IFA generate many peptide-specific cytokine producing CD4 T cells. We did not observe an increase in $OVA_{323-339}$ specific effector CD4 T cell generation upon co-administration of $HEL_{105-120}$ and $OVA_{323-339}$.

In two separate immunization protocols, cooperative effects between co-administered peptides have been observed. These cooperative effects were manifested through increases in the number of $HEL_{105-120}$-specific cytokine producing CD4 T cells in the spleen of mice immunized with multiple peptides compared to the numbers in the spleen of mice given $HEL_{105-120}$ alone. We wished to confirm that similar effects could be observed in the population of CD4 T cells that inhabit lymph nodes draining the site of IFA injection. We immunized mice with $HEL_{105-120}$ alone, with $HEL_{105-120}$ and $OVA_{323-339}$, with $HEL_{105-120}$ with three HEL peptides, or with the HEL protein itself, subcutaneously in IFA in the footpad. Seven or ten days post-immunization $HEL_{105-120}$-specific cytokine producing cells in the popliteal lymph node were enumerated by ELISpot assay. Figure 3.3.7 displays the results from this experiment. We observed similar patterns of enhancement in the generation of $HEL_{105-120}$-specific effector CD4 T cells as were observed in the spleens of mice immunized similarly.

Figure 3.3.6 *$OVA_{323-339}$ co-administered with $HEL_{105-120}$ increases the generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells*. BALB/c mice were immunized with either 10μg of $HEL_{105-120}$ alone (H), 10μg of $OVA_{323-339}$ alone (O), or 10μg of both peptides together (H+O), subcutaneously in IFA. Peptide-specific cytokine producing CD4 T cells in the spleen were enumerated ten days post-immunization. Each data point represents the total number of ELISpot forming cells detected in the spleen of a single mouse. These are pooled results from two three independent experiments with two to five mice per group (n = 5-12). P-values indicate the probability that the means of the indicated samples are not significantly different as assessed by unpaired, two-tailed, T tests.

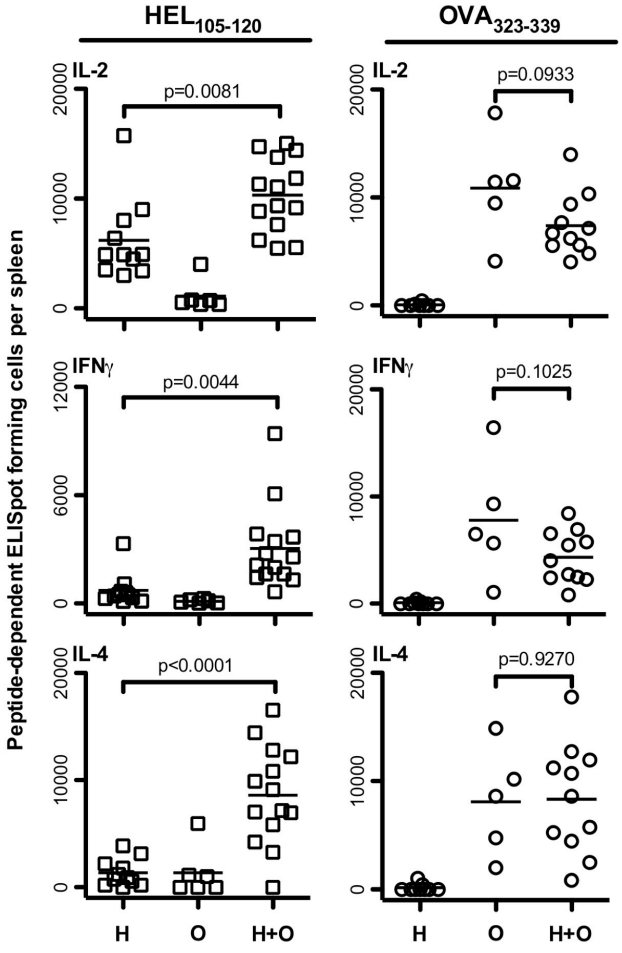
HEL105-120
OVA323-339
IL-2
p=0.0081
IL-2
p=0.0933
IFNγ
p=0.0044
IFNγ
p=0.1025
IL-4
p<0.0001
IL-4
p=0.9270
Peptide-dependent ELISpot forming cells per spleen
0
10000
20000
6000
12000
H
O
H+O

Figure 3.3.6

Figure 3.3.7 *The generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells in the draining popliteal lymph node upon immunization with various IFA preparations subcutaneously in the footpad*. BALB/c mice were immunized with either 10μg $HEL_{105-120}$ alone, 10μg each of $HEL_{105-120}$ and $OVA_{323-339}$ (H+O), 10 μg each of HEL_{11-25}, HEL_{48-63}, HEL_{74-96}, and $HEL_{105-120}$, or with HEL protein (molar equivalent to $HEL_{105-120}$), subcutaneously in IFA in the left hind footpad. On days seven and ten post-immunization, the ELISpot forming $HEL_{105-120}$-specific CD4 T cell in the popliteal lymph node were enumerated. Each data point represents the total number of ELISpot forming cells detected in the draining lymph node of a single mouse. This is the result of a single experiment with two to three mice per group (n = 2-3).

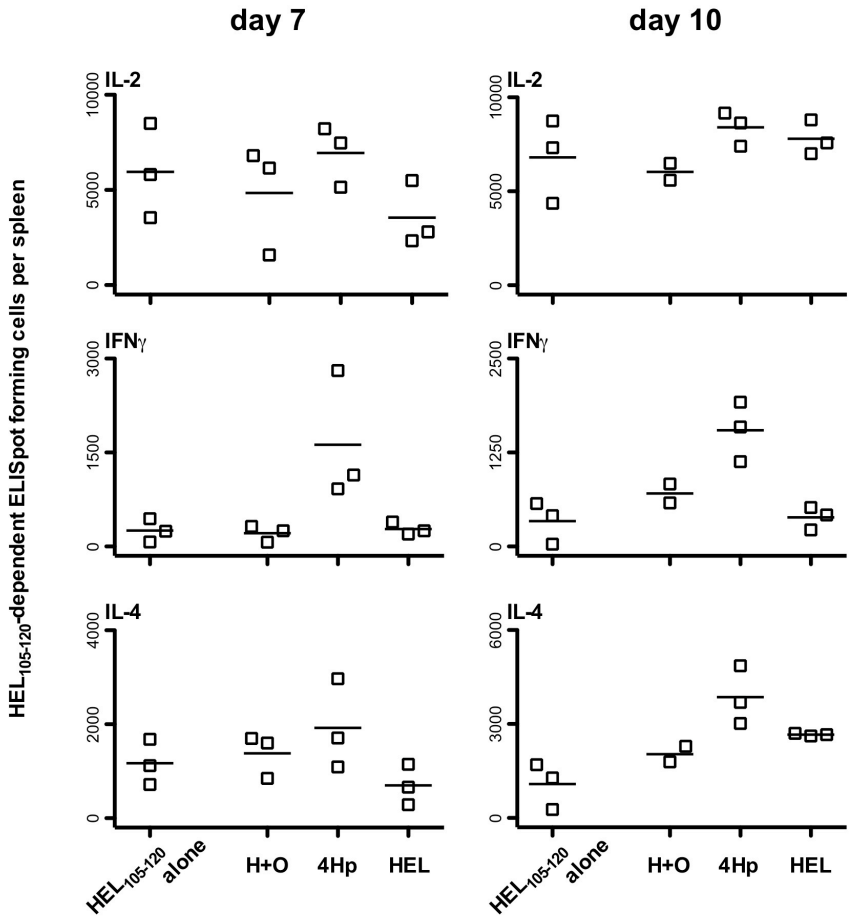
day 7
day 10
IL-2
IFNγ
IL-4
HEL105-120-dependent ELISpot forming cells per spleen
HEL105-120 alone
H+O
4Hp
HEL

Figure 3.3.7

We conclude that our observations made in the spleen of immunized mice probably represent well the responses generated in the draining lymph nodes following immunization with peptides in IFA. Due to ethical considerations, concerning the administration of IFA into the footpad, we avoided experimenting further with this type of protocol.

Three major possibilities exist that may explain the fact that $OVA_{323-339}$-specific CD4 effector T cell generation is not influenced by simultaneous administration of $HEL_{105-120}$ while $HEL_{105-120}$-specific effector generation is affected by the administration of $OVA_{323-339}$. Our preparation of $OVA_{323-339}$ may have been contaminated in such a way that it contained immunostimulatory molecules that were able to supersede the effects of cooperation between CD4 T cells. This possibility could also explain why co-administration of $OVA_{323-339}$ with $HEL_{105-120}$ increases $HEL_{105-120}$ effector generation. This possibility has been addressed in experiments described below, making this possibility somewhat unlikely. A second possibility is that the number of $OVA_{323-339}$-specific precursor CD4 T cells in BALB/c mice is greater than those specific for $HEL_{105-120}$ or these CD4 T cells are partially activated in our mice. This possibility would result in sufficient cooperation between $OVA_{323-339}$-specific CD4 T cells to generate optimal numbers of effector cells. The third possibility, which we consider to be unlikely, is that $HEL_{105-120}$ specific CD4 T cells are uniquely dependent on additional stimuli that $OVA_{323-339}$- and $LACK_{158-173}$-, and perhaps other peptide-specific CD4 T cells, are not.

3.4 Attempts to Mimic Cooperative Effects by Co-administration of Immunostimulatory Substances

We had demonstrated that the incorporation of another MHCII-binding peptides into IFA when immunizing with $HEL_{105-120}$ enhanced the activation of CD4 T cells specific for this peptide. Our hypothesis that the CD4 T cells specific for the additional peptides are activated by, and cooperatively enhance, the generation of $HEL_{105-120}$ specific CD4 T cells by co-presentation on APC was supported by these observations. However, despite our monitoring peptide preparations for sterility, it was possible that the effects observed were due a contamination of these peptides by bacteria or other stimulatory products, thus leading to enhanced activation of APC by their stimulation of TLR or other pattern recognition receptors. Finally, because we hypothesized that activation of APC by way of CD4 T cell-APC contacts may lead to enhanced peptide-specific effector CD4 T cell generation, we reasoned that if the cooperative effects, seen upon administration of multiple peptides in IFA, could be mimicked by the administration of a given immunostimulatory substance, that this would shed light on the mechanism by which CD4 T cells cooperate.

As mentioned above, CFA contains the remains of heat-killed mycobacteria. We knew that peptides or proteins in CFA are generally more immunogenic than when administered in IFA. This effect is due to various potent immunostimulation by microbial products normally borne by pathogens. These substances, often PAMPS, are detected by APC, such as DC and B cells, following their interaction with germline encoded pattern recognition receptors. However, the effect could also be partially due to cooperation of mycobacteria-specific CD4 T cells with antigen-specific CD4 T cells (see Figure 3.2.1).

Figure 3.4.1 The effect of *addition of Toll-like receptor ligands to immunizations of HEL protein*. All BALB/c mice were given 100μg of heat-aggregated HEL in saline intraperitoneally. In some cases the protein preparation was supplemented with either 5μg of *E. coli* lipopolysaccharide (+LPS), 5μg of c-class CpG ODN (+CpG), or 5μg of mycobacterial muramyl dipeptide (+MDP). The HEL-specific cytokine producing CD4 T cells in the spleen were enumerated ten days post-immunization. Each data point represents the total number of ELISpot-forming cells detected in the spleen of a single mouse. This is the result of a single experiment with three mice per group (n = 3). P-values represent the probability that the means of the two samples indicated are not different as assessed by unpaired, two-tailed T tests.

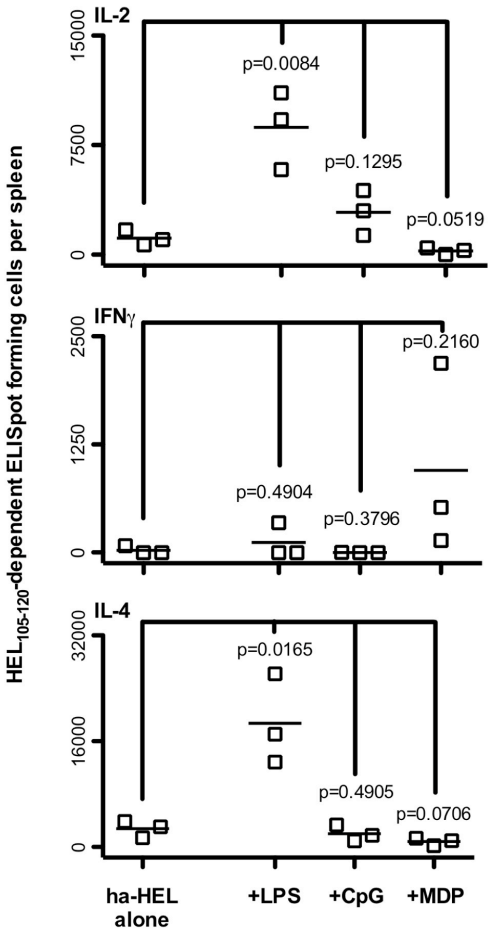
IL-2
15000
7500
0
p=0.0084
p=0.1295
p=0.0519
IFNγ
2500
1250
0
p=0.2160
p=0.4904
p=0.3796
IL-4
32000
16000
0
p=0.0165
p=0.4905
p=0.0706
HEL105-120-dependent ELISpot forming cells per spleen
ha-HEL alone
+LPS
+CpG
+MDP

Figure 3.4.1

Three of the most well-known PAMPS associated with mycobacteria are lipopolysaccharide (LPS) and unmethylated cytosine-guanine DNA sequences (CpG), and muramyl dipeptide (MDP), a component of the cell wall. The immunostimulatory properties of these substances have been well characterized. Activation of APC via TLR 4, 9, and 2/6, respectively, by these substances leads to enhanced presentation of antigen (Pasare and Medzhitov 2004; Moynagh 2005; O'Neill 2006). We sought to determine whether PAMPS could mimic the effect of cooperation seen upon administration of multiple peptides in IFA. We first determined whether LPS, CpG or MDP could enhance minimal responses to a protein antigen. As Figure 3.4.1 shows, 5μg *E. coli* LPS administered with 100μg of heat-aggregated HEL protein, intraperitoneally in saline, significantly enhanced the generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells. We found little evidence that CpG or MDP enhanced the generation of protein-specific immune responses when mice were vaccinated in this way and so the effects of these substances were not further investigated.

Given that LPS substantially enhances protein-specific CD4 effector T cell generation, when heat-aggregated HEL was given in saline, we wished to determine whether a similar level of LPS could enhance $HEL_{105-120}$ and HEL protein-specific cytokine-producing cell generation when administered in IFA. We found that LPS significantly increased the generation of HEL-peptide-specific effector CD4 T cells when it was given with *protein* antigen in IFA, but it did not effect the generation of $HEL_{105-120}$-specific CD4 T cells when this *peptide* was given in IFA (Figure 3.4.2). Based on this result, we concluded that activation of innate immune pathways by LPS was not sufficient to mimic T cell cooperation and thus, bacterial contamination of our peptide stocks was unlikely to have affected the generation of $HEL_{105-120}$-specific cytokine-producing cells in our previous experiments.

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Figure 3.4.2 *Addition of LPS to $HEL_{105-120}$ containing IFA preparations, in contrast to HEL protein containing IFA preparations, does not affect the generation of $HEL_{105-120}$ cytokine producing CD4 T cells, upon immunization*. BALB/c mice were immunized with either 10μg of $HEL_{105-120}$ or 100μg of soluble HEL protein in IFA, subcutaneously at the tail base. IFA preparations were either not further supplemented (saline) or were supplemented with LPS to achieve a final dose of 5μg per mouse (+LPS). The $HEL_{105-120}$-specific and HEL protein-specific cytokine producing CD4 T cells in the spleen were enumerated ten days post-immunization. Each data point represents the total number of ELISpot-forming cells detected in the spleen of a single mouse. These are pooled data from two to three independent experiments with three mice per group (n = 6-9). P-values represent the probability that the means of the two samples indicated are not different as assessed by unpaired, two-tailed T tests.

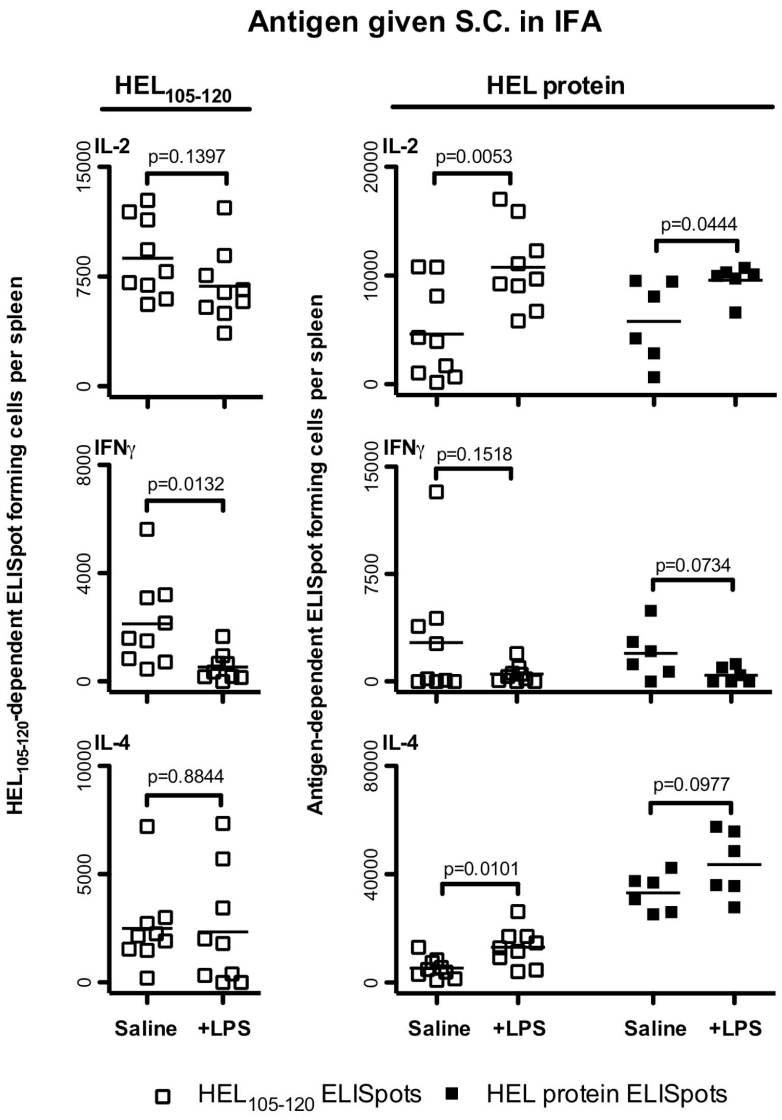

Antigen given S.C. in IFA
HEL105-120
HEL protein
IL-2
IFNγ
IL-4
p=0.1397
p=0.0132
p=0.8844
p=0.0053
p=0.0444
p=0.1518
p=0.0734
p=0.0101
p=0.0977
HEL105-120-dependent ELISpot forming cells per spleen
Antigen-dependent ELISpot forming cells per spleen
Saline
+LPS
HEL105-120 ELISpots
HEL protein ELISpots

Figure 3.4.2

To rule out the possibility that enhanced $HEL_{105-120}$-specific effector CD4 T cell generation, is solely due to the activation of APC we used an agonist antibody to CD40 to artificially activate APC in our assay. The CD40 receptor is a member of the TNF receptor superfamily and is constitutively expressed on DC and B cells. Upon the administration of this antibody, FGK45, resting APC, that normally inactivate T cells, become able to activate CD4 T cells by virtue of the upregulation of co-stimulatory molecules (Hawiger, Inaba et al. 2001). Anti-CD40 treated DC can also serve as effective initiators of cytotoxic T cell responses (Schoenberger, Toes et al. 1998). Echoing our findings with LPS, FGK45 did not significantly increase the numbers of $HEL_{105-120}$-specific effector CD4 T cells when $HEL_{105-120}$ peptide was given in IFA. However, when mice immunized with HEL protein in IFA were treated with anti-CD40 the HEL-specific cytokine producing cells in the spleen were significantly increased. It thus appears activation of APC, in the manner caused by CD40 ligation, cannot mimic T-cell cooperation. Interestingly, our results lead us to suggest that the presence of IFA alone leads to sufficient activation of the APC, such that single peptides given in IFA are not made more immunogenic by the additional presence of LPS, or stimulation by anti-CD40 agonistic antibody.

The finding that LPS and FGK45 served to enhance protein-specific, but not peptide-specific immune responses, is interesting. It is possible that these substances play a particularly important role in enhancing the presentation of protein derived peptides but not antigens that are already processed into peptides. Enhanced generation of protein-specific cytokine producing cells upon administration of LPS or anti-CD40 may be due to more efficient acquisition and processing of proteins, factors that may not be heavily involved in the presentation of soluble peptides given in IFA. Alternatively, increased APC activation by immunostimulatory substances may serve to enhance CD4 T cell responses in down-stream interactions that follow cooperation between CD4 T cells. In a preliminary experiment, we found no difference between mice given all four HEL peptides in IFA whether the immunization contained LPS or not (not shown). We therefore favor the notion that the effect of LPS or FGK45 in enhancing responses to HEL protein in IFA is due to more efficient presentation.

Figure 3.4.3 *Administration of an agonistic antibody to CD40 together with $HEL_{105-120}$ in IFA, in contrast with HEL protein in IFA, does not affect the generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells*. BALB/c mice were immunized with either 10μg of $HEL_{105-120}$ or 100μg of soluble HEL protein in IFA, subcutaneously at the tail base. Mice were injected with 100μg of either an isotype-matched control antibody (2A3) or a CD40 agonistic antibody (FGK45) intravenously at the time of immunization. The $HEL_{105-120}$-specific and HEL protein-specific cytokine producing CD4 T cells in the spleen were enumerated ten days post-immunization. Each data point represents the total number of ELISpot-forming cells detected in the spleen of a single mouse. These are pooled data from one to two independent experiments with three mice per group (n = 3-6). P-values represent the probability that the means of the two samples indicated are not different as assessed by unpaired, two-tailed T tests.

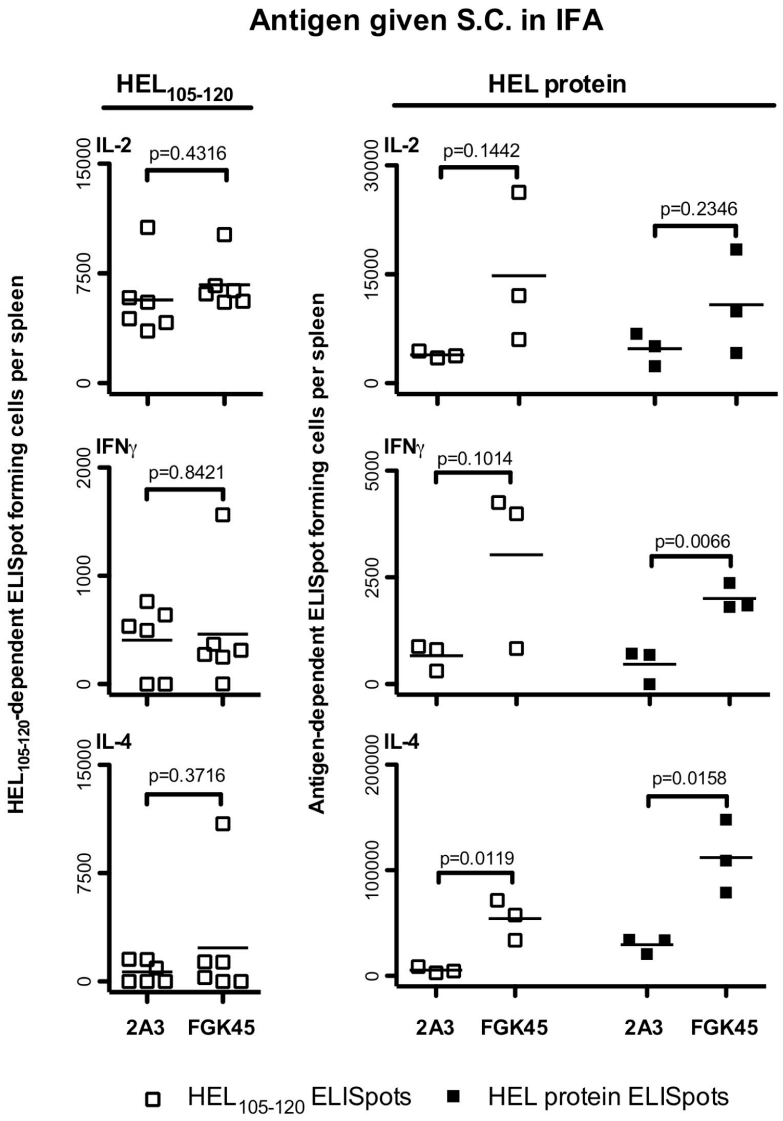

Antigen given S.C. in IFA
HEL105-120
HEL protein
IL-2
IFNγ
IL-4
p=0.4316
p=0.8421
p=0.3716
p=0.1442
p=0.2346
p=0.1014
p=0.0066
p=0.0119
p=0.0158
HEL105-120-dependent ELISpot forming cells per spleen
Antigen-dependent ELISpot forming cells per spleen
2A3
FGK45
HEL105-120 ELISpots
HEL protein ELISpots

Figure 3.4.3

Having been unsuccessful in enhancing the generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells by immunizing with a single peptide in IFA and additional substances aimed at activating APC, we decided to address whether directly stimulating CD4 T cells through cell surface receptors, known to augment the activation of the T cell itself, could enhance the generation of peptide-specific effector CD4 T cells. CD28, the classical co-stimulatory receptor, and OX40 were initially identified as such targets. As outlined in the sections 1.2 and 1.5, stimulation through either CD28 or OX40, mediates potent enhancement in the generation of CD4 effector T cells and their survival. We undertook experiments similar to those outlined above to address the potential role of these molecules in our experimental system.

Our initial pilot experiments showed that administration of an agonist antibody to CD28 did not affect the generation of peptide-specific effector CD4 T cells upon administration of $HEL_{105-120}$ alone in IFA (not shown). This was consistent with our previous experiments wherein LPS and FGK45 also did not enhance the generation of $HEL_{105-120}$-specific effector cells; one of the major downstream effects of CD40 stimulation is the activation of APC to express high-levels of B7 molecules (CD80 and CD86). We did not pursue stimulation through CD28 further. However, we observed potent and significant increases in the levels of peptide-specific cytokine secreting cells generated in mice given an agonistic antibody to OX40, called OX86, whether they were immunized with HEL protein in IFA or with $HEL_{105-120}$ alone in IFA (Figure 3.4.4 A and B). We also determined that this effect was dependent on immunization with an appropriate peptide at the time of OX86 administration (Figure 3.4.4 C and D). The effects of the OX40 agonist were potent and specific. This finding is consistent with the finding of others that OX40 is only up-regulated upon TCR ligation and that OX86 can be employed to enhance immunity to protein vaccinations and tumor cells (Gramaglia, Weinberg et al. 1998; Piconese, Valzasina et al. 2008).

Taking the above findings together, we conclude that the cooperative effect, observed upon co-administration of multiple MHCII-binding peptides, on the generation of $HEL_{105-120}$-specific effector CD4 T cells, is likely not due to non-specific activation of APC presenting the peptide.

Figure 3.4.4 *Administration of an agonistic antibody to OX40, together with $HEL_{105-120}$ or HEL protein in IFA, positively affects the generation of $HEL_{105-120}$ cytokine producing CD4 T cells*. (A and B) BALB/c mice were immunized with either 10μg of $HEL_{105-120}$ or 100μg of soluble HEL protein in IFA, at the tail base. (C and D) BALB/c mice were immunized with either 10μg of $HEL_{105-120}$ or 10μg of HEL_{11-25} in IFA, at the tail base. Mice were treated with 100μg of either an isotype-matched control antibody (HRPN) or an OX40 agonistic antibody (OX86) intravenously at the time of immunization. (A and B) The $HEL_{105-120}$-specific and HEL protein-specific cytokine producing CD4 T cells in the spleen were enumerated ten days post-immunization. (C and D) Both $HEL_{105-120}$ and HEL_{11-25}-specific cytokine producing CD4 T cells in the spleen were enumerated ten days post-immunization. Each data point represents the total number of ELISpot-forming cells detected in the spleen of a single mouse. These are pooled data from one to two independent experiments with three mice per group (n = 3-6). P-values represent the probability that the means of the two samples indicated are not different as assessed by unpaired, two-tailed T tests.

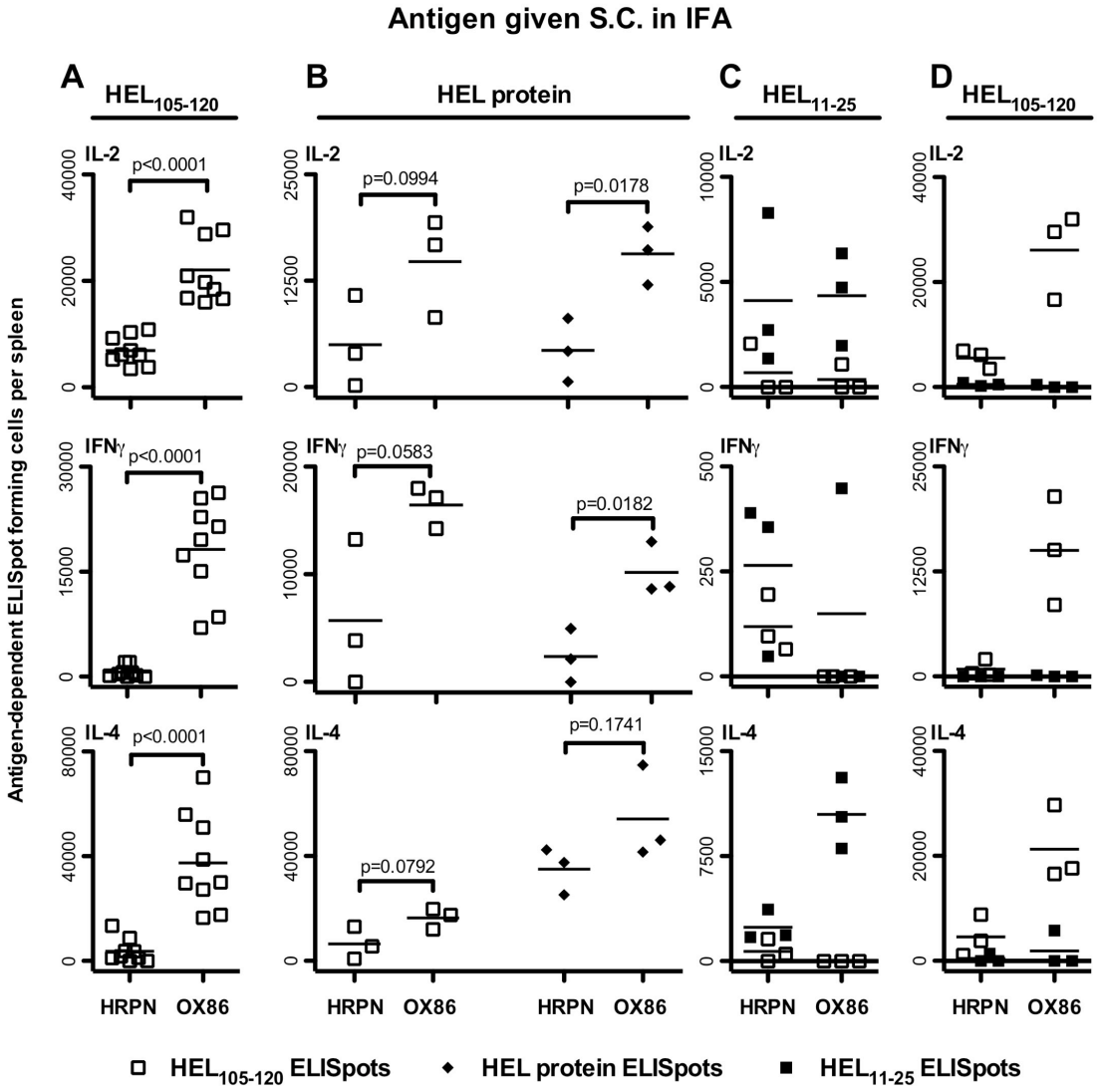

Antigen given S.C. in IFA
A
HEL105-120
B
HEL protein
C
HEL11-25
D
HEL105-120
Antigen-dependent ELISpot forming cells per spleen
IL-2
IFNγ
IL-4
p<0.0001
p=0.0994
p=0.0178
p=0.0583
p=0.0182
p=0.1741
p=0.0792
HRPN
OX86
HEL105-120 ELISpots
HEL protein ELISpots
HEL11-25 ELISpots

Figure 3.4.4

Furthermore, because peptide-specific responses can be readily increased by the administration of OX86, we hypothesized that a critical signal, whether it occurs through stimulation of OX86 or another molecule on the surface of CD4 T cells, fails to be generated upon administration of $HEL_{105-120}$ alone but is generated when multiple peptides are co-administered. As co-administration of multiple peptides leads to the activation of multiple populations of CD4 T cells, we propose that activation of these populations leads either directly or indirectly to the expression of certain cell surface ligands on APC or on the T cells themselves that serve to enhance mutual activation and effector function of other co-activated CD4 T cells.

3.5 Direct Investigation of the Underlying Mechanisms of CD4 T cell Cooperation in Intact Mice

Questions about the phenotypes and functions of specific populations of APC and peptide-specific CD4 T cells are particularly difficult to address in this experimental setting. Because we did not have a way to specifically identify the APC that were presenting peptides following immunization, or to track the specific CD4 T cells generated or their very scarce precursors, further analysis of these cells was precluded. We subsequently developed another experimental system aimed at addressing the cellular and molecular mechanisms of CD4 T cell cooperation. The results of experiments undertaken in this setting are outlined in Chapter 4 of this thesis. However, there were some critical aspects of CD4 T cell cooperation that could be further addressed within the context of the system outlined above.

We hypothesized that multiple antigen-specific CD4 T cells, primed following presentation of peptides in our IFA immunizations, cooperated to enhance activation of T-cells specific for $HEL_{105-120}$. If this were true, then CD4 T cells specific for other peptides should be present following immunization. To directly address whether CD4 T cells specific for other peptides given along with $HEL_{105-120}$ were required for the cooperative effect we selectively removed them by tolerization before immunization. In our previous experiments we found that $HEL_{105-120}$ and $OVA_{323-339}$-specifc CD4 T cells appear to cooperate, in the generation of $HEL_{105-120}$-specific CD4 effector T cells, when

these peptides were given together in IFA. To assess whether $OVA_{323-339}$-specific CD4 T cells were required for this cooperation we removed the $OVA_{323-339}$-specific CD4 T cells from mice by administration of high-doses of soluble $OVA_{323-339}$, intravenously, two weeks prior to immunization. High dose administration of soluble peptide is known to result in the functional removal of CD4 T cells specific for a given peptide by forcing them into an anergic state or into apoptosis (Kearney, Pape et al. 1994; Peters, Kroeger et al. 2009). Figure 3.5.1 shows the results of experiments of this type. It can clearly be seen that the cooperative effect between $OVA_{323-339}$ and $HEL_{105-120}$ in the generation of $HEL_{105-120}$-specific cytokine producing effector CD4 T cells is completely abrogated in $OVA_{323-339}$ tolerant mice. This loss of cooperation corresponds with the lack of generation $OVA_{323-339}$-specific effector CD4 T cells in these mice, indicating that they were indeed tolerant of $OVA_{323-339}$. We further confirmed that $OVA_{323-339}$-specific CD4 T cells were required for cooperation by replacing the anergic/deleted endogenous $OVA_{323-339}$-specific cells with CD4 T cells from DO11.10 mice. These CD4 T cells express a transgene-encoded TCR that recognizes $OVA_{323-339}$ in the context of $I\text{-}A^d$. Adoptive transfer of DO11.10 CD4 T cells clearly reconstituted both the generation of $OVA_{323-339}$-specific cytokine producing cells and restored the cooperative effect seen upon administration of $HEL_{105-120}$ and $OVA_{323-339}$ together in IFA. This result is strong evidence that co-activated CD4 T cells do cooperate *in vivo* with one another in achieving optimal generation of effector CD4 T cells.

Our previous finding that administration of OX86, the OX40 agonistic antibody, potently stimulated peptide-specific CD4 T cells when mice were concurrently immunized, suggested that OX40 signaling *could* be involved in CD4 T cell cooperation. However, this finding does not address whether OX40-OX40L signaling is involved in cooperative interactions between populations of CD4 T cells. To directly determine whether OX40-OX40L signaling played a significant role in CD4 T cell cooperative responses, we immunized mice with $HEL_{105-120}$ alone or with $HEL_{105-120}$ with three HEL peptides together in IFA, in the presence or absence of a non-cytolytic blocking antibody to OX40L.

Figure 3.5.1 *Enhancement of $HEL_{105-120}$-specific CD4 effector generation by co-administration of $HEL_{105-120}$ and $OVA_{323-339}$ requires CD4 T cells specific for $OVA_{323-339}$.* BALB/c mice were either treated with saline (N) or made tolerant to $OVA_{323-339}$ by administration of high amounts of $OVA_{323-339}$ intravenously (Tol) as described in Chapter 2. Fourteen days later, 10^5 $OVA_{323-339}$-specific purified CD4 T cells from DO11.10 mice were seeded into some tolerized mice (Tol + DO11.10) intravenously. All mice were then immunized with either 10μg of $HEL_{105-120}$ alone (H), or 10μg of both $HEL_{105-120}$ and $OVA_{323-339}$ together (H+O), subcutaneously in IFA. Peptide-specific cytokine producing CD4 T cells in the spleen were enumerated ten days post-immunization. Each data point represents the total number of ELISpot forming cells detected in the spleen of a single mouse. These are pooled results from two to four independent experiments with three mice per group (n = 6-12). P-values represent the probability that the means of the two samples indicated are not different as assessed by unpaired, two-tailed T tests.

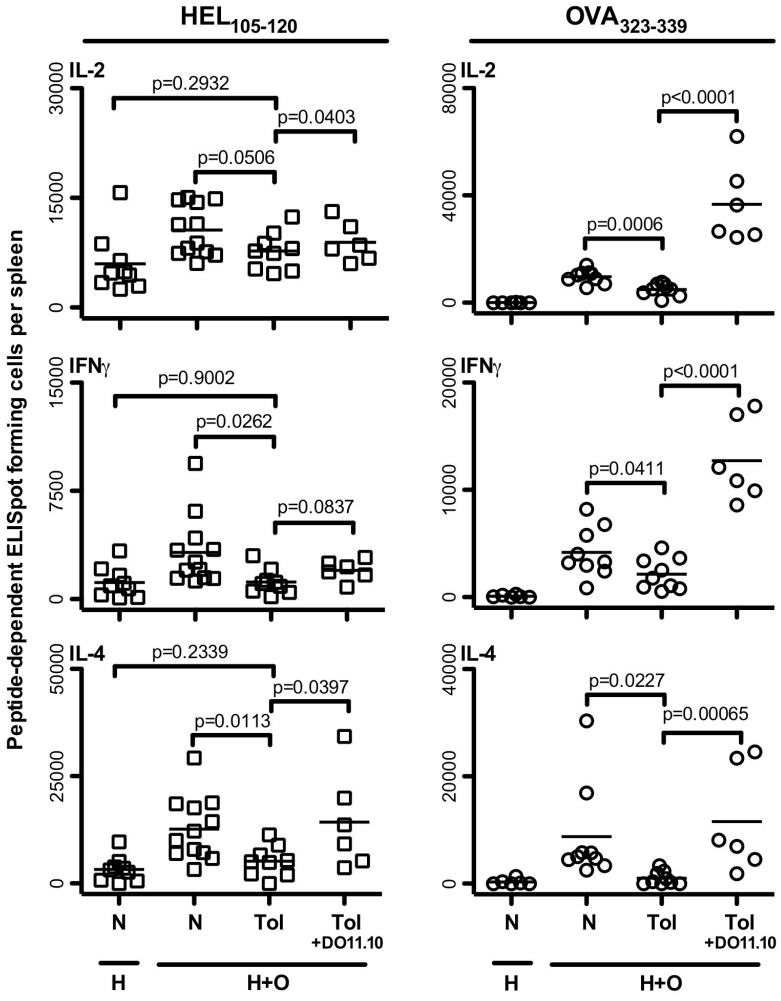

HEL105-120
OVA323-339
IL-2
IFNγ
IL-4
Peptide-dependent ELISpot forming cells per spleen
p=0.2932
p=0.0403
p=0.0506
p<0.0001
p=0.0006
p=0.9002
p=0.0262
p=0.0837
p=0.0411
p<0.0001
p=0.2339
p=0.0397
p=0.0113
p=0.0227
p=0.00065
N
N
Tol
Tol +DO11.10
H
H+O

Figure 3.5.1

As can be clearly seen in Figure 3.5.2, blocking OX40L had a potent effect on the generation of $HEL_{105-120}$ specific cytokine-producing CD4 T cells upon immunization with all four HEL peptides. However, no effect was observed on the minimal $HEL_{105-120}$ responses seen upon immunization with $HEL_{105-120}$ alone. These results are displayed as an index of cooperative effect in panel B. Whereas the number of $HEL_{105-120}$-specific cytokine-producing CD4 T cells is increased between 2.5 and 20-fold in mice given all four HEL peptides compared with mice immunized with $HEL_{105-120}$ alone, the cooperative enhancement is severely impaired in the presence of the blocking antibody to OX40L. This result strongly supports the hypothesis that OX40-OX40L interactions are involved in CD4 T cell cooperation. It appears that they are particularly important in mediating the enhanced generation of effector CD4 T cells, seen upon cooperation, and not simply critical for the activation of CD4 T cells in a general way.

The OX40-OX40L interaction had been identified as critical to cooperative responses. Our previous work on CD4 T cell cooperation (Peters, Kroeger et al. 2009) led us to conclude that, in some cases, CD4 T cell cooperation is required for optimal generation of cytokine producing CD4 effector T cells. Evidence for this conclusion came from experiments in BALB/c mice made tolerant to $HEL_{105-120}$. Critically, these mice fail to respond to $HEL_{105-120}$ *and all other HEL peptides* upon administration with HEL protein on ALUM. The generation of CD4 effector T cells specific for the other HEL peptides could be rescued by immunizing with HEL coupled to OVA, thus replacing the $HEL_{105-120}$-specific cooperating CD4 T cells with an OVA-specific CD4 T cell population. Given our finding that OX40 stimulation mimics, and blocking OX40L abrogates, the effects of CD4 T cell cooperation (Figures 3.4.4 and 3.5.2), it was natural to address whether OX40 stimulation, on its own, could rescue the generation of HEL-peptide specific CD4 effector cell generation in $HEL_{105-120}$ tolerant mice immunized with HEL on ALUM.

Figure 3.5.2 *Blocking OX40L abrogates the effect of cooperation between HEL-peptide-specific CD4 T cells*. (A) BALB/c mice were immunized with $HEL_{105-120}$ or $HEL_{105-120}$ + 3 HEL peptides as in Figure 3-4, and were treated with 100μg of either an isotype-matched control (iso. cont.) antibody or an OX40L blocking antibody (OX40L blocker), intravenously at the time of immunization. $HEL_{105-120}$-specific ELISpot forming cells in the spleen were enumerated on day 10 post-immunization. (B) The data in panel *A* is displayed as cooperative effect (fold enhancement) of $HEL_{105-120}$-specific CSCs between mice immunized with $HEL_{105-120}$ alone or with $HEL_{105-120}$ + 3 HEL peptides together in IFA. P-values represent the probability that the means of the two samples indicated are not different as assessed by unpaired, two-tailed T tests. These are pooled results from three independent experiments with three mice per group (n = 9).

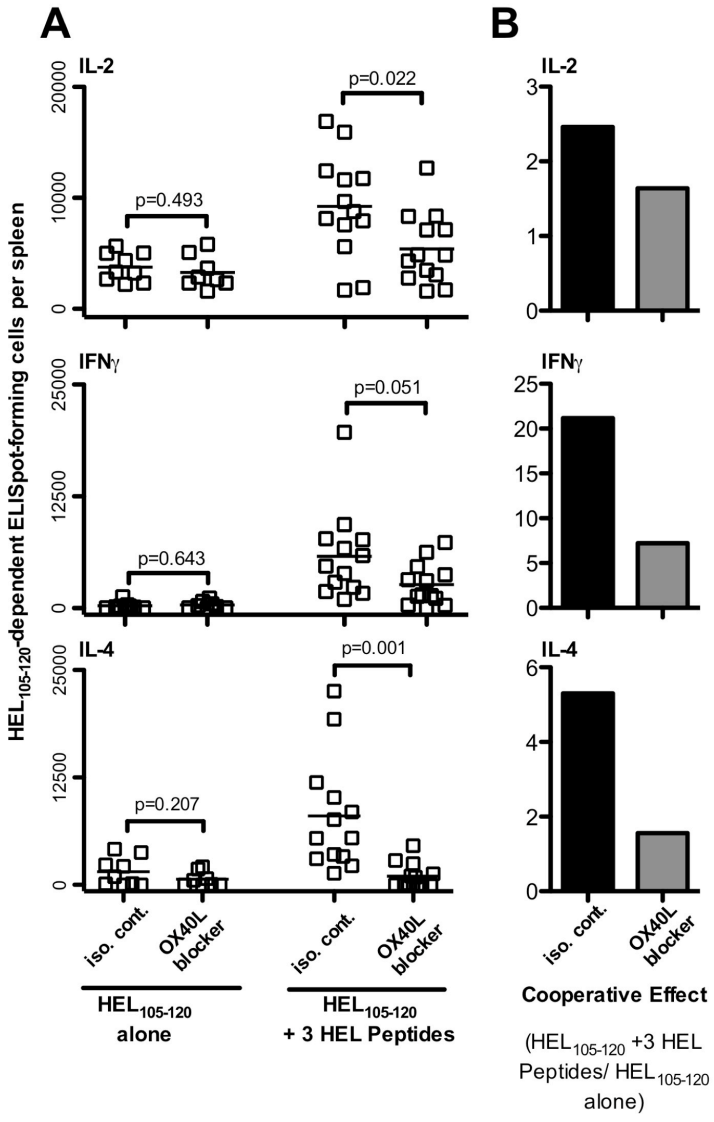

A
B
IL-2
IFNγ
IL-4
p=0.493
p=0.022
p=0.643
p=0.051
p=0.207
p=0.001
HEL105-120-dependent ELISpot-forming cells per spleen
iso. cont.
OX40L blocker
HEL105-120 alone
HEL105-120 + 3 HEL Peptides
Cooperative Effect
(HEL105-120 +3 HEL Peptides/ HEL105-120 alone)

Figure 3.5.2

Figure 3.5.3 shows the results of an experiment that addresses this question. The responses to the HEL_{11-25}, HEL_{48-63} and HEL_{74-96} peptides are lost in $HEL_{105-120}$ tolerant animals upon immunization with HEL protein on ALUM. Significantly, these responses are rescued upon stimulation with an agonistic antibody to OX40. However, it appears that treatment with anti-OX40 antibody rescued the generation of $HEL_{105-120}$-specific CD4 T cells as well, a result that is not surprising given that others have published similar findings (Bansal-Pakala, Jember et al. 2001). Given that the level of minor peptide-specific effector T cells generated in $HEL_{105-120}$ tolerized mice was much greater than in normal mice, and our findings that minor-peptide-specific CD4 T effector cells can be stimulated directly by anti-OX40 (Figure 3.4.4 C), we suggest that the effect of agonist antibody treatment on the generation of CD4 T cells specific for minor HEL peptides, was a combination of cooperation resulting from the rescue of the $HEL_{105-120}$-specific CD4 T cells and the direct action of the agonistic antibody on minor peptide-specific CD4 T cells.

Figure 3.5.3 *Stimulation of HEL peptide-specific CD4 T cells through OX40 rescues their activation in mice made tolerant to* $HEL_{105\text{-}120}$. BALB/c mice were treated with saline or were treated with soluble $HEL_{105\text{-}120}$, as described in Chapter 2. Fourteen days later all mice were challenged with 100μg of heat-aggregated HEL on ALUM intraperitoneally. Some mice were treated with 100μg of an isotype-matched control antibody while some tolerant mice were treated with 100μg of an OX40 agonist antibody intravenously at the time of immunization. The cytokine producing CD4 T cells in the spleen, specific for four different HEL peptides were enumerated by optimized ELISpot assay ten days post-immunization. Each data point represents the total number of ELISpot forming cells detected in the spleen of a single mouse. These are pooled results from three independent experiments with three mice per group (n = 9). P-values represent the probability that the means of the two samples indicated are not different as assessed by unpaired, two-tailed T tests.

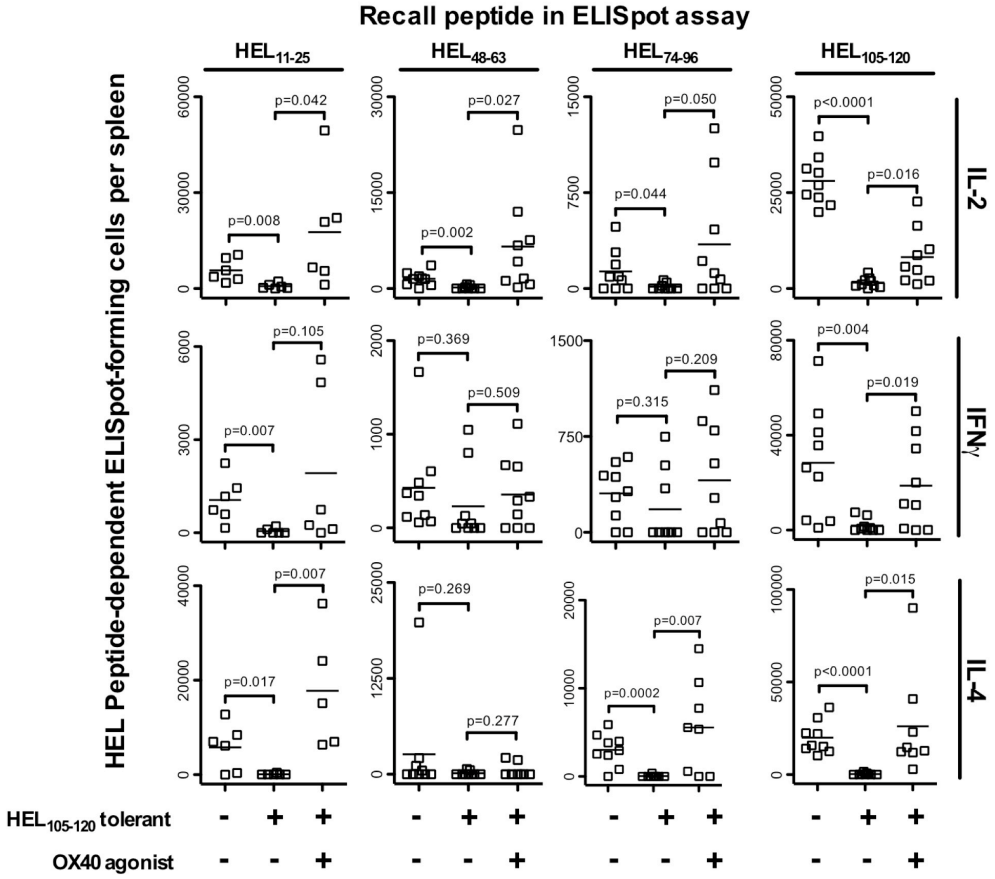

Recall peptide in ELISpot assay
HEL11-25
HEL48-63
HEL74-96
HEL105-120
HEL Peptide-dependent ELISpot-forming cells per spleen
IL-2
IFNγ
IL-4
p=0.042
p=0.008
p=0.027
p=0.002
p=0.050
p=0.044
p<0.0001
p=0.016
p=0.105
p=0.007
p=0.369
p=0.509
p=0.209
p=0.315
p=0.004
p=0.019
p=0.007
p=0.017
p=0.269
p=0.277
p=0.007
p=0.0002
p=0.015
p<0.0001
HEL105-120 tolerant
OX40 agonist

Figure 3.5.3

3.6 Conclusions

We had initially hypothesized that CD4 T cells, activated at the same time, could cooperate with one another, leading to enhancement of their proliferation and cytokine production, i.e. their effector function. We found that CD4 T cells specific for mycobacterium-derived peptides, for three peptides of HEL, and for $OVA_{323-339}$, could cooperatively enhance the generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells. This cooperative enhancement could not be mimicked by LPS administration or by systemic APC activation by an agonistic antibody to CD40. We therefore conclude that CD4 T cell cooperation most likely enhances CD4 T cell activation beyond signals delivered by APC activated through LPS or CD40 ligation. We found that CD4 T cells, specific for the $OVA_{323-339}$ peptide, were required to enhance the generation of $HEL_{105-120}$-specific effector CD4 T cells when both peptides were given together in IFA. We conclude that our observations result from cooperation between CD4 T cells. In two separate experimental systems, OX40 stimulation mimics the effects of CD4 T cell cooperation. Given these findings, in addition to the ability of OX40L blockade to abrogate the effect of CD4 T cell cooperation, we conclude that the OX40-OX40L interaction is likely critical in the cooperation between populations of CD4 T cells.

As noted above, many questions about the cellular and molecular mechanisms of CD4 T cell cooperation remained following the completion of these studies. We therefore undertook the development of another experimental system that would allow us to address questions such as the nature of the APC that mediates cooperation. This work is described in Chapter 4.

4.0 CHAPTER 4 - OBSERVATIONS AND MECHANISMS OF CD4 T CELL COOPERATION IN VIVO: II ADOPTIVE TRANSFER OF PEPTIDE-PULSED APC

4.1 Introduction

As we have shown in the previous chapter, CD4 T cell populations recognizing distinct peptides can cooperate with one another to enhance their own activation following immunization with multiple peptides that bind MHCII. The experimental protocols outlined in Chapter 3 yielded significant results and allowed us to conclude that cooperation between CD4 T cells, differing in TcR specificity, is likely a biologically relevant phenomenon, not solely an artifact of our experimental system. We also concluded that cooperation between CD4 T cell populations requires signaling through OX40-OX40L interactions and that the effects of cooperation cannot be mimicked by co-administration with LPS or an agonist antibody to CD40. However, a major problem with protocols that use IFA to formulate peptides injected into mice is that, upon injection, little can be done to identify the cellular and molecular interactions that occur to induce CD4 T cell activation and subsequent cooperation. The nature of the APC involved in cooperation, whether the same APC must present both peptides simultaneously, and how OX40L co-stimulatory signals mediate CD4 T cell cooperation are remaining questions, with important implications for understanding the biology of CD4 T cell cooperation. Thus we sought to develop a new experimental system that would allow us to address these questions.

We hypothesized that, upon administration of soluble peptides in IFA, these peptides are presented by MHCII-bearing APC following uptake and processing of peptide-containing micelles or following exogenous loading of empty MHCII molecules.

We reasoned that if a population of APC could be loaded with single or multiple peptides *in vitro*, and then adoptively transferred into mice, they may function to mediate CD4 T cell activation and cooperation in a way similar to endogenous APC. The advantage of employing this experimental model is that specific APC subpopulations can be isolated prior to transfer, loaded with defined peptides, analyzed by flow-cytometry or biochemical methods prior to injection, and tracked after injection. The use of this system allowed me to investigate the underlying cellular and molecular mechanisms involved in cooperative CD4 T cell interactions *in vivo*.

4.2 Establishing an Experimental System for Adoptive Transfer of APC

Many reports describe the exogenous loading of APC populations with antigen prior to adoptive transfer. This loading is commonly referred to as "antigen pulsing". Typically, APC, such as DC, are pulsed with peptide or protein antigens in culture, washed and then injected subcutaneously or intravenously to generate immune responses. Given that these protocols have been established and employed to shed light on a variety of immunological phenomena, we were confident that such a pulsing system could lead to significant results in our own hands.

Because CD4 T cell cooperation, in mice immunized with IFA emulsions containing peptides, is mediated by endogenous APC, we wished to employ a similar set of APC for our pulsing studies. The spleen is a convenient source of APC subpopulations, including B cells and various subpopulations of DC. We first used unfractionated splenocytes, pulsed with peptides, as APCs in subsequent experiments.

Resting APC are often found to be tolerogenic (see section 1.2). We wished to avoid inducing tolerance as a complicating factor in our experiments and so attempted to activate APC in cultures of splenocytes by supplementing these cultures with LPS. LPS is a known ligand for TLR-4 in mice, and upon binding, induces activation of both myeloid DC and B cells. These cells rapidly acquire enhanced antigen-presenting capability that correlates with the up-regulation of co-stimulatory molecules such as CD86. Figure 4.2.1 shows the cell surface phenotype, as assessed by flow-cytometry, of splenocytes cultured for approximately 24 hours with or without 1μg/mL *E. coli* LPS in the media.

Figure 4.2.1 *Overnight culture in the presence of LPS increases CD86 expression by splenocytes*. BALB/c splenocytes were prepared as single cell suspension and re-suspended at a concentration equivalent to two spleens per 3mL of RPMI media without FCS supplement. Three mL of this cell suspension was plated in 60mm tissue culture dishes with and without 1μg/mL *E. coli* LPS. Following overnight culture, cells were harvested and stained with fluorophore-conjugated monoclonal antibodies to the indicated surface protein. Stained cells were analyzed by flow-cytometry. Black histograms show the level of fluorescence of cells stained with the appropriate antibody. Grey, dotted, histograms show the level of fluorescence of cells stained with a matched fluorophore-conjugated isotype-matched control antibody. The percentages of cells falling within the gated region within each profile are shown. Data presented are representative of results from two independent experiments.

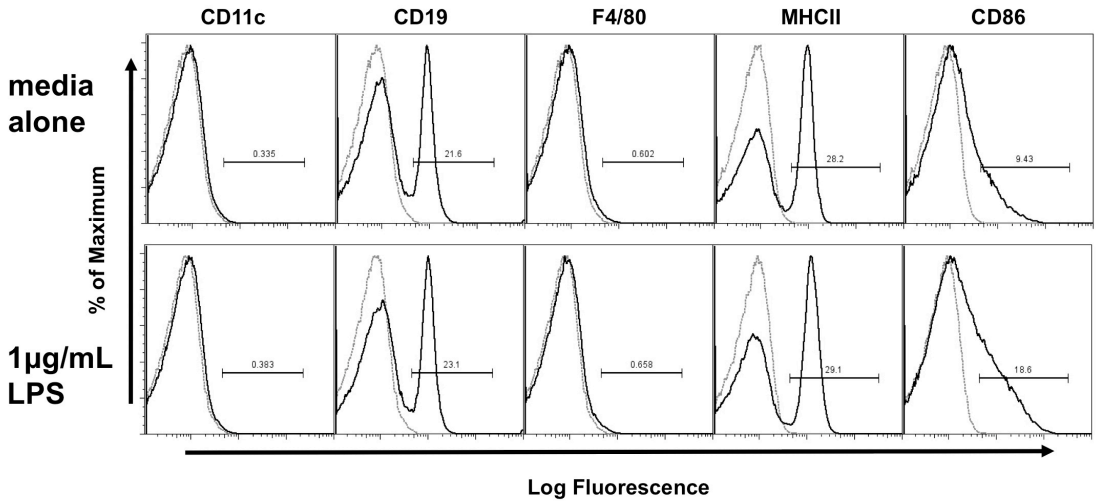

CD11c
CD19
F4/80
MHCII
CD86
media alone
1µg/mL LPS
% of Maximum
0.335
21.6
0.602
28.2
9.43
0.383
23.1
0.658
29.1
18.6
Log Fluorescence

Figure 4.2.1

The relative proportions of APC populations do not change substantially between cultures containing or not containing LPS. The level of CD86 expression increased when splenocytes were cultured with LPS. Given the considerations that resting APC are often tolerogenic, the finding that CD86 expression increased in splenocytes cultured with LPS, and our previous findings that LPS activation of APC *in vivo* does not affect CD4 T cell cooperation, we always employed LPS in our subsequent APC cultures.

After analyzing the phenotype of LPS-activated cultured splenocytes, we wanted to determine whether LPS stimulated splenocytes could function as APCs in adoptive transfer experiments with peptides. We first investigated whether cultured splenocytes, injected subcutaneously into the hind footpad, would migrate to the draining lymph node, the site of antigen presentation. Overnight-cultured splenocytes were labeled with carboxy-fluorocein succinimidyl ester (CFSE), to allow us to detect splenocytes after transfer. CFSE-labeled cells were injected subcutaneously into either the footpad or lower leg of normal BALB/c mice. We observed that the numbers of $CFSE^+$ cells present in the popliteal lymph nodes (LN) peaked at 48h post-injection (data not shown). As can be seen in Figure 4.2.2A, the $CFSE^+$ cells that reach the LN constitute a small fraction of the total lymph node population that was isolated. We determined that CFSE labeled cells expressing CD11c (conventional DC), CD19 (B cells), MHCII, and CD86 reached the draining lymph node following adoptive transfer (Figure 4.2.2B). Thus the possibility that peptide pulsed, cultured splenocytes could deliver antigen to the draining lymph node was confirmed.

We next examined whether peptide-pulsed, cultured splenocytes could, in fact, present antigen to CD4 T cells *in vivo* following adoptive transfer. To achieve this, we took advantage of a well-described methodology for detecting antigen-induced proliferation in CD4 T cells. That is, when CD4 T cells experience antigen presentation through ligation of the TCR by peptide-MHCII complexes for which they have affinity, they begin to divide. We CFSE labeled splenocytes from DO11.10 mice that contain CD4 T cells that express a transgene-encoded TCR with affinity for $OVA_{323-339}$ in the context of $I\text{-}A^d$. These labeled splenocytes were then injected intravenously into BALB/c mice. One day later, syngeneic splenocytes cultured for 24h in the presence or absence of

50μM $OVA_{323-339}$, were injected into these mice. This high peptide concentration was chosen to facilitate rapid loading of empty MHCII molecules. Six days later, the spleens and lymph nodes of injected mice were harvested and stained for DO11.10 TCR with a clonotype-specific fluorophore-conjugated antibody. Flow cytometry was used to monitor DO11.10 TCR^+ cells proliferation, by measuring CFSE labeling intensity, and clonal expansion by measuring the percentage of DO11.10 TCR^+ cells in the lymph nodes and spleen. As can be seen in Figure 4.2.3, only when adoptively transferred splenocytes were pulsed with $OVA_{323-339}$ was there division and accumulation of CFSE-labeled DO11.10 CD4 T cells. This effect was observed only in the draining lymph nodes at the time of analysis.

Figure 4.2.2 *Cultured splenocytes labeled with CFSE migrate to the popliteal lymph node following subcutaneous footpad injection*. BALB/c splenocytes were cultured overnight as described in Chapter 2 in the presence of 1μg/mL LPS and without exogenous peptide. All cells were harvested and labeled with 5μM CFSE. After thorough washing, 10^7 stained splenocytes were re-suspended in 50μL Leibovitz media and injected subcutaneously into the footpad and lower leg of BALB/c mice. After 48 hours, the popliteal lymph nodes of mice were harvested and stained with fluorophore-conjugated antibodies to detect the indicated cell surface proteins. Stained cells were analyzed by flow-cytometry (A) CFSE positive cells are shown gated from total lymph node cells. (B) The expression of individual cell surface proteins is shown for CFSE positive cells. Black histograms show the relative frequency of fluorescence of cells stained with the indicated antibody. Grey, dotted, histograms show the level of fluorescence of cells stained with a matched fluorophore-conjugated isotype-matched control antibody. Percentages of cells falling within the gated region of the total number of cells within the given plot are shown. These profiles are representative of results from three independent experiments.

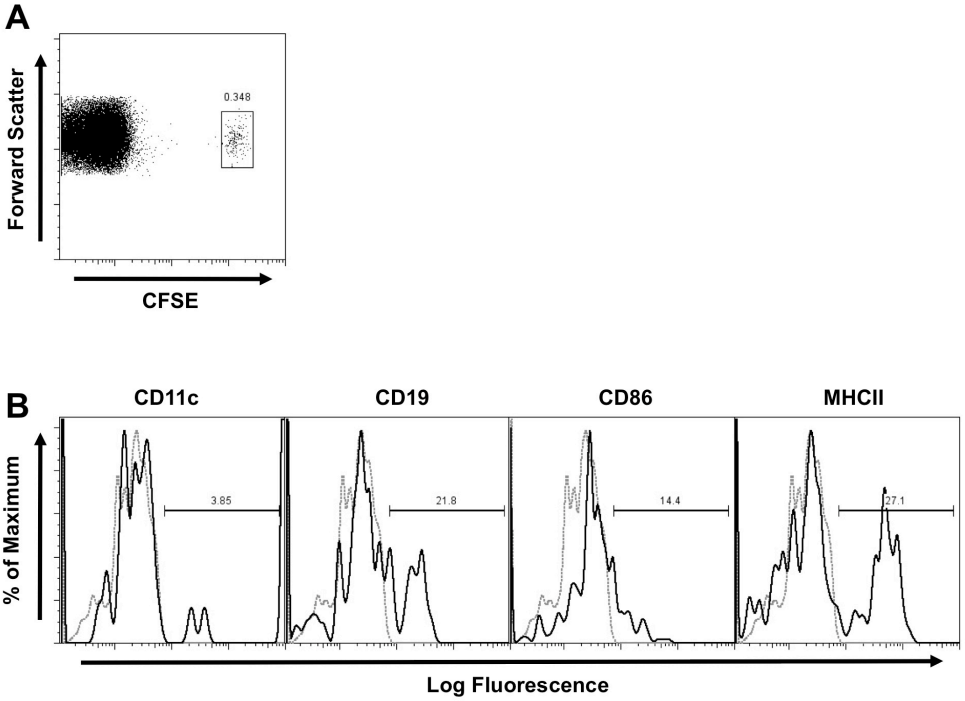

A
Forward Scatter
0.348
CFSE
B
CD11c
CD19
CD86
MHCII
% of Maximum
3.85
21.8
14.4
27.1
Log Fluorescence

Figure 4.2.2

Taken together, these results indicate that following injection into the footpad the peptide pulsed splenocytes migrated to the draining lymph nodes, and presented antigen to CD4 T cells. We subsequently investigated whether these splenocytes mediated cooperation between CD4 T cell populations. Because we had previously observed that simultaneous administration of $OVA_{323-339}$ and $HEL_{105-120}$ in IFA resulted in cooperative effects on CD4 T cells responses, and because these peptides do not bind to the same MHCII molecules, we used these peptides to investigate whether peptide-pulsed APC could mediate T cell cooperation.

Splenocytes, pulsed with $OVA_{323-339}$, and injected into mice, presented the peptide to CD4 T cells in the draining lymph nodes. However, we did not know whether $OVA_{323-339}$ pulsed APCs cells could also present $HEL_{105-120}$. Furthermore, it was not clear whether overnight peptide pulsing was essential for APC loading, or if a shorter time would be sufficient. We carried out an experiment to address these questions. Taking advantage of the increased generation of effector CD4 T cells upon secondary stimulation, we were able to determine that administration of 10^7 $HEL_{105-120}$-pulsed splenocytes likely resulted in primary stimulation of endogenous CD4 T cells (Figure 4.2.4). Moreover, we found significant increases in secondary $HEL_{105-120}$-specific cytokine producing CD4 T cell levels only when splenocytes were cultured in the presence of $HEL_{105-120}$ for 24 hours.

Figure 4.2.3 *$OVA_{323-339}$-pulsed splenocytes present peptide to CD4 T cells following subcutaneous injection*. Splenocytes from DO11.10 mice, containing CD4 T cells that recognize $OVA_{323-339}$ in the context of I-A^d, were labeled with 5μM CFSE and10^7 labeled splenocytes were injected intravenously into BALB/c mice. Twenty-four hours later these mice were injected subcutaneously in the footpad and lower leg with BALB/c splenocytes cultured overnight, in the presence or absence of 50μM $OVA_{323-339}$ (as described in Chapter 2). Six days post-injection, draining popliteal lymph nodes (DLN) and spleens from injected mice were harvested and stained with a fluorophore-conjugated antibody to the DO11.10 TCR expressed by transgenic CD4 T cells. The level of CFSE staining and numbers of DO11.10 positive cells were assessed by flow cytometry. Percentages of cells falling within the gate out of the total number of cells within the given plot are shown. These results are representative of two independent experiments.

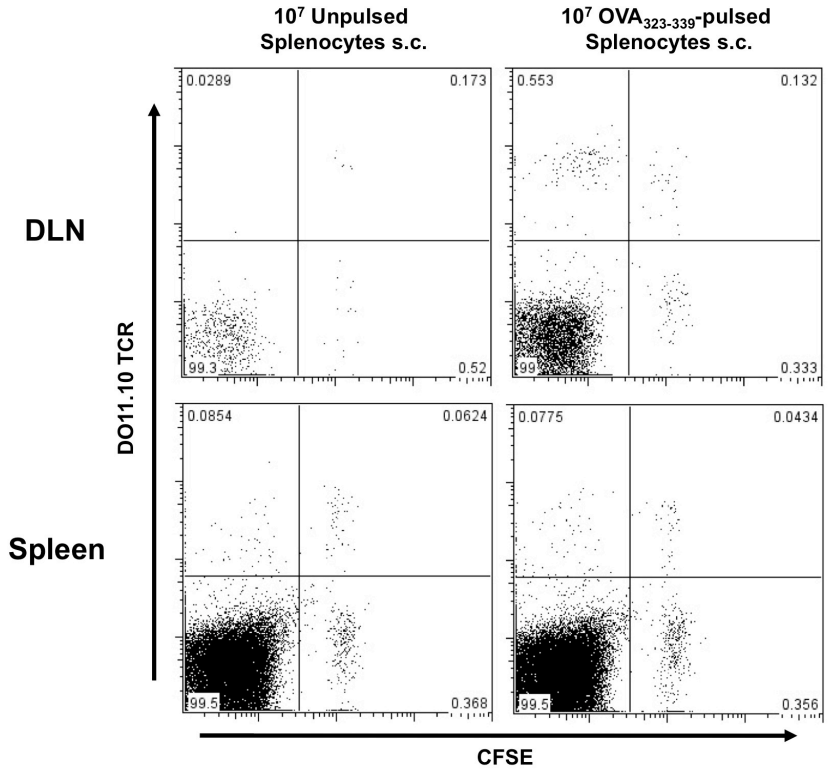

10^7 Unpulsed Splenocytes s.c.
10^7 OVA323-339-pulsed Splenocytes s.c.
DLN
Spleen
DO11.10 TCR
CFSE
0.0289
0.173
99.3
0.52
0.553
0.132
99
0.333
0.0854
0.0624
99.5
0.368
0.0775
0.0434
99.5
0.356

Figure 4.2.3

Figure 4.2.4 *Subcutaneous footpad injection of $HEL_{105-120}$-pulsed splenocytes results in accumulation of $HEL_{105-120}$-specific cytokine producing CD4 T cells that enhance secondary responses to $HEL_{105-120}$.* BALB/c splenocytes were cultured for 24 hours, as described in Chapter 2, with 50μM $HEL_{105-120}$ for the indicated times. After harvesting and extensive washing, 10^7 pulsed splenocytes in 50μL Leibovitz media were injected into the footpad and lower leg of normal BALB/c mice. Some mice were injected with Leibovitz media alone to serve as controls (N). Fourteen days after injection, mice were injected subcuatneously at the tail base with 10μg $HEL_{105-120}$ in CFA, Ten days post-injection, the $HEL_{105-120}$-specific cytokine producing cells in the spleen were enumerated by ELISpot. Each data point represents the number of specific ELISpot-forming cells in the spleen of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. This is the result of a single experiment with three mice per group (n = 3).

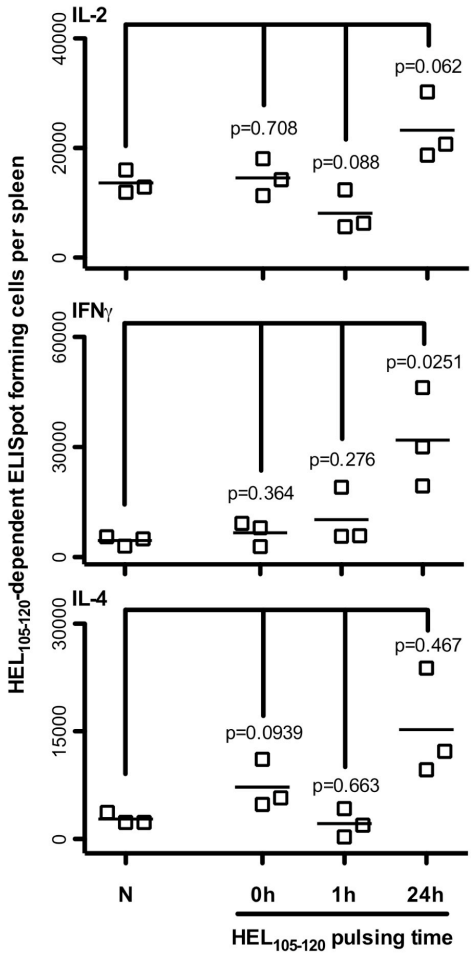

IL-2
IFNγ
IL-4
HEL105-120-dependent ELISpot forming cells per spleen
40000
20000
0
60000
30000
0
30000
15000
0
p=0.062
p=0.708
p=0.088
p=0.0251
p=0.276
p=0.364
p=0.467
p=0.0939
p=0.663
N
0h
1h
24h
HEL105-120 pulsing time

Figure 4.2.4

Having established that antigen presentation to endogenous CD4 T cell populations occurs upon injection of $HEL_{105-120}$ or $OVA_{323-339}$-pulsed splenocytes, we replicated the previous experiments that demonstrated cooperation between two peptide-specific CD4 T cell populations. Figure 4.2.5 shows the results of a single experiment in which mice were injected with either 10^7 splenocytes pulsed with $HEL_{105-120}$ alone, $OVA_{323-339}$ alone, or both peptides together. Ten days after this injection the number of $HEL_{105-120}$ and $OVA_{323-339}$-specific cytokine producing CD4 cells in the draining popliteal lymph node were enumerated. Very low and variable numbers of peptide-specific effector CD4 T cells were detected. However, we did not observe statistically significant differences and it appeared that the number of $HEL_{105-120}$-specific cytokine producing CD4 T cells was higher, in mice given splenocytes pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$, than in mice given splenocytes pulsed with $HEL_{105-120}$ alone. We hypothesized that the low responses observed may be due to the inefficiency inherent in our experimental protocol and subsequently attempted to improve the efficiency of CD4 T cell activation.

We had shown when $HEL_{105-120}$ and $OVA_{323-339}$ are given together in IFA, the generation of effector CD4 T cells specific for $OVA_{323-339}$ is not significantly affected by the presence of $HEL_{105-120}$. Therefore, we thought that we might improve the efficiency of CD4 cooperation, in generating $HEL_{105-120}$-specific cytoking producing cells, without compromising the potential to observe converse effects, by seeding mice with exogenous $OVA_{323-339}$-specific CD4 T cells. We therefore seeded normal mice with varying numbers of splenocytes from DO11.10 mice, prior to the injection of peptide-pulsed APC. We found that increasing the numbers of $OVA_{323-339}$-specific CD4 T cells had a potentially observable effect on the generation of $HEL_{105-120}$-specific cytokine producing effector CD4 T cells, although the differences did not reach statistical significance (Figure 4.2.6). We hypothesized that, even in the presence of increased numbers of $OVA_{323-339}$-specific CD4 T cells, the generation of peptide specific effector CD4 T cells was limited. This limitation may be due to failure of peptide-pulsed APC to sustain the presentation of those peptides over time. When peptides are given in IFA they are likely to be continuously presented over the course of the generation of the immune response since IFA depots can be found under the skin of immunized mice weeks after injection (data not shown).

Figure 4.2.5 *A single subcutaneous injection of peptide-pulsed splenocytes is insufficient to elicit significant primary peptide-specific effector CD4 T cell responses*. BALB/c splenocytes were cultured overnight, as described in Chapter 2, in the presence of either 50μM $HEL_{105-120}$, 50μM $OVA_{323-339}$, or 50μM of both peptides. Following harvest and extensive washing, 10^7 of pulsed splenocytes in 50μL Leibovitz media were injected into the footpad and lower leg of normal BALB/c mice. Ten days post-injection, the $HEL_{105-120}$ and $OVA_{323-339}$-specific cytokine producing cells in the draining popliteal lymph node (DLN) were enumerated by ELISpot in mice given only $HEL_{105-120}$-pulsed splenocytes (H), only $OVA_{323-339}$-pulsed splenocytes (O), and in mice given splenocytes pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$ (H+O). Each data point represents the number of specific ELISpot-forming cells in the popliteal lymph node of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. This is the result of a single experiment with three mice per group (n = 3).

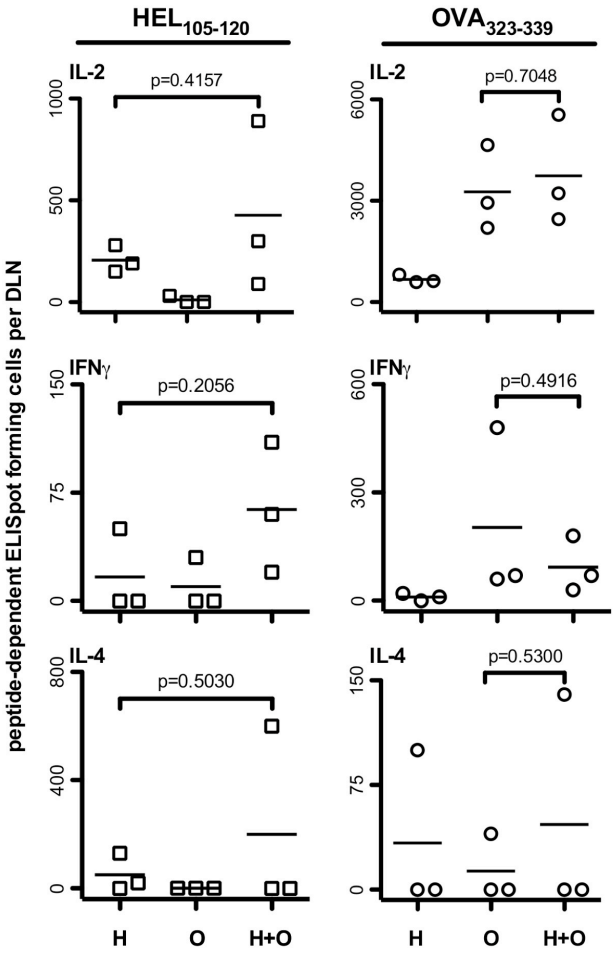

HEL105-120
OVA323-339
IL-2
p=0.4157
p=0.7048
IFNγ
p=0.2056
p=0.4916
IL-4
p=0.5030
p=0.5300
peptide-dependent ELISpot forming cells per DLN
H
O
H+O

Figure 4.2.5

Figure 4.2.6 *Mice seeded with DO11.10 splenocytes appear to support enhanced generation of $OVA_{323-339}$ and $HEL_{105-120}$-specific effector CD4 T cell generation upon injection of splenocytes pulsed with both peptides*. One day prior to injection of pulsed splenocytes, BALB/c mice were injected with DO11.10 splenocytes which contain approximately 10% CD4+ DO11.10+ T cells. BALB/c splenocytes were cultured overnight, as described in Chapter 2, in the presence of both 50μM $HEL_{105-120}$ and 50μM $OVA_{323-339}$. Following harvest and extensive washing, 10^7 peptide pulsed splenocytes in 50μL Leibovitz media were injected into the footpad and lower leg of BALB/c mice previously injected with DO11.10 splenocytes. Ten days post-injection, the $HEL_{105-120}$ and $OVA_{323-339}$-specific cytokine producing cells in the draining popliteal lymph node (DLN) were enumerated by ELISpot assay. Each data point represents the number of specific ELISpot-forming cells in the popliteal lymph node of a single mouse. This is the result of a single experiment with three mice per group (n = 3).

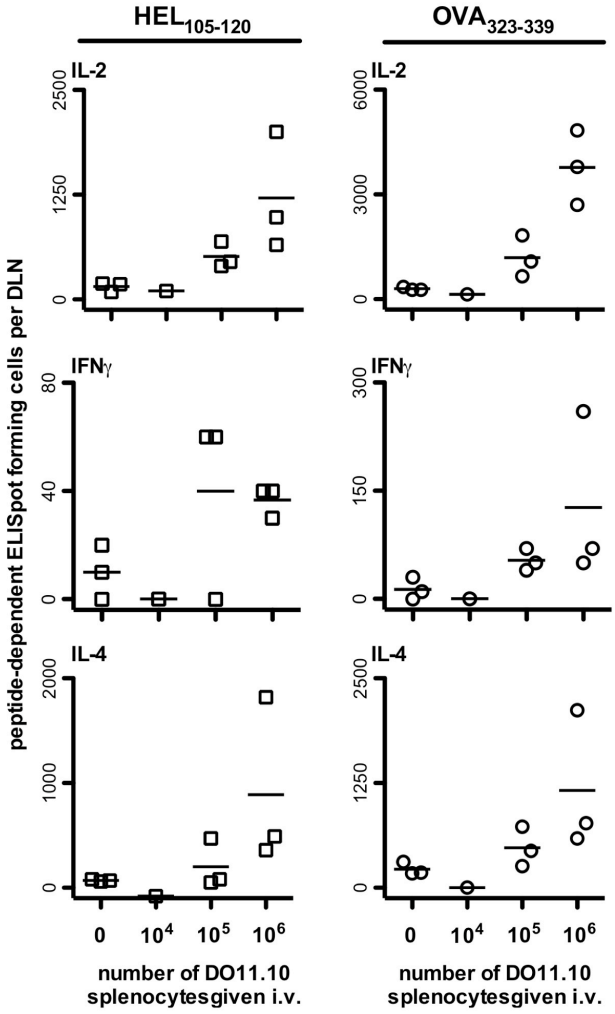

HEL105-120
OVA323-339
IL-2
IL-2
IFNγ
IFNγ
IL-4
IL-4
peptide-dependent ELISpot forming cells per DLN
0
1250
2500
3000
6000
40
80
150
300
1000
2000
0
10^4
10^5
10^6
number of DO11.10 splenocytesgiven i.v.
number of DO11.10 splenocytesgiven i.v.

Figure 4.2.6

We decided to provide more sustained antigen presentation by giving repeated injections of peptide-pulsed APC. As can be seen in Figure 4.2.7, three injections of $HEL_{105-120}$ and $OVA_{323-339}$-pulsed APC, three days apart, resulted in substantial increases in the levels of peptide-specific effector CD4 T cells generated nine days after the initial injection. Furthermore, there was a clear dose dependent response in the number of $OVA_{323-339}$-specific cytokine producing cells generated and, importantly, the level of $HEL_{105-120}$-specific effector CD4 T cells was dependent on the number of CD4-positive DO11.10 cells seeded into mice prior to the first injection. The finding that $HEL_{105-120}$-specific effector cells were more efficiently generated when more $OVA_{323-339}$ CD4 T cells were present, provided direct evidence that cooperation occured between these two CD4 T cell populations in the activation of $HEL_{105-120}$-specific CD4 T cells.

Since the injection of 10^5 CD4-positive DO11.10 cells into mice appeared to result in optimal generation of $HEL_{105-120}$-specific responses, we repeated the experiment shown in Figure 4.2.7 with that number of DO11.10 CD4 T cells. Figure 4.2.8 shows the pooled results of these repeated experiments. A clear cooperative effect in $HEL_{105-120}$-specific effector CD4 T cell generation is seen in mice injected with splenocytes pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$ when compared to mice injected with splenocytes pulsed with $HEL_{105-120}$ alone. Consistent with our earlier observations, only a marginal effect on the generation of $OVA_{323-339}$-specific effector CD4 T cells was observed in mice injected with splenocytes pulsed with both peptides. However, interpretation of this observation is complicated by the fact that all mice were injected with 10^5 $OVA_{323-339}$-specific CD4 T cells prior to adoptive transfer of peptide-pulsed APC.

We therefore established an experimental system, employing adoptively transferred peptide-pulsed APC populations to elicit peptide-specific CD4 T cell activation, which resulted in cooperative responses between CD4 T cells specific for $HEL_{105-120}$ and $OVA_{323-339}$, replicating our earlier findings employing peptides administered in IFA. We subsequently employed this same basic experimental setup to address mechanistic questions surrounding the phenomenon of CD4 T cell cooperation.

Figure 4.2.7 *Three subcutaneous injections of $HEL_{105-120}$ and $OVA_{323-339}$ peptide-pulsed splenocytes induce significant peptide-specific effector CD4 T cell responses in DO11.10-seeded mice*. One day prior to injection of pulsed splenocytes, BALB/c mice were injected with the indicated number of MACS purified $CD4^+$ DO11.10 T cells. BALB/c slpenocytes were cultured overnight, as described in Chapter 2, in the presence of 50μM of both $HEL_{105-120}$ and $OVA_{323-339}$. Following harvest and extensive washing, 10^7 peptide-pulsed splenocytes in 50μL Leibovitz media were injected into the footpad and lower leg of normal BALB/c mice on experimental days 0, 3, and 6. Nine days after the initial injection of pulsed splenocytes, the $HEL_{105-120}$ and $OVA_{323-339}$-specific cytokine producing cells in the draining popliteal lymph node (DLN) were enumerated by ELISpot assay. Each data point represents the number of specific ELISpot-forming cells in the popliteal lymph node of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. This is the result of a single experiment with three mice per group (n = 3).

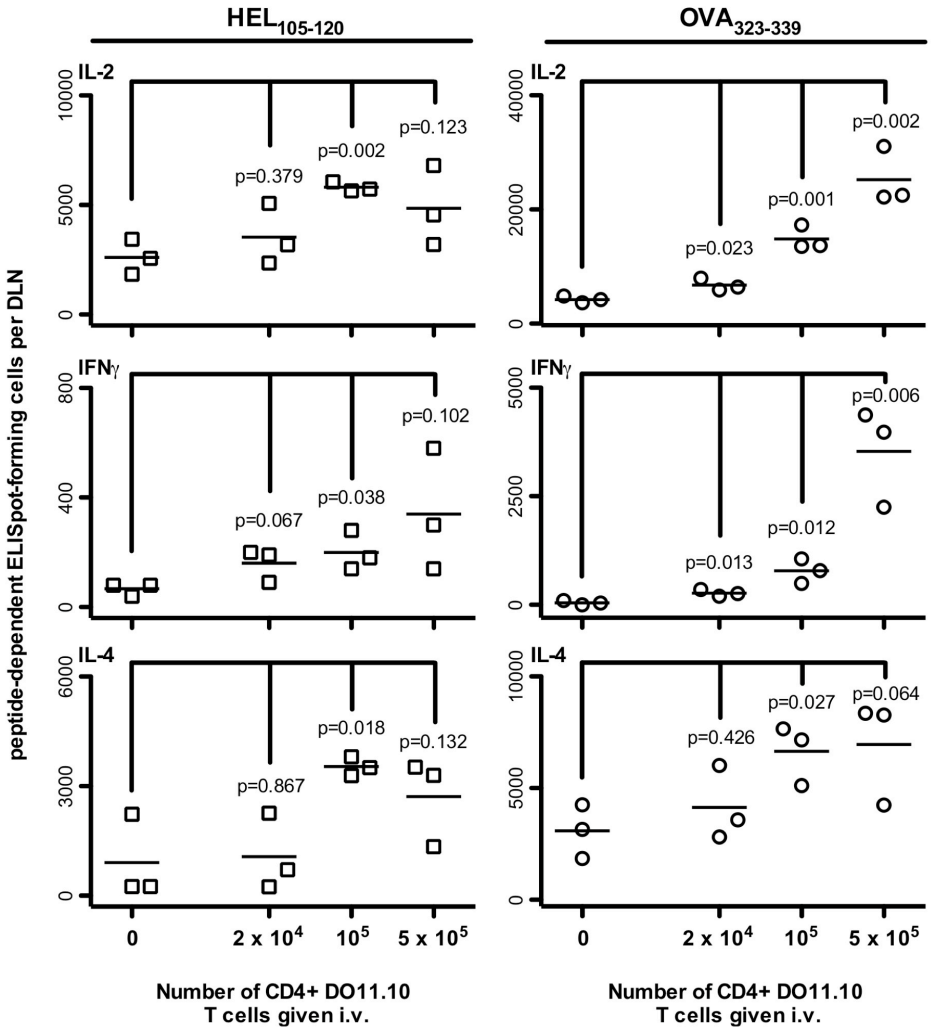

HEL105-120
OVA323-339
IL-2
IFNγ
IL-4
peptide-dependent ELISpot-forming cells per DLN
p=0.379
p=0.002
p=0.123
p=0.023
p=0.001
p=0.002
p=0.067
p=0.038
p=0.102
p=0.013
p=0.012
p=0.006
p=0.867
p=0.018
p=0.132
p=0.426
p=0.027
p=0.064
0
2 x 10^4
10^5
5 x 10^5
Number of CD4+ DO11.10
T cells given i.v.

Figure 4.2.7

Figure 4.2.8 *Administration of $HEL_{105-120}$ and $OVA_{323-339}$-pulsed splenocytes to DO11.10-seeded mice results in significant cooperative enhancement of $HEL_{105-120}$-specific effector CD4 T cell generation*. One day prior to injection of pulsed splenocytes, BALB/c mice were injected with 10^5 MACS purified CD4+ DO11.10+ T cells. BALB/c splenocytes were cultured overnight, as described in Chapter 2, in the presence of either 50μM $HEL_{105-120}$ alone, 50μM $OVA_{323-339}$ alone, or 50μM of both peptides. Following harvest and extensive washing, 10^7 of peptide-pulsed splenocytes in 50μL Leibovitz media were injected subcutaneously into the footpad and lower leg of BALB/c mice on days 0, 3, and 6. Nine days after the initial injection of pulsed splenocytes, the $HEL_{105-120}$ and $OVA_{323-339}$-specific cytokine producing cells in the spleens and draining popliteal lymph nodes (DLN) were enumerated by ELISpot in mice injected with only $HEL_{105-120}$-pulsed splenocytes (H), only $OVA_{323-339}$-pulsed splenocytes (O), and in mice injected with splenocytes pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$ (H+O). Each data point represents the number of specific ELISpot-forming cells in the popliteal lymph node of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. Results presented are pooled data from three to five independent experiments with three mice per group (n = 9-15).

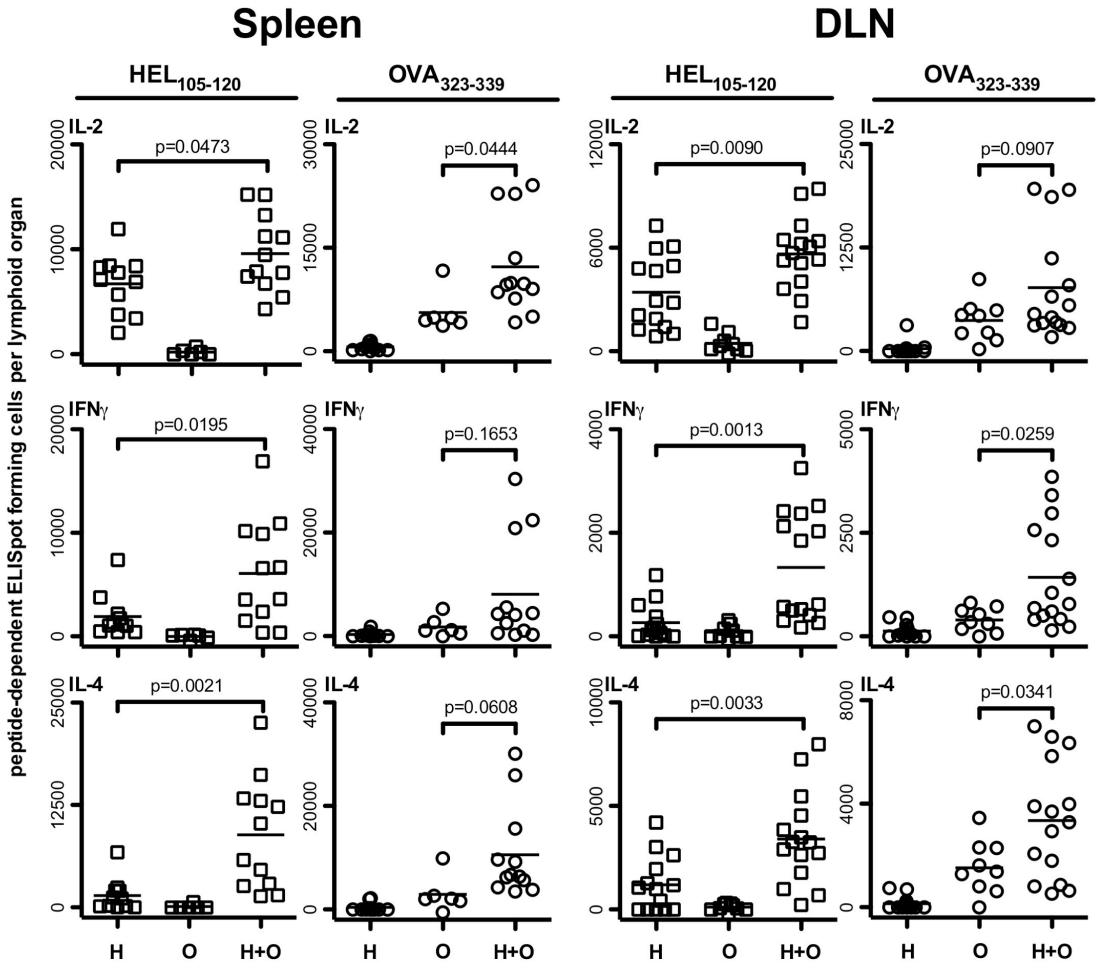

Spleen
DLN
HEL105-120
OVA323-339
peptide-dependent ELISpot forming cells per lymphoid organ
IL-2
IFNγ
IL-4
p=0.0473
p=0.0444
p=0.0090
p=0.0907
p=0.0195
p=0.1653
p=0.0013
p=0.0259
p=0.0021
p=0.0608
p=0.0033
p=0.0341
H
O
H+O

Figure 4.2.8

4.3 Cellular Mechanisms of CD4 T cell Cooperation

We confirmed that significant CD4 T cell cooperation can occur between two distinct peptide-specific populations when these peptides are presented in association with MHCII molecules on adoptively transferred APC. More-specifically, we demonstrated that $OVA_{323-339}$-specific CD4 T cells enhanced the frequency of $HEL_{105-120}$ specific CD4 T cells when both peptides are presented by APCs pulsed with peptide at the same time. However, the question remained as to whether this cooperation occurred between CD4 T cells activated by the same APC or whether antigen presentation by different APC within the same lymph node was sufficient to support T cell cooperation. The answer to this question has implications for the physiological function of CD4 T cell cooperation as outlined in section 1.3. If cooperation generally occurs between CD4 T cells activated by the same APC, then the peptides presented will normally be derived from antigens that are physically or functionally associated. It is easy to envisage situations where linkage between antigens occurs; linkage of peptide epitopes may result from their presence within the primary sequence of a single protein, a complex of proteins, or indeed an entire microorganism that is taken up and digested within a single APC, particularly if the process of antigen uptake is specific. If cooperation between CD4 T cells generally does not require presentation of two or more peptides on the same APC, then there is no requirement for linkage. The implication of finding that CD4 T cells cooperate via an unlinked mechanism would be that "bystander" interactions, occurring between T cells responding to unlinked interactions, would allow populations of CD4 T cells to influence one another, upon their simultaneous interaction within the same lymph node, for example. If this type of interaction was common, it would be difficult to envisage how the activation and phenotype of CD4 T cells could be exquisitely controlled. We therefore undertook experiments designed to determine whether CD4 T cell cooperation occurs through linked or bystander interactions between CD4 T cells.

Figure 4.3.1. *Cooperation between $HEL_{105-120}$ and $OVA_{323-339}$-specific CD4 T cells requires simultaneous presentation of both peptides by the same APC*. BALB/c mice were seeded with 10^5 MACS purified CD4+ DO11.10 T cells one day prior to injecting mice three times (as in Figure 4.2.8) with 10^7 splenocytes pulsed with either $HEL_{105-120}$ alone (H), $OVA_{323-339}$ alone (O), both $HEL_{105-120}$ and $OVA_{323-339}$ (H+O), or co-injection of 10^7 H and O splenocytes. The peptide-specific cytokine producing cells in the spleen and draining lymph nodes of these mice were enumerated by ELISpot on day nine after the first injection. Each data point represents the number of specific ELISpot-forming cells in the lymphoid organ of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. Data were pooled from two to three independent experiments with three mice per group (n = 6-9).

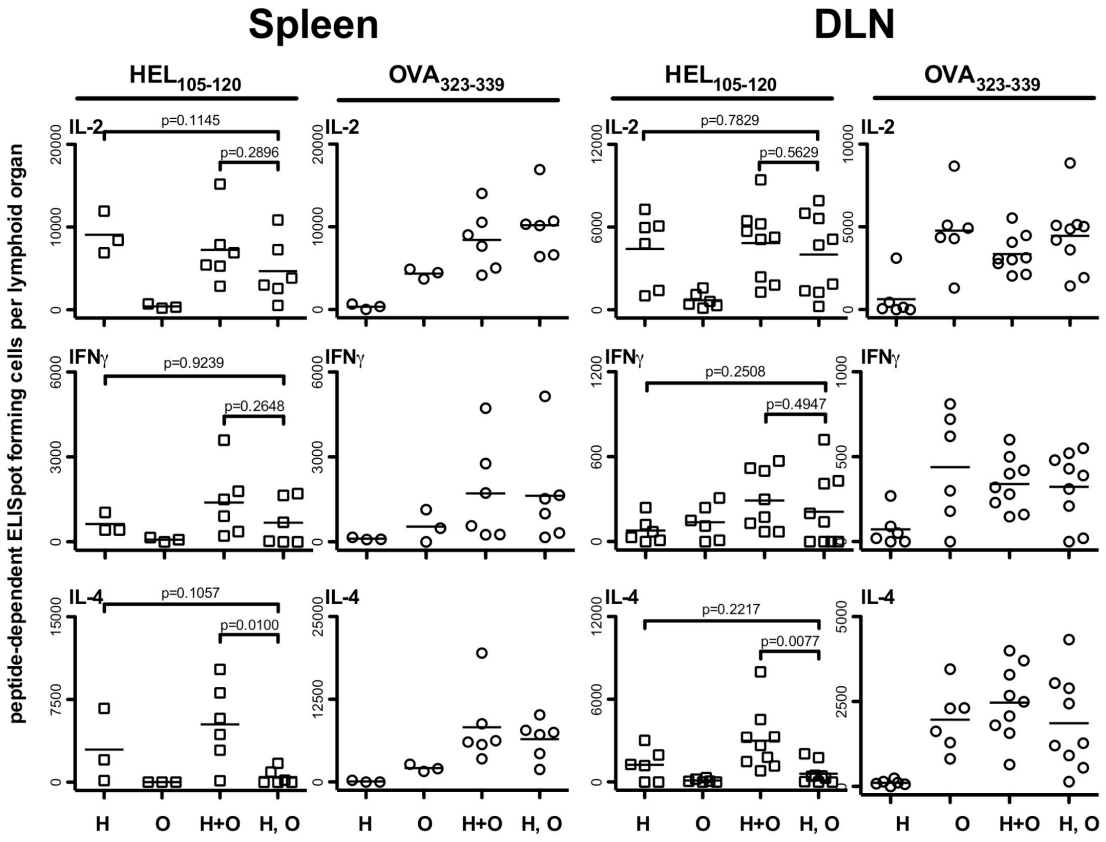

Spleen
DLN
HEL105-120
OVA323-339
peptide-dependent ELISpot forming cells per lymphoid organ
IL-2
IFNγ
IL-4
p=0.1145
p=0.2896
p=0.9239
p=0.2648
p=0.1057
p=0.0100
p=0.7829
p=0.5629
p=0.2508
p=0.4947
p=0.2217
p=0.0077
H
O
H+O
H, O

Figure 4.3.1

As can be seen from Figure 4.3.1, the cooperative generation of IL-4 producing effector CD4 T cells, observed upon administration of dual peptide-pulsed APC, requires presentation of $HEL_{105-120}$ and $OVA_{323-339}$ on the same APC. The generation of $HEL_{105-120}$-specific cytokine producing cells in mice given 10^7 APC pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$-pulsed was compared to mice co-injected with 10^7 $HEL_{105-120}$-pulsed APCs and 10^7 $OVA_{323-339}$-pulsed APCs. The number of total APCs injected was changed to ensure that the number of specific peptide-MHCII complexes available was not different between groups. No cooperative effect was evident in mice given APC pulsed separately with single peptides. The level of $HEL_{105-120}$-specific cells generated in mice given APC loaded with $HEL_{105-120}$ alone was not different from the number generated in mice given APC pulsed with $HEL_{105-120}$ and $OVA_{323-339}$ separately. Thus, we can conclude that CD4 T cell cooperation normally occurs primarily through linked recognition of peptides.

We next investigated the nature of the APC that mediates T cell cooperation. Because the spleen contains many different potential APC types, a fundamental question that arose from our findings was: Is CD4 T cell cooperation mediated by a specific type of APC? If so, what APC is it? Differences in the physiological roles for different types of APC are a function of their differential uptake of antigen, their presence in different physiological niches, and differential expression and timing of processing and presentation machinery and co-stimulatory molecules. To put it simply, different APC are expected to have different roles in the activation of CD4 T cells because they are different. Alternatively, there could be no specific APC requirement for cooperation between CD4 T cells, in which case all populations of APC should mediate cooperative enhancement of CD4 effector T cells equally well. We therefore attempted to determine whether a certain APC type, contained within our pulsed spleen population, preferentially mediated cooperation amongst CD4 T cells.

Dendritic cells, in particular conventional DC and plasmacytoid DC, are seen as the most potent activators of naïve T cells. Both of these cell types are found within the spleen (Villadangos and Schnorrer 2007). In order to determine whether splenic DCs have a role in mediating cooperation between CD4 T cells, we selectively isolated them from cultured, pulsed, splenocytes and assessed the generation of $HEL_{105-120}$-specific CD4 T cells, following injection, when these DC were pulsed with $HEL_{105-120}$ alone or with

both $HEL_{105-120}$ and $OVA_{323-339}$. Figure 4.3.2 displays data pooled from three such experiments. While the splenocytes remaining, after DC isolation by MACS, appear to have retained the ability to mediate cooperation, DC enriched APC appear unable to mediate enhancement of the activation of $HEL_{105-120}$-specific CD4 T cells when pulsed with both peptides. It is clear that the DC enriched APC, given in proportion to the numbers isolated by MACS from the total population of splenocytes, elicit substantial $HEL_{105-120}$ and $OVA_{323-339}$-specific effector CD4 T cell generation. However, even when the numbers of DC enriched APC were increased five-fold, enhancement in the generation of $HEL_{105-120}$-specific cytokine producing cells, by cooperative interaction, was not observed.

Our analysis of the cell surface phenotype of DC-enriched APC was hindered by the presence of cell debris in the isolated populations. A large proportion of these cells bear CD11c and MHCII (Figure 4.3.3). Cells that expressed MHCII, but not CD11c were observed. Our protocol allowed for selection of splenic plasmacytoid DC. We assume that at least some of the MHCII expressing, $CD11c^-$ cells were plasmacyotid DC as virtually none of the cells isolated expressed CD19 indicating that there were few contaminating B cells. Given that these cells efficiently activated CD4 T cells even when given at levels nearly 100-fold lower than pulsed-splenocyte APC, we believe that the majority of these cells were splenic DC. From these findings we conclude that while DC potently activate CD4 T cells but appear unable to mediate CD4 T cell cooperation.

B cells are a major population of APC in the spleen. These cells are at least ten-fold more prevalent than DC in our pulsed spleen preparations. Their numbers, and their well-described ability to present antigen to CD4 T cells, made it likely that B cells were activators of T cells in our experimental system. We tested whether purified B cells could mediate cooperation between CD4 T cells. After overnight pulsing with $HEL_{105-120}$ alone or with $HEL_{105-120}$ and $OVA_{323-339}$ together, B cells were purified from the remaining splenocytes by negative selection. The resulting untouched B cells, pulsed with one peptide or two, were injected into the footpad of mice seeded with 10^5 CD4+ DO11.10 cells, as per our standard protocol. On the ninth day after the initial injection, we enumerated the peptide-specific cytokine producing cells in the draining lymph nodes and spleens of injected mice.

Figure 4.3.2 *Enriched splenic DCs do not mediate cooperation between $HEL_{105-120}$ and $OVA_{323-339}$-specific CD4 T cell populations*. BALB/c mice were seeded with 10^5 MACS purified CD4+ DO11.10 T cells one day prior to injecting splenocytes cultured as described in Chapter two in the presence of 50μM $HEL_{105-120}$ alone or in the presence of both 50μM $HEL_{105-120}$ and 50μM $OVA_{323-339}$. After harvesting and washing, splenic DC populations were isolated by positive selection using pan-DC-specific MACS beads. Selection resulted in the isolation of roughly 2% of the pulsed splenocyte population. Both DC depleted and DC enriched (DC+) peptide-pulsed cells were injected in proportion to the standard injection of 10^7 (10^7 and $2x10^5$ cells respectively). In one experiment the number of DC enriched cells was increased five-fold (10^6 DC+). These injections were given on days 0, 3, and 6. On day 9 after the first injection the $HEL_{105-120}$ and $OVA_{323-339}$-specific cytokine-producing cells in the spleens, and draining popliteal lymph nodes (DLN), were enumerated by ELISpot, in mice given $HEL_{105-120}$-pulsed APC (H), or $HEL_{105-120}$ and $OVA_{323-339}$-pulsed APC (H+O). Each data point represents the number of specific ELISpot-forming cells in the lymphoid organ of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. The data presented are pooled values from one to three independent experiments with three mice per group (n = 3-9).

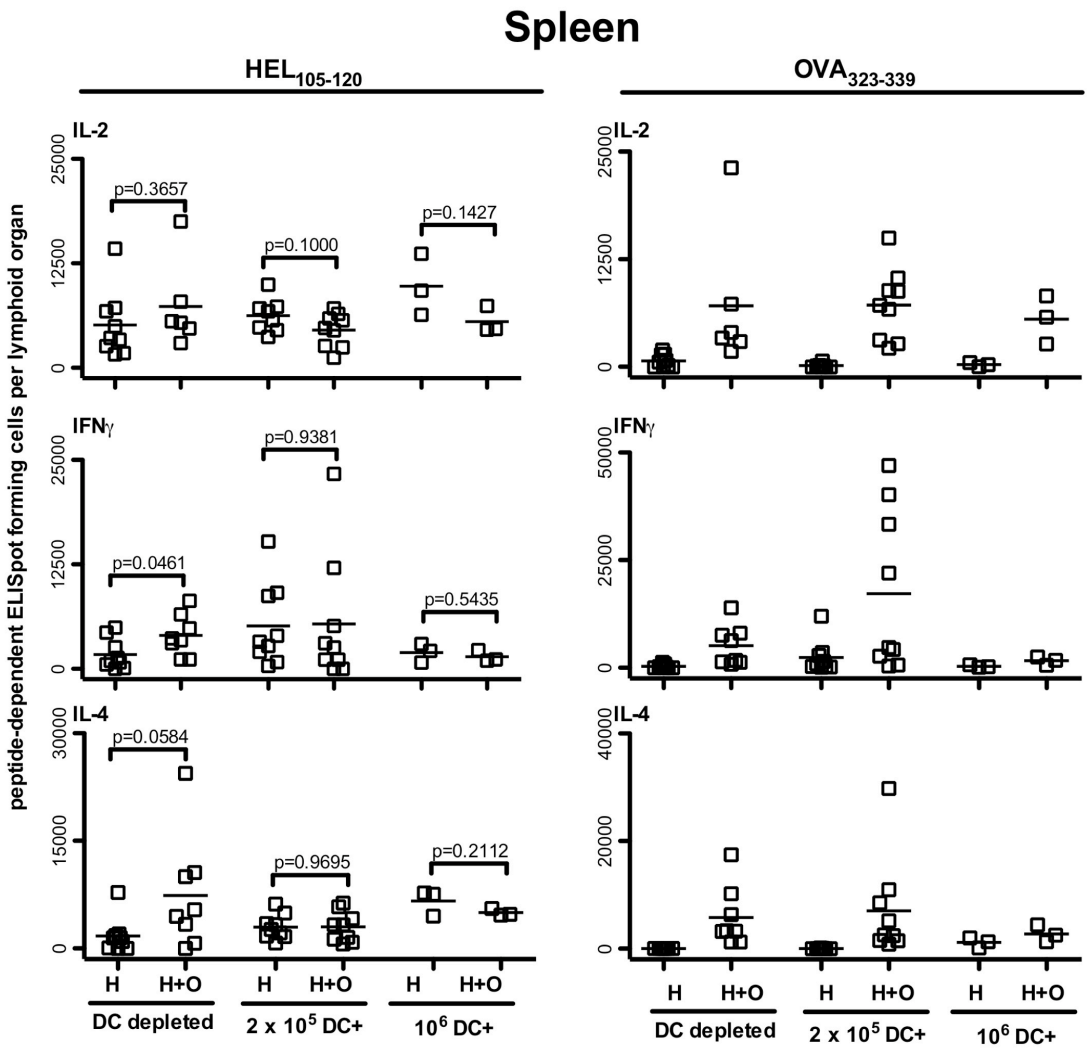

Spleen
HEL105-120
OVA323-339
IL-2
IFNγ
IL-4
peptide-dependent ELISpot forming cells per lymphoid organ
p=0.3657
p=0.1000
p=0.1427
p=0.0461
p=0.9381
p=0.5435
p=0.0584
p=0.9695
p=0.2112
H
H+O
DC depleted
2 x 10^5 DC+
10^6 DC+

Figure 4.3.2a

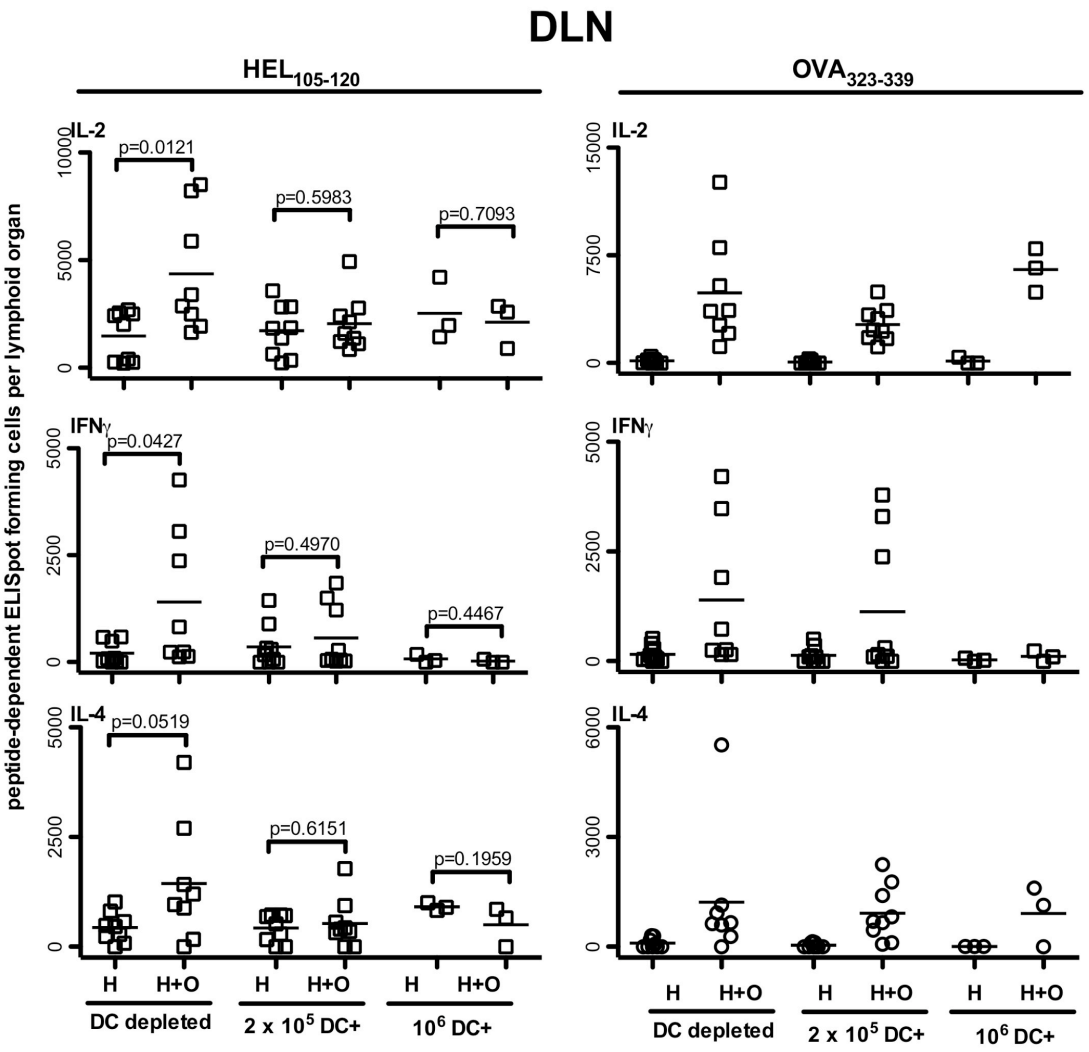

DLN
HEL105-120
OVA323-339
peptide-dependent ELISpot forming cells per lymphoid organ
IL-2
IFNγ
IL-4
p=0.0121
p=0.5983
p=0.7093
p=0.0427
p=0.4970
p=0.4467
p=0.0519
p=0.6151
p=0.1959
H
H+O
DC depleted
2 x 10^5 DC+
10^6 DC+

Figure 4.3.2b

Figure 4.3.3 *Surface phenotype of the DC-enriched population isolated from cultured splenocytes*. Splenocytes were cultured and DCs isolated by MACS as in Figure 4.3.2. DC enriched cells were then separated from cell debris by centrifugation over a Ficol-paque gradient. The remaining cells were stained with fluorophore-conjugated antibodies specific for the indicated cell surface markers. Assessment of the expression of these markers by flow-cytometry on DC-enriched cells is shown. Density plots gated on events that had forward and side scatter properties of cells are shown. Percentages of cells falling within the quadrant out of the total number of cells within the given plot are shown. Data presented are representative of results from two independent experiments.

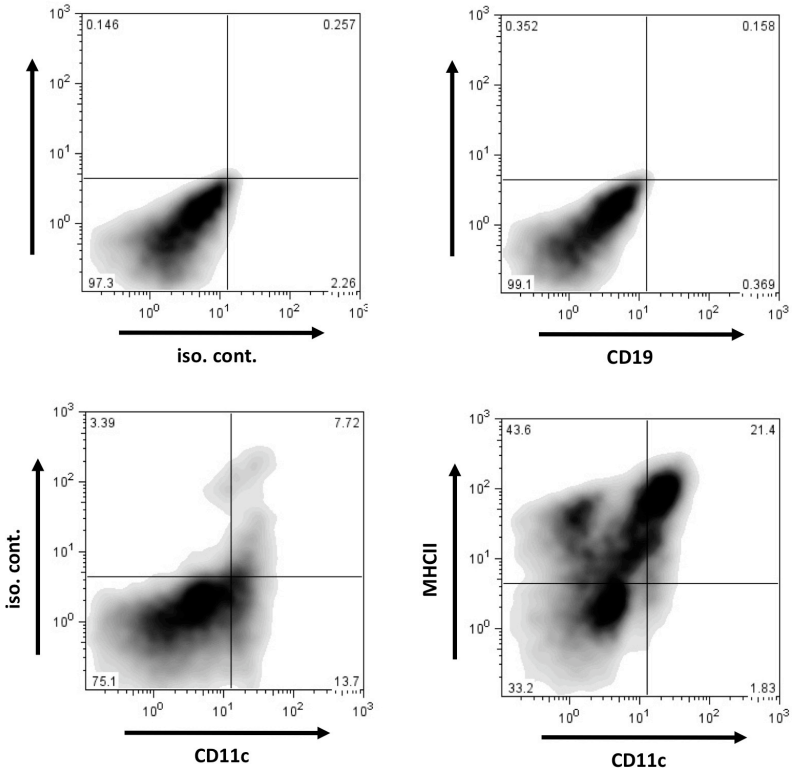

0.146
0.257
97.3
2.26
iso. cont.
0.352
0.158
99.1
0.369
CD19
3.39
7.72
75.1
13.7
iso. cont.
CD11c
43.6
21.4
33.2
1.83
MHCII
CD11c
Log Fluorescence

Figure 4.3.3

We observed clear cooperative effects in the generation of $HEL_{105-120}$-specific IFNγ and IL-4 producing CD4 T cells between mice given B cells pulsed with $HEL_{105-120}$ alone and those given B cells pulsed with $HEL_{105-120}$ and $OVA_{323-339}$ (Figure 4.3.4). Subsequently, we administered B cells pulsed with $OVA_{323-339}$ alone to assess whether, when B cells were the sole APC presenting peptide, cooperative responses could be observed in the generation of $OVA_{323-339}$-specific effector cells. Consistent with our previous findings, cooperation was not observed in the generation of $OVA_{323-339}$-specific responses. We determined that the B cells employed in these studies were at least 95% pure and expressed both MHCII and CD86 (Figure 4.3.5). We conclude from these findings that B cells are able to mediate cooperation between CD4 T cells.

Our findings here indicate that CD4 T cell cooperation is mediated primarily by APC presenting peptides in a linked fashion and that these APC are normally B cells. These findings are consistent with what is known about the antigen-presenting role of B cells. Taken as a whole, these findings have important implications for the physiology of CD4 T cell cooperation. If our findings reflect are biologically relevant, they suggest that cooperative responses generally occur between CD4 T cells that recognize peptides bound to MHCII molecule on the same B cell. Since B cells are antigen-specific APC, mainly acquiring protein antigen by way of the BCR, CD4 T cell cooperation should normally occur between CD4 T cells specific for peptides that are derived from physically linked foreign proteins. The implications of this in the generation of normal and of abnormal immune responses will be discussed further in Chapters 5 and 6.

Figure 4.3.4 *Purified B cells mediate cooperation between $HEL_{105-120}$ and $OVA_{323-339}$-specific CD4 T cells*. BALB/c mice were injected with 10^5 MACS purified CD4+ DO11.10 T cells one day prior to injection. Splenocytes from BALB/c mice were cultured as described in Chapter two in the presence of 50μM $HEL_{105-120}$ alone or in the presence of both 50μM $HEL_{105-120}$ and 50μM $OVA_{323-339}$. After harvesting and washing, the splenic B cells were isolated by negative selection with the MACS B cell isolation kit. Selection resulted in the isolation of roughly 35% of the pulsed splenocytes. B cells were injected in proportion to the standard injection of 10^7, with 3.5 x 10^6 per mouse. On day 9 after the first injection the $HEL_{105-120}$ and $OVA_{323-339}$-specific cytokine-producing cells in the spleens, and draining popliteal lymph nodes (DLN), were enumerated by ELISpot, in mice given $HEL_{105-120}$-pulsed B cells (H), or $HEL_{105-120}$ and $OVA_{323-339}$-pulsed B cells (H+O). Each data point represents the number of specific ELISpot-forming cells in the lymphoid organ of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. Data presented were pooled from one to three independent experiments with three mice per group (n = 3-9).

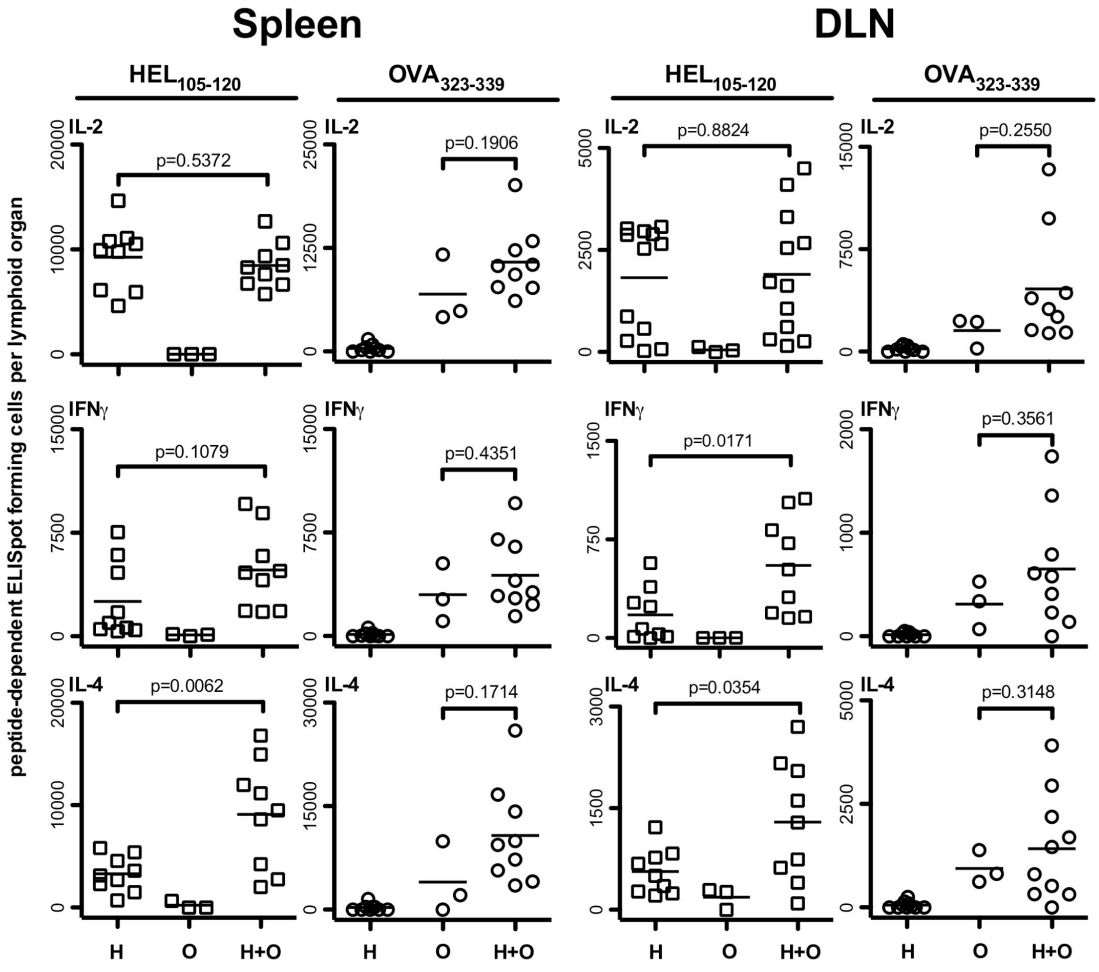

Spleen
DLN
HEL105-120
OVA323-339
HEL105-120
OVA323-339
peptide-dependent ELISpot forming cells per lymphoid organ
IL-2
p=0.5372
IL-2
p=0.1906
IL-2
p=0.8824
IL-2
p=0.2550
IFNγ
p=0.1079
IFNγ
p=0.4351
IFNγ
p=0.0171
IFNγ
p=0.3561
IL-4
p=0.0062
IL-4
p=0.1714
IL-4
p=0.0354
IL-4
p=0.3148
H
O
H+O

Figure 4.3.4

Figure 4.3.5 *Phenotype of MACS purified, peptide pulsed, B cells*. Splenocytes were cultured and the B cells isolated as in Figure 4.3.4. B cells were then stained with fluorophore-conjugated antibodies specific for the indicated cell surface markers. Grey, dotted, histograms show the level of fluorescence of cells stained with a matched fluorophore-conjugated isotype-matched control antibody. Proportions (% of total) of cells falling within the indicated gates are shown. Data presented are representative of results from three independent experiments.

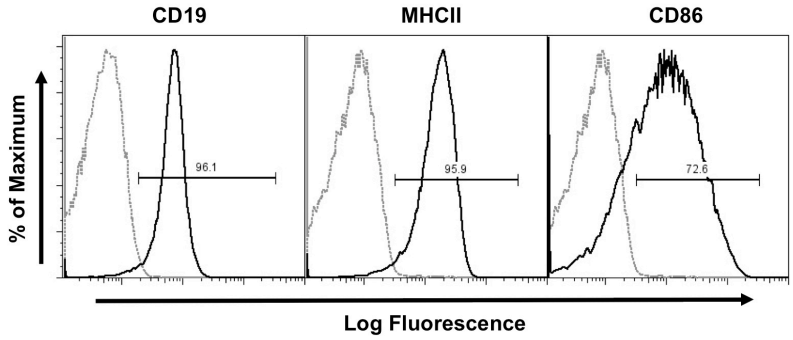


Figure 4.3.5

4.4 Molecular Mechanisms of CD4 T cell Cooperation

Our data supported the conclusion that B cells are the APC that normally mediate cooperation between CD4 T cells *in vivo*. We decided to investigate the molecular interactions involved in this B cell-mediated cooperation. Because cooperation was found to occur only when $HEL_{105-120}$ and $OVA_{323-339}$ were presented on the same APC we hypothesized that the cooperation between CD4 T cells involved antigen-specific activation of B cells by CD4 T cells, leading to their enhanced ability to activate other CD4 T cells. Classically, CD40L (CD154), borne by activated CD4 T cells is known to potently activate B cells (Croft, Bradley et al. 1994; Jaiswal, Dubey et al. 1996; Croft, Joseph et al. 1997; Evans, Munks et al. 2000) and increase their ability to present antigen to CD4 T cells. Moreover, CD40 stimulation of other APC, such as DC, has been shown to license these cells to more potently activate CD8 T cells (Ridge, Di Rosa et al. 1998; Schoenberger, Toes et al. 1998). We hypothesized that CD40-CD40L interactions could be also be involved in CD4 T cell cooperation.

To address whether CD40 expressed by B cells was involved in cooperation between CD4 T cell populations, we investigated whether the need for $OVA_{323-339}$-specific CD4 T cell activation, in the generation of optimal $HEL_{105-120}$-specific effector CD4 T cells, could be replaced by treatment of the B cells with an agonistic antibody to CD40 (FGK45). We therefore pulsed MACS-purified B cells with $HEL_{105-120}$ in the presence of 1ng/mL FGK45. The results of a single experiment of this type are shown in Figure 4.4.1. We found that while the generation of $HEL_{105-120}$-specific cytokine producing cells in mice given B cells pulsed with $HEL_{105-120}$ in the presence of FGK45 was not as potent as when mice were given B cells pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$, there was nevertheless a significant increase in the numbers of $HEL_{105-120}$-specific effector CD4 T cells generated when compared with mice given B cells pulsed with $HEL_{105-120}$ alone. These results suggested that CD40 stimulation may play a role in CD4 T cell cooperation, and therefore we chose to pursue this further. Curiously, the inclusion of an antibody known to aggregate the BCR appeared to reverse the effect of FGK45. Despite the low numbers of mice, this finding is interesting and, in retrospect, may be due to inactivation of B cells by this antibody. We did not further address this possibility.

Figure 4.4.1 *Peptide-pulsed B cells, activated in vitro by anti-CD40 antibody, elicit increased numbers of $HEL_{105-120}$-specific effector CD4 T cells than B cells not activated with anti-CD40.* MACS purified B cells were cultured overnight in the presence of either 50μM $HEL_{105-120}$ alone, 50μM each of $HEL_{105-120}$ and $OVA_{323-339}$, 50μM $HEL_{105-120}$ with 1ng/mL FGK45 (agonist antibody to CD40), or 50μM $HEL_{105-120}$ with 1ng/mL FGK45 and 1μg/mL soluble anti-immunoglobulin antibody. After harvest and extensive washing, BALB/c mice, seeded with 10^5 CD4+ DO11.10 T cells, were injected with 3.5 x 10^6 cultured B cells on days 0, 3, and 6, subcutaneously in the footpad and lower leg. On day 9 after the first injection, the $HEL_{105-120}$ and $OVA_{323-339}$-specific cytokine-producing cells in the spleens were enumerated by ELISpot assays, in mice injected with either $HEL_{105-120}$-pulsed B cells (H), $HEL_{105-120}$ and $OVA_{323-339}$-pulsed B cells (H+O), $HEL_{105-120}$ with FGK45 pulsed B cells (H + αCD40), or $HEL_{105-120}$ with FGK45 and anti-immunoglobulin (H+ αCD40+ αIg). Each data point represents the number of specific ELISpot-forming cells in the lymphoid organ of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. These are the results of a single experiment with three mice per group (n = 3).

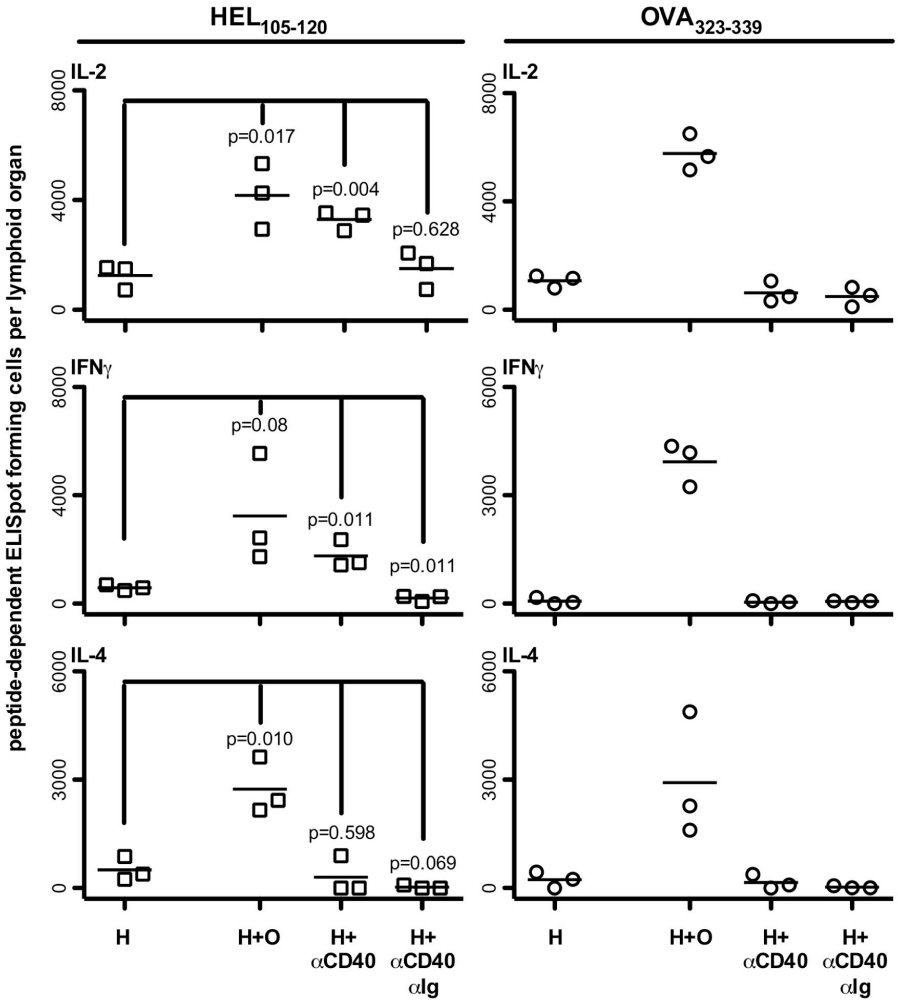

HEL105-120
OVA323-339
IL-2
IFNγ
IL-4
peptide-dependent ELISpot forming cells per lymphoid organ
p=0.017
p=0.004
p=0.628
p=0.08
p=0.011
p=0.011
p=0.010
p=0.598
p=0.069
H
H+O
H+ αCD40
H+ αCD40 αIg

Figure 4.4.1

To determine whether CD40 stimulation could replace the need for $OVA_{323-339}$-specific T cells for the enhanced generation of $HEL_{105-120}$-specific effector CD4 T cells, we decided to undertake the following experiment. APC were cultured overnight in the presence of MACS purified CD4 T cells from WT, as a negative control, or DO11.10 transgenic mice, and in the presence of $OVA_{323-339}$. We hypothesized that these culture conditions would result in the antigen-specific activation of DO11.10 CD4 T cells, leading to the corresponding activation of the B cells presenting $OVA_{323-339}$. In identical parallel cultures, we stimulated B cells with 10μg/mL FGK45 (a concentration commonly employed to activate B cells *in vitro* (Godfrey, Fagnoni et al. 1994)). As a negative control, APC were cultured in the presence of an isotype-matched control antibody. After an initial 24 hours of culture, the splenocytes from these cultures were harvested, washed briefly, and re-suspended in fresh media containing 50μM $HEL_{105-120}$. After overnight pulsing, the B cells from these cultures were purified by negative selection using MACS and were assessed for their activation status by flow cytometry. Figure 4.4.2 shows the activated phenotype of B cells cultured with DO11.10 CD4 T cells and $OVA_{323-339}$ and of B cells cultured with FGK45. These cells express increased levels of CD86 and have increased cell size compared to their corresponding isotype control mAb-treated B cells. Interestingly, B cells activated by specific CD4 T cells and those activated by FGK45 appear very similar in their expression of CD86 and cell size.

We next assessed whether $HEL_{105-120}$-pulsed, purified, B cells, activated in culture as above, enhance the generation of HEL_{105}-specific effector cells upon adoptive transfer. We observed significant increases in $HEL_{105-120}$-specific IL-4 producing effector CD4 T cell generation in mice that were injected with B cells primed with FGK45 compared to mice given B cells treated with control antibody (Figure 4.4.3). We did not observe significant increases in IL-4 producing $HEL_{105-120}$-specific CD4 T cells in mice given B cells activated in vitro by DO11.10 T cells. There was, however, a trend in the same direction as was observed in mice given FGK45-activated B cells. It appears that CD4 T cell cooperation can be partially replaced by CD40 ligation on B cells. We therefore concluded that the CD40-CD40L interaction may contribute to cooperation between populations of CD4 T cells.

Figure 4.4.2 *Phenotype of purified B cells isolated from peptide-pulsing cultures under activating conditions*. MACS-purified B cells were cultured for 24 hours under the conditions shown at a final density of 10^6/well in a 96-well V-bottom plate. These cultures contained either 10^5 MACS purified wild-type CD4 T cells with 1μM $OVA_{323-339}$, 10^5 DO11.10 CD4 T cells with 1μM $OVA_{323-339}$, 10μg/mL of the isotype-matched control antibody 2A3, or 10μg/mL FGK45. All cells were harvested washed and re-plated in 3mL fresh media with 50μM HEL and cultured overnight in 60mm tissue culture dishes. After this overnight culture B cells were isolated by MACS negative selection and stained for the surface markers indicated with fluorophore-conjugated antibodies. Assessment, by flow-cytometry, of the proportion of cells that express these markers of the total population of isolated cells is shown. Grey, dotted, histograms show the level of fluorescence of cells stained with a matched fluorophore-conjugated isotype-matched control antibody. Data presented are representative of values from two independent experiments.

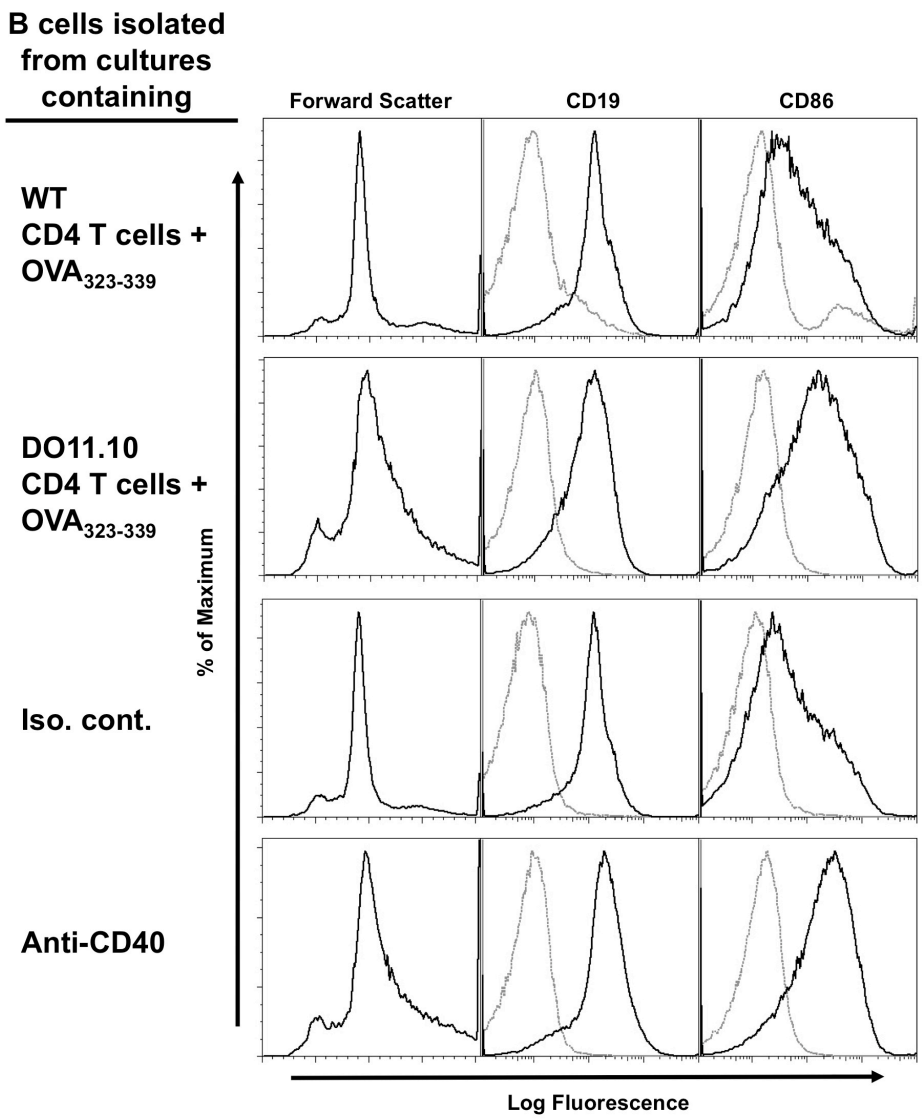

B cells isolated from cultures containing
Forward Scatter
CD19
CD86
WT CD4 T cells + OVA323-339
DO11.10 CD4 T cells + OVA323-339
Iso. cont.
Anti-CD40
% of Maximum
Log Fluorescence

Figure 4.4.2

Figure 4.4.3 *Peptide-pulsed B cells, activated by anti-CD40 antibody in vitro, elicit increased numbers of $HEL_{105-120}$-specific effector CD4 T cells than B cells incubated with isotype-control antibody*. Total spleen cells were cultured for 24 hours at a final density of 10^6/well in a 96-well V-bottom plate. Splencoytes were co-cultured with either 10^5 MACS purified WT CD4 T cells with 1μM $OVA_{323-339}$, 10^5 DO11.10 CD4 T cells with 1μM $OVA_{323-339}$, 10μg/mL of the isotype-matched control antibody 2A3, or 10μg/mL FGK45. All cells were harvested washed and re-plated in 3mL fresh media with 50μM HEL and cultured overnight in 60mm tissue culture dishes. After this overnight culture B cells were isolated by MACS negative selection and 3.5 x 10^6 cells were injected subcutaneously in the footpad and lower leg of each BALB/c mouse,. This procedure was repeated twice more such that injections were given on days 0, 3, and 6. On day 9 after the first injection, the $HEL_{105-120}$-specific cytokine-producing cells in the spleens and draining lymph nodes (DLN) were enumerated by ELISpot, in mice given B cells cultured with WT CD4 T cells (WT), B cells cultured with DO11.10 CD4 T cells (DO11.10). B cells cultured with control antibody (iso. cont.), and B cells cultured with FGK45 (FGK45). Each data point represents the number of specific ELISpot-forming cells in the lymphoid organ of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. Data presented are values pooled from two independent experiments with three mice per group (n = 6).

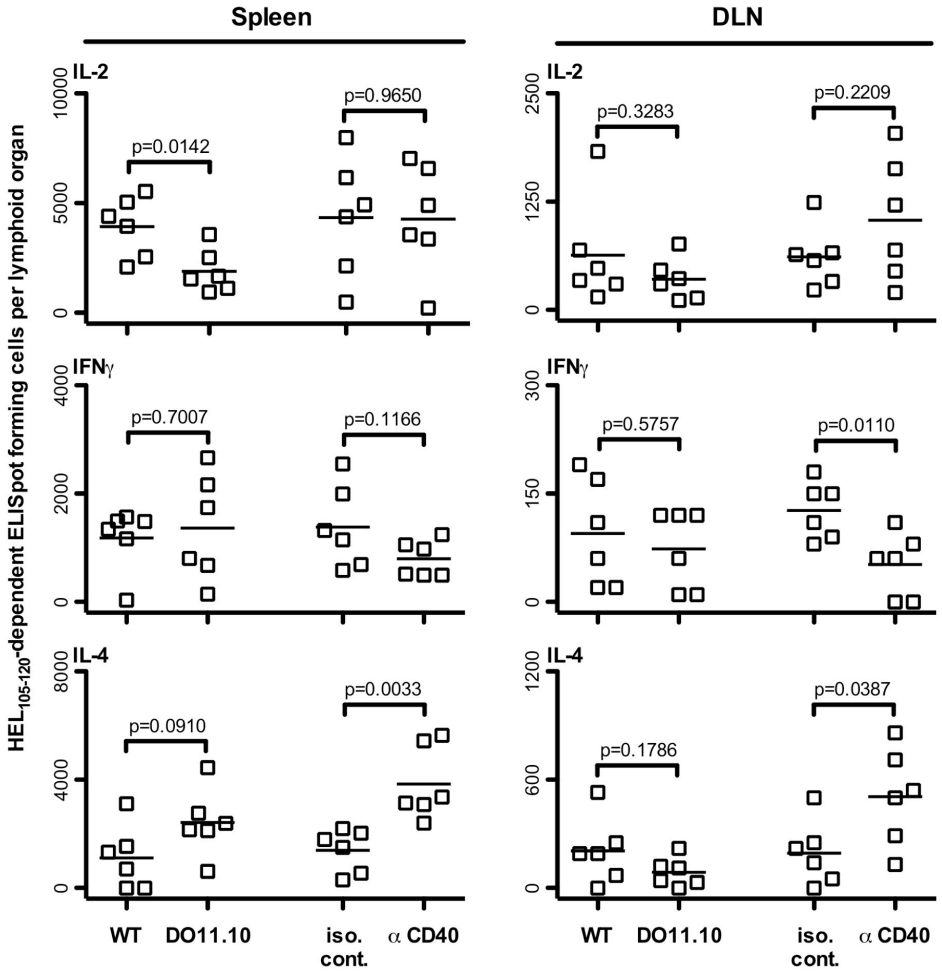
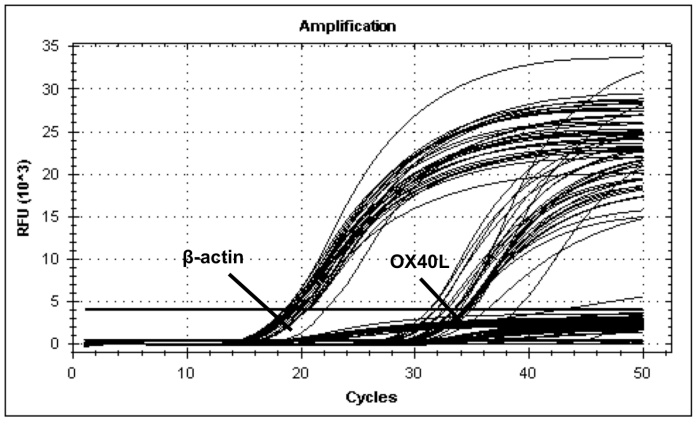

Spleen
DLN
IL-2
IFNγ
IL-4
p=0.0142
p=0.9650
p=0.3283
p=0.2209
p=0.7007
p=0.1166
p=0.5757
p=0.0110
p=0.0910
p=0.0033
p=0.1786
p=0.0387
HEL105-120-dependent ELISpot forming cells per lymphoid organ
WT
DO11.10
iso. cont.
α CD40

Figure 4.4.3

In our previous studies we identified the OX40-OX40L interaction as critical in cooperative responses between CD4 T cells. Thus it was logical to investigate whether this interaction also played a role in CD4 T cell cooperation mediated by pulsed B cells. Because we were able to replace the need for $OVA_{323-339}$-specific CD4 T cell activation for the enhanced generation of $HEL_{105-120}$-specific effector CD4 T cells with FGK45, we investigated whether this interaction induced the expression of OX40L in B cells. Examination of B cells, activated in culture, for OX40L protein expression was not successful. B cell staining with antibodies specific for OX40L was not detectable by either flow cytometry or western blots when using the same monoclonal antibody clone. However, it was not clear whether these techniques were not sufficiently sensitive to detect OX40L on pulsed B cells. We therefore examined the expression of OX40L mRNA in activated B cell cultures using quantitative real time polymerase chain reaction (qRTPCR). Figure 4.4.4. presents the results of the qRT-PCR experiment. It is clear that B cells, when cultured using the experimental conditions examined, expressed equivalent amounts of OX40L transcripts. This finding, while somewhat surprising, indicated to us that perhaps preferential priming of CD4 T cells, by B cells, which appear to express OX40L at similar levels regardless of their state of activation, could be a general mechanism of CD4 T cell cooperation. Alternatively, the culture conditions employed in our experiment may have influenced the expression of OX40L mRNA in our B cells. Moreover, we cannot rule out the possibility that post-transcriptional regulation of OX40L message occurred in these B cells. We attempted to determine whether OX40L expression is induced on APC presenting $OVA_{323-339}$ to DO11.10 CD4 T cells *in vivo*. Total splenocytes were pulsed with $HEL_{105-120}$ alone or with $HEL_{105-120}$ and $OVA_{323-339}$ together, labeled with CFSE, and injected into the footpad of mice seeded with 10^5 DO11.10 CD4 T cells. 48 hours after this injection, the DLN of three individual mice per group were pooled and the CFSE+ cells from these lymph nodes were sorted with a fluorescence activated cell sorter (FACS). Figure 4.4.5A demonstrates that the CFSE+ T cells were purified from approximately 0.3% of the total lymph node, to over 40% purity. A very small number, 10^4, of CFSE+ cells were isolated and thus yielded small amounts of mRNA. We performed qRTPCR on samples of cDNA derived from 10^4 CFSE+ cells.

Figure 4.4.4 *Expression of OX40L mRNA in B cells activated by CD4 T cells or anti CD40 agonistic antibody*. Total spleen cells were cultured for 24 hours at a final density of 10^6/well in a 96-well V-bottom plate. These cultures contained either 10^5 MACS purified WT CD4 T cells with 1μM $OVA_{323-339}$, 10^5 DO11.10 CD4 T cells with 1μM $OVA_{323-339}$, 10μg/mL of the isotype-matched control antibody 2A3, or 10μg/mL FGK45. All cells were harvested washed and re-plated in 3mL fresh media with 50μM HEL and cultured overnight in 60mm tissue culture dishes. After this overnight culture B cells were isolated by MACS negative selection and the mRNA was isolated by passage over QIAGEN RNAeasy spin columns. Copy DNA was prepared, employing the BioRad iScript kit. Sequences corresponding to roughly 400bp of the primary sequence of mouse β-actin, and mouse OX40L were amplified in triplicate in the presence of EvaGreen dye to allow detection of amplified DNA. (A) Raw data from qRTPCR run showing observed Ct in all wells. β-actin and OX40L cDNA-dependent fluorescence clustered tightly, as indicated. (B) Relative gene expression of OX40L, based on ΔΔCt and normalized to control treated B cells is shown.

A



B

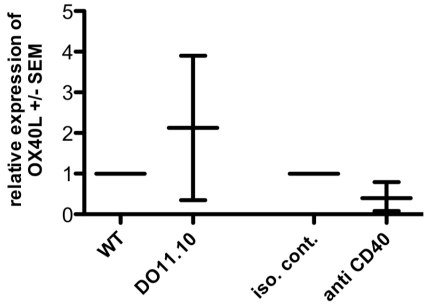


Figure 4.4.4

We were able to confirm the expression of β-actin as a positive control, however OX40L mRNA expression in these cells was detected at very high Ct values (Figure 4.4.5B). In some cases, the signal from template-negative control wells, a result of primer dimer formation, was higher than in experimental wells indicating that the levels of OX40L cDNA in our samples was exceedingly low. Moreover, the quantity of cDNA generated was very small, precluding extensive analysis. Because of the complexity of this type of experiment, we did not further pursue these findings. It is possible that at the time point investigated OX40L expression had not yet been induced on APC. Though these experiments were possibly complicated by the tight regulation of expression of OX40L, this type of experiment may be a viable way to analyze the phenotype of adoptively transferred APC in the future. In any case, we sought to obtain functional data on the role of OX40L in our system when peptides were presented only by pulsed, purified B cells.

With some evidence that OX40L was expressed by B cells, and that ligation of CD40 on these B cells is sufficient to allow for increased generation of $HEL_{105-120}$-specific effector cells when pulsed B cells are adoptively transferred, we sought functional evidence that OX40-OX40L interactions were involved in cooperation between populations of CD4 T cells. We first confirmed that stimulation with a monoclonal antibody specific for OX40 could enhance the generation of peptide-specific effector CD4 generation when peptides were presented by pulsed APC. As shown in Figure 4.4.6, administration of the OX40 agonistic antibody OX86 potently and significantly increased the generation of peptide-specific cytokine producing cells in mice injected with peptide-pulsed APC in. It was clear that ligation of OX40 increased the generation of these cells substantially above the levels seen in control antibody-treated mice. We observed decreases in the numbers of effector cells in the DLN, possibly due to efflux of highly activated CD4 T cells. Thus, it appears that signaling through OX40 is not saturated during CD4 T cell cooperation.

We next performed an experiment to address whether OX40-OX40L interactions are critically involved in the generation of cooperative responses between CD4 T cells when B cells present antigen. We duplicated our experiments employing $HEL_{105-120}$-pulsed or $HEL_{105-120}$ and $OVA_{323-339}$-pulsed MACS purified B cells in the presence or absence of a blocking antibody for OX40L.

Figure 4.4.5. *Fluorescence activated cell sorting of peptide-pulsed APC 48 hours after injection and assessment of OX40L mRNA expression*. Splenocytes were cultured overnight in the presence of either 50μM $HEL_{105-120}$ alone or 50μM $OVA_{323-339}$ and $HEL_{105-120}$. After washing, the remaining cells from each culture were labeled with CFSE and 10^7 were injected subcutaneously into the hind footpad of three Balb/c mice that were seeded, one day before, with 10^5 DO11.10 CD4 T cells. 48h following this injection, the draining popliteal lymph nodes, from mice of each group, were harvested and pooled. These lymph node cells were then sorted by a fluorescence activated cell sorter based on CFSE. (A) Pre- and post-sort proportions of CFSE+ cells from lymph nodes. (B) After cell sorting, mRNA was isolated from CFSE+ enriched populations by passage over QIAGEN RNAeasy spin columns. cDNA libraries were prepared, employing the BioRad iScript kit. Sequences corresponding to roughly 400bp of the primary sequence of mouse β-actin, and mouse OX40L were amplified in triplicate the presence of EvaGreen dye to allow detection of amplified DNA.

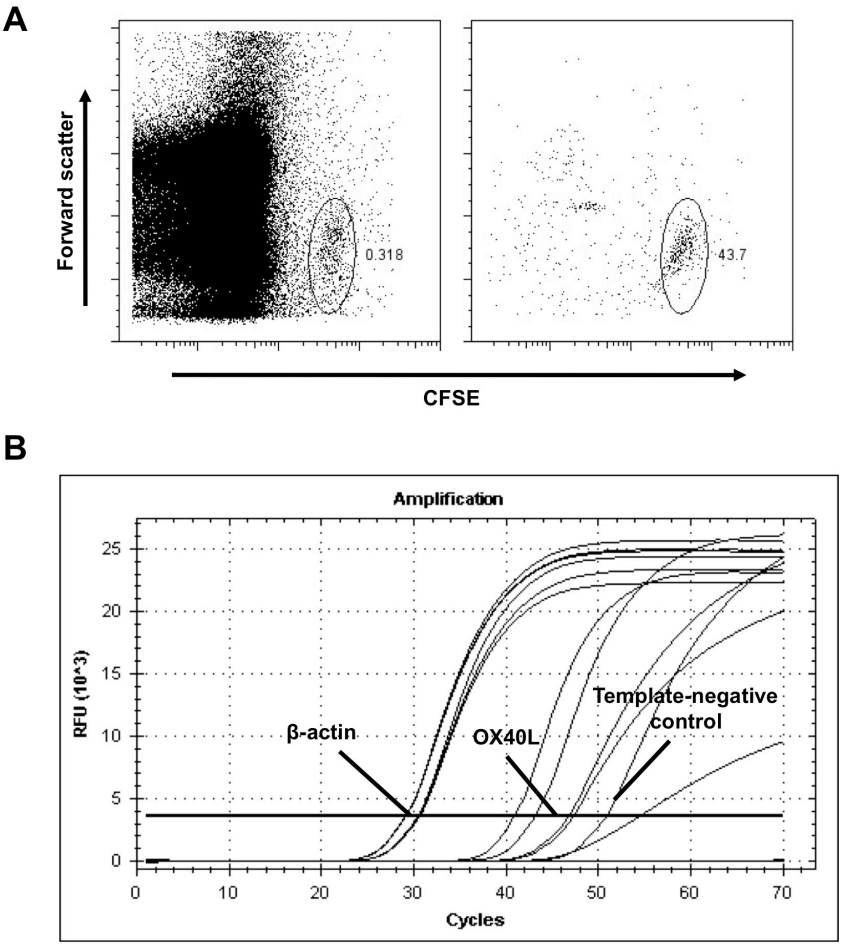

A
Forward scatter
0.318
43.7
CFSE
B
Amplification
RFU (10^3)
25
20
15
10
5
0
0
10
20
30
40
50
60
70
Cycles
β-actin
OX40L
Template-negative control

Figure 4.4.5

Figure 4.4.6 *The generation of $HEL_{105-120}$-specific effector CD4 T cells by peptide-pulsed APC is enhanced by an agonistic antibody to OX40*. One day prior to injection of pulsed splenocytes, normal BALB/c mice were seeded with 10^5 MACS purified CD4+ DO11.10+ T cells. BALB/c splenocytes were cultured overnight, as described in Chapter 2, in the presence of 50μM $HEL_{105-120}$ alone, or 50μM of each of $HEL_{105-120}$ and $OVA_{323-339}$. Following harvest and extensive washing, 10^7 peptide-pulsed splenocytes in 50μL Leibovitz media were injected subcutaneously into the footpad and lower leg of normal BALB/c mice on days 0, 3, and 6. At the time when pulsed splenocyte were injected the mice were also injected intravenously with 50μg of either an isotype-matched control antibody (iso. cont.) or with the OX40 agonistic antibody (OX40 agonist). Nine days after the initial injection of pulsed splenocytes, the $HEL_{105-120}$ and $OVA_{323-339}$-specific cytokine producing cells in the spleens and draining popliteal lymph nodes (DLN) were enumerated by ELISpot in mice given only $HEL_{105-120}$-pulsed splenocytes (H), and in mice given splenocytes pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$ (H+O). Each data point represents the number of specific ELISpot-forming cells in the popliteal lymph node of individual mice. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. These are the results of a single experiment with three mice per group (n = 3).

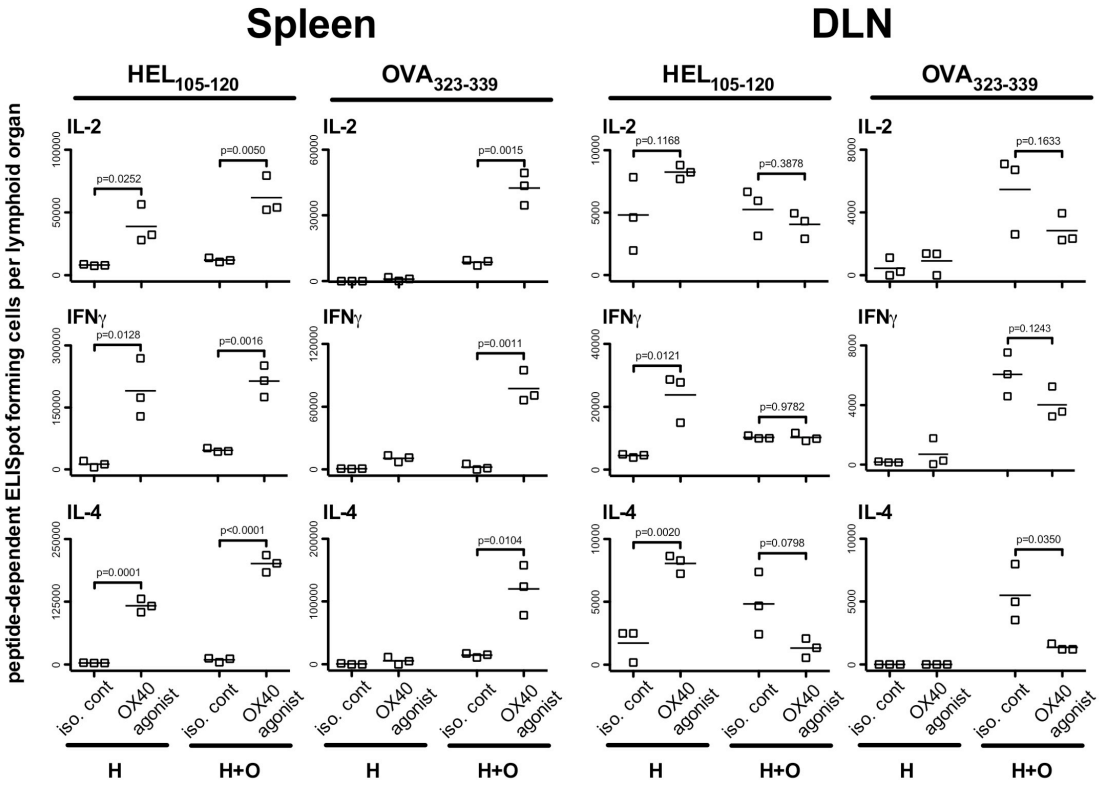

Spleen
DLN
HEL105-120
OVA323-339
HEL105-120
OVA323-339
peptide-dependent ELISpot forming cells per lymphoid organ
IL-2
IFNγ
IL-4
p=0.0252
p=0.0050
p=0.0015
p=0.1168
p=0.3878
p=0.1633
p=0.0128
p=0.0016
p=0.0011
p=0.0121
p=0.9782
p=0.1243
p=0.0001
p<0.0001
p=0.0104
p=0.0020
p=0.0798
p=0.0350
iso. cont
OX40 agonist
H
H+O

Figure 4.4.6

We observed not only a complete abrogation of cooperative generation of $HEL_{105-120}$-specicfic responses mediated by B cells pulsed with both peptides, but also a severe limitation in the generation of CD4 T cells specific for either peptide (Figure 4.4.7). We conclude that when B cells are the sole APC presenting peptide, that OX40-OX40L interactions are critical for the generation of effector CD4 T cells. This finding contrasts with our earlier findings that OX40-OX40L interactions only are critical for the cooperative enhancement of effector CD4 T cells. When four HEL peptide-specific CD4 T cell populations cooperate to enhance the generation of $HEL_{105-120}$-specific CD4 effector T cells, blocking OX40L blocked only the cooperation dependent component of T cell activation and not activation of individual peptide-specific T cell populations.. However, when B cells are the sole APC loaded with peptide, OX40L is critically important for the generation of all peptide-specific cytokine-producing CD4 T cells. Taking these two findings together, we conclude that a major role for B cells as APC, in activating CD4 T cells, is to mediate cooperation between them. Whether this corresponds with B cell effector function, such as antibody production, has not been addressed.

Figure 4.4.7. *OX40L blockade significantly impairs the generation of peptide-specific cytokine producing effector CD4 T cells following injection of peptide-pulsed purified B cells*. One day prior to injection of pulsed B cells, BALB/c mice were injected with 10^5 MACS purified CD4+ DO11.10+ T cells. BALB/c splenocytes were cultured overnight, as described in Chapter 2, in the presence of either 50μM $HEL_{105-120}$ alone or 50μM of both $HEL_{105-120}$ and $OVA_{323-339}$. Following splenocyte harvest, B cells were isolated by MACS negative selection and 3.5 x 10^6 peptide-pulsed B cells in 50μL Leibovitz media were injected subcutaneously into the footpad and lower leg of normal BALB/c mice on days 0, 3, and 6. With each B cell injection, mice were injected intravenously with either 50μg of an isotype-matched control antibody (iso. cont.) or with an OX40 blocking antibody (OX40 blocker). Nine days after the initial injection of pulsed splenocytes, the $HEL_{105-120}$ and $OVA_{323-339}$-specific cytokine producing cells in the spleens and draining popliteal lymph nodes (DLN) were enumerated by ELISpot for mice given only $HEL_{105-120}$-pulsed B cell (H), and in mice injected with B cells pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$ (H+O). Each data point represents the number of specific ELISpot-forming cells in the spleen and popliteal lymph node of individual mice. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. Data presented are values pooled from three independent experiments with three mice per group (n = 9).

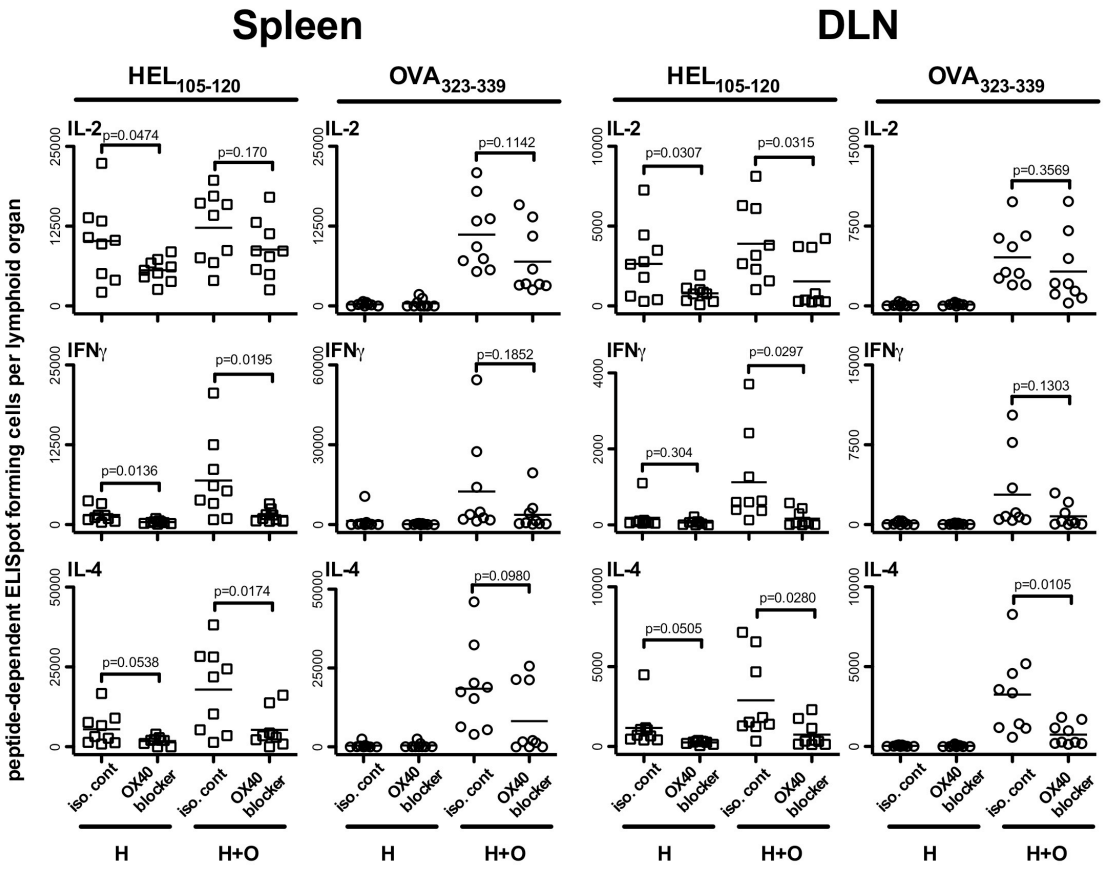

Spleen
DLN
HEL105-120
OVA323-339
HEL105-120
OVA323-339
peptide-dependent ELISpot forming cells per lymphoid organ
IL-2
IFNγ
IL-4
p=0.0474
p=0.170
p=0.1142
p=0.0307
p=0.0315
p=0.3569
p=0.0136
p=0.0195
p=0.1852
p=0.304
p=0.0297
p=0.1303
p=0.0538
p=0.0174
p=0.0980
p=0.0505
p=0.0280
p=0.0105
iso. cont
OX40 blocker
H
H+O

Figure 4.4.7

4.5 Functional Consequences of CD4 T cell Cooperation

Through the findings reported in this and previous chapters, we describe observations and give insights into the cellular and molecular mechanisms involved in cooperation between CD4 T cell populations that give rise to increased generation of effector cells. We have demonstrated that cooperation between $OVA_{323-339}$ and $HEL_{105-120}$-specific populations results in enhanced generation of primary $HEL_{105-120}$-specific responses. We chose to assess whether these enhanced primary immune responses could potentially have functional consequences in terms of immune responses.

A hallmark of the adaptive immune response is the ability to generate immunological memory. Upon secondary exposure to the same pathogen, or experimental antigen "memory" lymphocytes respond to this antigen again often inducing secondary immune responses that are of greater magnitude and the response occurs more quickly than the primary response. CD4 effector T cells can transition into memory cells and be maintained over time to facilitate these rapid secondary immune responses of greater magnitude. The quality of this secondary response is often associated with factors that influence effector CD4 T cell generation during the primary phase (Pepper and Jenkins). Thus we investigated whether cooperative enhancement of $HEL_{105-120}$ specific effector CD4 T cells during the primary response would result in enhanced secondary immune responses.

To address whether cooperation between $OVA_{323-339}$ and $HEL_{105-120}$ specific CD4 T cells, mediated by dual peptide pulsed B cells, resulted in enhanced memory, we undertook the following experiment. BALB/c mice were first injected with CD4 T cells from DO11.10 mice and after 24 h were injected with MACS purified B cells pulsed with $OVA_{323-339}$ alone, $HEL_{105-120}$ alone, or $HEL_{105-120}$ and $OVA_{323-339}$ together, as per our standard protocol. These mice were left for seven weeks, sufficient time for effector CD4 transition to memory cells. We then challenged these mice with HEL protein and assessed the characteristics of the secondary immune response.

Figure 4.5.1 *Cooperative enhancement of $HEL_{105-120}$-specific effector CD4 T cell generation results in increased $HEL_{105-120}$-specific effector cell responses following secondary immunization*. One day prior to injection of pulsed splenocytes, BALB/c mice were seeded with 10^5 MACS purified CD4+ DO11.10+ T cells. BALB/c slpenocytes were cultured overnight, as described in Chapter 2, in the presence of either 50μM $OVA_{323-339}$ alone, 50μM $HEL_{105-120}$ alone, or 50μM of both $HEL_{105-120}$ and $OVA_{323-339}$. Following splenocyte harvest, B cells were isolated by MACS negative selection and 3.5 x 10^6 peptide-pulsed B cells in 50μL Leibovitz media were injected subcutaneously into the footpad and lower leg of BALB/c mice on days 0, 3, and 6. Seven weeks later all mice were injected intraperitoneally with 100ug heat-aggregated HEL protein in saline. Ten days post-challenge the $HEL_{105-120}$-specific cytokine producing cells in the spleen of mice given $OVA_{323-339}$-pulsed B cells (O), $HEL_{105-120}$-pulsed B cells (H), and $OVA_{323-339}$ and $HEL_{105-120}$-pulsed B cells (H+O) were assessed by ELISpot. Each data point represents the number of specific ELISpot-forming cells in the spleen of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. The data presented are values pooled from three independent experiments with two to three mice per group (n = 8).

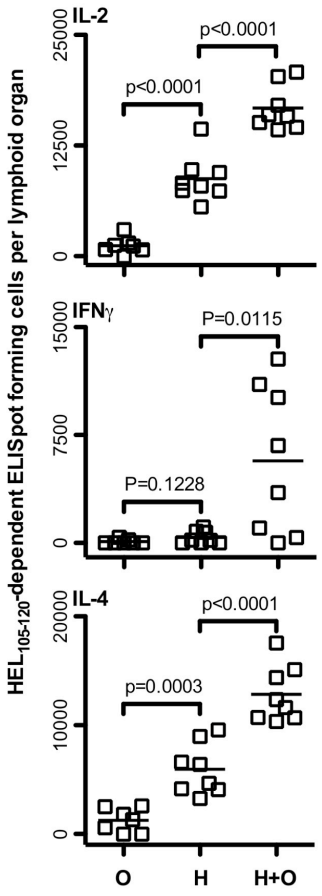

IL-2
p<0.0001
p<0.0001
25000
12500
0
IFNγ
P=0.0115
P=0.1228
15000
7500
0
IL-4
p<0.0001
p=0.0003
20000
10000
0
HEL105-120-dependent ELISpot forming cells per lymphoid organ
O
H
H+O

Figure 4.5.1

We observed very little $HEL_{105-120}$-specific cytokine production in mice primed with $OVA_{323-339}$-pulsed B cells. Those mice that had been primed with $HEL_{105-120}$-pulsed B cells generated moderate numbers of IL-2 and IL-4 producing $HEL_{105-120}$-specific effector cells following secondary immunization. Strikingly, mice that were primed with B cells pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$ produced significantly greater numbers of $HEL_{105-120}$-specific IFNγ and IL-4 producing effector CD4 T cells following secondary immunization with HEL protein (Figure 4.5.1).

These observations support the conclusion that CD4 cell cooperation during the primary immune response resulted in enhanced memory CD4 T cells and significantly increased anamnestic responses. This functional outcome is consistent with our finding that OX40-OX40L interactions are critical in CD4 T cell cooperation as others have shown that OX40 stimulation of effector CD4 T cells resulted in enhanced T cell survival and memory cell commitment (see section 1.5).

4.6 Conclusions

In summary, I developed an experimental model involving peptide-pulsing of APC *in vitro* and the adoptive transfer of peptide-pulsed APC to investigate functional cooperation between CD4 T cell subpopulations. We demonstrated that for cooperation to occur between two peptide-specific subpopulations of CD4 T cells, both peptides must be presented by the same APC. We observed that this APC could be a B cell and that splenic DCs, under the conditions used, did not efficiently mediate cooperation between CD4 T cells. We confirmed with this system that OX40-OX40L interactions were critical for CD4 T cells cooperation; these interactions were vital for effector CD4 T cell generation when B cells alone were loaded with peptide. Finally, we show that enhanced generation of primary CD4 T cells through CD4 T cell cooperation can support enhanced secondary immune responses.

Taking our results together, we conclude that CD4 T cell cooperation, when B cells function as the APC, has the potential to influence the magnitude of both primary and secondary immune responses.

5.0 CHAPTER 5. INVESTIGATIONS OF THE MECHANISMS OF TOLERANCE INDUCTION IN CD4 T CELLS

5.1 Introduction

While the immune system is effective in defending animals from invasion by pathogens it also has the potential to cause serious damage to endogenous tissues. Thus, maintenance of tolerance to self-components is of central importance for survival in animals that possess immune systems. This importance was recognized by early immunologists, like Paul Ehrlich, who were aware that antibodies had the capacity to cause lysis of host erythrocytes and that in certain pathological situations, antibodies that lead to erythrocyte lysis arise endogenously. As discussed in section 1.2, maintenance of self-tolerance in T cells occurs by a number of mechanisms. Mature, peripheral, CD4 T cells that encounter peptide-MHCII complexes, for which their TCR has affinity, results in either activation or inactivation of these cells depending on additional information relayed to the CD4 T cell. The additional information required, termed the second signal, controls the fate of CD4 T cells that encounter antigen. When the critical second signal is relayed to CD4 T cells, they become activated; if this signal is not relayed to the CD4 T cell, it is inactivated, undergoing apoptosis or becoming anergic, removing itself from the T cell repertoire. Therefore, the circumstances that control the availability of the critical second signal determine the fate of CD4 T cells upon antigen encounter.

The above-described paradigm for how the activation-inactivation decision is made in CD4 T cells is known as the two-signal hypothesis. The most pervasive current view, for how the second signal is controlled, stems from the adaptation of the two-signal hypothesis by Lafferty and Cunningham (Lafferty and Cunningham 1975). This model holds that certain antigen-presenting cell types possess heightened levels of co-stimulatory molecules on their surface, and that these co-stimulatory molecules are the

necessary and sufficient providers of the critical second signal for T cell activation. Numerous observations, made over the past forty years, support this model. However, within our laboratory, there have been some who propound a somewhat different model for how a critical second signal arises during the generation of immune responses. The two-step, two-signal model, put forward by Bretscher in 1999, proposes that the initial interaction of CD4 T cells with APC, like DC, activated in such a way that they express high-levels of co-stimulatory molecules, is in itself insufficient to result in full activation of CD4 T cells. In this model the critical second signal for CD4 T cells, thought to result in full activation and acquisition of effector function, is generated uniquely upon the interaction of two or more CD4 T cells. Additionally, the interaction of these CD4 T cells, as envisaged by Bretscher, is mediated by a B cell to which both T cells bind.

As I have demonstrated in the previous two chapters, CD4 T cells having different antigen specificities, appear to interact with one another, leading to an enhancement of CD4 effector function. I have also shown that B cells play a critical role in mediating this cooperative enhancement. However, our data do not indicate that cooperation, amongst CD4 T cells, is essential for the generation of effector CD4 T cells as we did not observe situations where no effector CD4 T cells were generated upon immunization. Implicit in the two-step, two-signal model is that the result of failing to achieve a cooperative interaction between CD4 T cells is inactivation of these cells. Again our data, shown in the previous chapters, do not appear to support this aspect of the hypothesis. However, it is possible that cooperation amongst CD4 T cells, specific for the same peptide, occurs in responses to single peptides, thereby leading to cooperation that is not detectible by our experimental design. Furthermore, it is possible that the low-level effector CD4 T cell generation, seen upon immunization with single peptides is reflective of a state of intermediate CD4 T cell activation wherein these cells may produce effector cytokine, but are on the pathway towards inactivation.

Formal attempts to address the predictions made on the two-step, two-signal model led to some interesting observations on the mechanisms of CD4 T cell tolerance and a dramatic change in our thinking on the mechanisms controlling CD4 T cell tolerance. Below, I present findings that indicate that CD4 T cell cooperation could be involved in the activation/inactivation decision of CD4 T cells that bind self-antigens.

5.2 Tolerance Induction by Administration of High-Dose Peptide in Naive CD4 T cells and Attempts to Prevent it

Based on the two-step, two-signal model for the primary activation of CD4 T cells, one would predict that CD4 T cells that fail to undergo a cooperative interaction, following TCR ligation, will be inactivated and eventually undergo cell death. Conversely, if, under similar conditions, the CD4 T cell encounters signals mediated through CD4 T cell activation of a B cell, then it should become a fully activated effector cell. Therefore, it would seem that activation, as a consequence of CD4 T cell cooperation, would be dominant over tolerance induction. That is, cooperation should be able to rescue CD4 T cells from apoptosis caused by interaction with antigen in a tolerogenic fashion. This was our working model at the outset of these studies.

In order to address whether CD4 T cell cooperation could rescue CD4 T cells from becoming deleted in response to antigen, we developed a protocol that would normally induce tolerance of peptide specific CD4 T cells. We found that the administration of high doses of $HEL_{105-120}$ in IFA was tolerogenic, this is in accordance with other studies (Murphy, Heimberger et al. 1990; Kearney, Pape et al. 1994; Peters, Kroeger et al. 2009). Figure 5.2.1 shows an example of a titration of $HEL_{105-120}$ given in IFA to mice. Although the tolerance was only seen clearly in the generation of IL-2-producing cells, we found that inactivation of $HEL_{105-120}$-specific CD4 T cells was readily accomplished by administration of this peptide in IFA. A dose of approximately 200μg given intraperitoneally was needed to achieve reliable tolerance.

Having established a protocol wherein reliable peptide-specific CD4 T cell unresponsiveness could be established, we attempted to rescue the CD4 T cells by arranging for cooperative events to occur. Figure 5.2.2 displays the results of such an attempt. Mice were given 200μg of $HEL_{105-120}$ intraperitoneally and were also immunized with $HEL_{105-120}$ together with the three other HEL peptides responded to normally in BALB/c mice. This latter immunization normally results in fairly robust activation of CD4 T cells specific for $HEL_{105-120}$ (see Chapter 3 of this thesis).

Figure 5.2.1 *A Titration of $HEL_{105-120}$, given intraperitoneally in IFA, with a subsequent $HEL_{105-120}$ in CFA challenge*. Normal BALB/c mice were injected intraperitoneally with 200μL of IFA emulsion containing graduated doses of $HEL_{105-120}$, or were not injected (N). Fourteen days after this initial injection, all mice were challenged with 10μg $HEL_{105-120}$ in CFA. Ten days post-challenge, the $HEL_{105-120}$-specific cytokine-producing cells, in the spleen of challenged mice, were enumerated by ELISpot. Each data point represents the number of specific ELISpot-forming cells in the spleen of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. This is the result of a single representative experiment with three mice per group (n = 3) of at least three similar experiments.

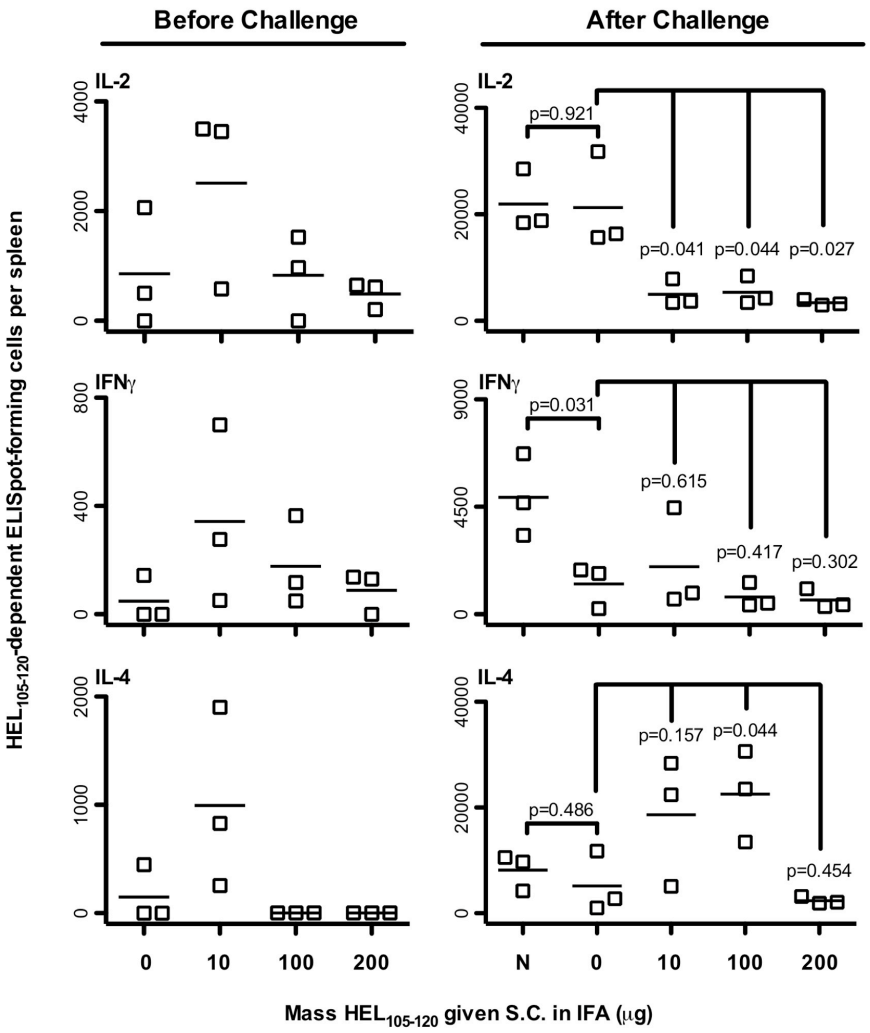

Before Challenge
After Challenge
IL-2
IFNγ
IL-4
p=0.921
p=0.041
p=0.044
p=0.027
p=0.031
p=0.615
p=0.417
p=0.302
p=0.157
p=0.044
p=0.486
p=0.454
0
2000
4000
20000
40000
400
800
4500
9000
1000
N
10
100
200
HEL105-120-dependent ELISpot-forming cells per spleen
Mass HEL105-120 given S.C. in IFA (μg)

Figure 5.2.1

Control mice, immunized with four HEL peptides in IFA subcutaneously and given only IFA intraperitoneally exhibit the expected enhanced generation of $HEL_{105-120}$ specific cytokine-producing T cells, at the time when these were enumerated, during the primary response to administered peptides, ten days post-immunization. However, all mice that were treated with 200μg of $HEL_{105-120}$ showed no sign of peptide-specific effectors cells on day 10. Following challenge with HEL protein on ALUM, we observed a similar pattern on $HEL_{105-120}$-specific effector CD4 T cells with the exception of mice that received four HEL peptides subcutaneously at the same time as a tolerogenic dose of $HEL_{105-120}$ intraperitoneally. These mice had more $HEL_{105-120}$-specific cytokine-producing CD4 T cells, on average, than did mice given $HEL_{105-120}$ alone subcutaneously with 200μg of $HEL_{105-120}$ intraperitoneally. Thus, while these results did not reach statistical significance, it appears that this protocol may have resulted in rescuing of at least some CD4 T cells specific for $HEL_{105-120}$ that would have normally become inactivated.

However, the above experiment is not without its complications. An alternative interpretation of the results shown in Figure 5.2.2, on more conventional theories of CD4 T cell activation, is that the CD4 T cells that recognize $HEL_{105-120}$ on APC from lymph nodes draining the subcutaneous IFA depot are activated and sequestered within those lymph nodes, where they may continually see the antigen in an immunogenic way. If this is the case, then these cells are not rescued, *per se*, but are sheltered from experiencing antigen in a tolerogenic context. Because of this difficulty, we decided that in order to test whether additional substances, given at the time of tolerance induction, could rescue $HEL_{105-120}$-specific CD4 T cells, they should be directly incorporated into IFA containing a high dose of $HEL_{105-120}$. We undertook numerous experiments attempting to rescue $HEL_{105-120}$-specific CD4 T cells from becoming inactivated by high-doses of the peptide. However, we were not able to observe rescuing. For brevity, I will not describe the results of these studies, save one.

Since we had not been able to rescue or prevent inactivation of $HEL_{105-120}$-specific CD4 T cells in mice injected with IFA containing 200μg of this peptide, we chose to investigate whether we could rescue the cells by introducing the peptide in CFA. As we have shown, CFA contains mycobacterial antigens that can elicit cooperating CD4 T cells (Figure 1.2.1).

Figure 5.2.2. *Partial prevention of tolerance in mice given four HEL peptides, together in IFA, and high-dose $HEL_{105-120}$ in IFA*. All normal BALB/c mice were injected with two IFA emulsions, one at the tail base, and the other intraperitoneally. Peptides incorporated into these emulsions were 10μg $HEL_{105-120}$ (H), 10μg each of HEL_{11-25}, HEL_{48-63}, HEL_{74-96}, and $HEL_{105-120}$ (4Hp), or 200μg $HEL_{105-120}$ (200μg H). Some mice were sacrificed on day ten post-injection for enumeration of the $HEL_{105-120}$-specific cytokine producing cells in the spleen. The remaining mice were challenged with 100μg heat-aggregated HEL on ALUM on day fourteen post-injection. Ten days post-challenge, the $HEL_{105-120}$-specific cytokine-producing cells, in the spleen of challenged mice, were enumerated by ELISpot. Each data point represents the number of specific ELISpot-forming cells in the spleen of a single mouse. This is the result of a single experiment with three mice per group (n = 3).

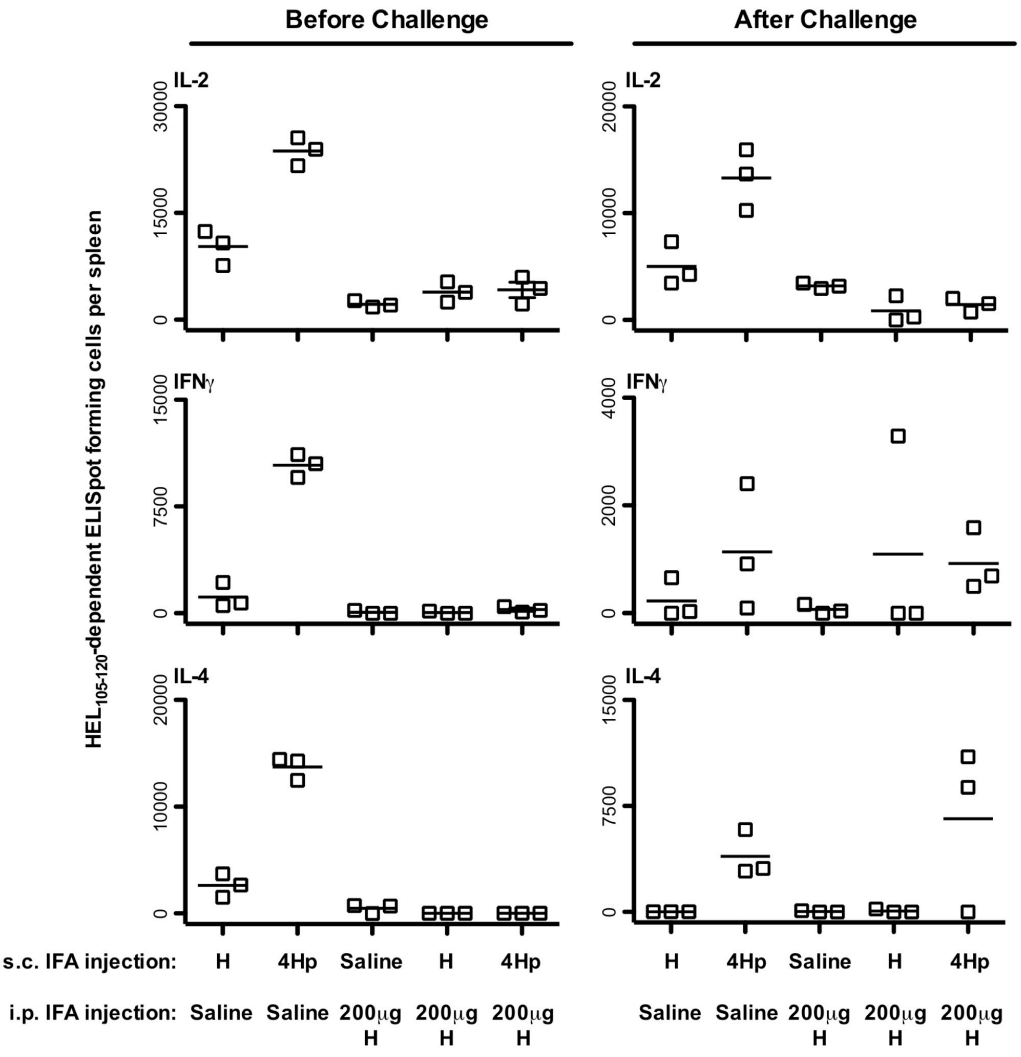

Before Challenge
After Challenge
IL-2
IFNγ
IL-4
HEL105-120-dependent ELISpot forming cells per spleen
s.c. IFA injection: H 4Hp Saline H 4Hp
i.p. IFA injection: Saline Saline 200μg H 200μg H 200μg H

Figure 5.2.2

Moreover, CFA contains many TLR ligands and other inflammatory mediators capable of activating APC. We reasoned that, whether one subscribes to conventional theories for the activation of CD4 T cells, or to the two-step, two-signal model, both would predict that high doses of the peptide, administered in CFA, would cause activation of $HEL_{105-120}$-specific CD4 T cells. As can be seen in Figure 5.2.3, administration of 200μg of $HEL_{105-120}$ in CFA intraperitoneally to BALB/c mice renders them tolerant of this peptide. We did not observe activation of peptide-specific CD4 T cells in primary instances in similarly injected mice (not shown). From these results we conclude that one of two possible phenomenon occur when high-dose peptide is administered in CFA. Either CD4 T cells, never having been activated, are inactivated directly, which seems unlikely given the properties of CFA, or CD4 T cells are activated by peptide in CFA and are *subsequently* inactivated. The latter possibility seemed to defy conventional knowledge. T cells were once thought to be subject to induction of tolerance only prior to activation. As it turns out, a number of studies have demonstrated that inactivation of CD4 T cells in virtually any state of activation is readily achievable by administration of antigen in a tolerogenic fashion (Critchfield, Racke et al. 1994; Higgins, Mihalyo et al. 2002; Steptoe, Ritchie et al. 2007; Kenna, Thomas et al. 2008; Doan, McNally et al. 2009; Kenna, Waldie et al. 2010; Nasreen, Waldie et al. 2010) (see section 1.2 for a discussion of this).

Figure 5.2.3. *High dose $HEL_{105\text{-}120}$, given subcutaneously in CFA, is tolerogenic*. BALB/c mice either with CFA/saline, or with CFA containing 200μg $HEL_{105\text{-}120}$, subcutaneously at the tail base. Fourteen days post-injection, all mice were challenged with 100μg heat-aggregated HEL on ALUM, intraperitoneally. Ten days post-challenge, the $HEL_{105\text{-}120}$-specific cytokine-producing cells, in the spleen of challenged mice, were enumerated by ELISpot. Each data point represents the number of specific ELISpot-forming cells in the spleen of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. This is the result of a single representative experiment, of two similar experiments, with six mice per group (n = 6).

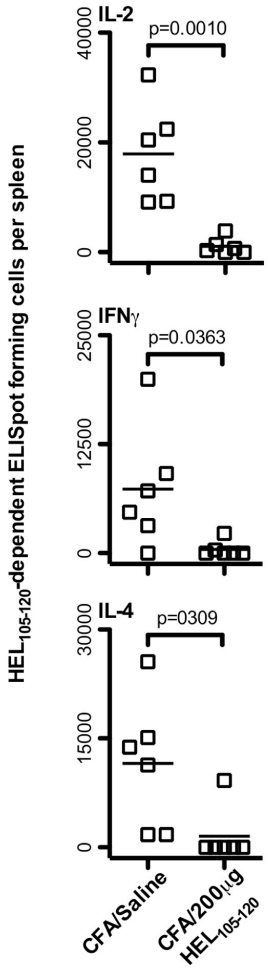

IL-2
p=0.0010
40000
20000
0
IFNγ
p=0.0363
25000
12500
0
IL-4
p=0309
30000
15000
0
HEL105-120-dependent ELISpot forming cells per spleen
CFA/Saline
CFA/200μg HEL105-120

Figure 5.2.3

5.3 Tolerance Induction in Activated CD4 T Cells by High-Dose Peptide

The finding that high doses of peptide, given in IFA or CFA, consistently led to inactivation of $HEL_{105-120}$-specific CD4 T cells in our hands, gave us reason to hypothesize that fully activated CD4 effector CD4 T cells could be inactivated by administration of high doses of peptide. We think that differential diffusion rates of the peptide vs the inflammatory mediators might be responsible for T cell inactivation. CD4 T cells specific for $HEL_{105-120}$ would be initially activated at the injection site by APC presenting this peptide that had been stimulated by the inflammatory molecules contained in CFA. The activated T cells would then circulate to distal sites where peptide is presented, having diffused throughout the body, but would be inactivated by "immature, or inactive" APC, if the inflammatory mediators had not diffused throughout the body. We wished to test the hypothesis as directly as possible. In our primary experiment, we attempted in inactivate activated CD4 T by the administration of high dose peptide in vivo. We activated CD4 T cells in vivo, by injecting the footpad with low doses of $HEL_{105-120}$, in CFA. From previous work, we knew seen that day ten was the peak of the generation of $HEL_{105-120}$-specific cytokine-producing cells in the spleen following immunization in this way. We reasoned that by the peak of effector cell generation, most $HEL_{105-120}$-specific CD4 precursor T cells would have been recruited into the effector pool. We therefore chose, first, to attempt to inactivate $HEL_{105-120}$-specific CD4 T cells at the peak of their effector phase. Beginning on day ten post-immunization, mice were given either saline or a standard tolerization dose of 300, 100, 100μg $HEL_{105-120}$, intravenously, every five days. On the fourteenth day following the first tolerization injection, mice were challenged with heat-aggregated HEL on ALUM. Ten days post-challenge, we enumerated the HEL105-120 peptide specific cytokine producing cells in the spleen of the putatively tolerized mice. We observed that mice that were treated with high tolerization doses of $HEL_{105-120}$ were almost completely unable to generate cytokine producing CD4 T cells specific for the peptide (Figure 5.3.1). The number of $HEL_{105-120}$ specific cytokine producing cells in the spleens of control-treated mice was significantly greater than the number in peptide-treated mice. From this finding, we conclude that effector CD4 T cells are sensitive to inactivation by high-dose peptide.

Figure 5.3.1. *Inactivation of activated $HEL_{105-120}$-specific CD4 T cells can be induced by high-dose peptide given at the peak of the effector phase*. All BALB/c mice were injected with 10μg $HEL_{105-120}$ in CFA, subcutaneously in the hind footpad. Ten days later, mice were treated intravenously with saline alone (saline) or with 300μg of $HEL_{105-120}$ in saline (Tol). Five and ten days after this first treatment, all mice were treated intravenously with either saline (saline) or with 100μg $HEL_{105-120}$ (Tol). Twenty-five days after the initial CFA injection, mice were challenged with 100μg heat-aggregated HEL on ALUM intraperitoneally. Ten days post-challenge, the $HEL_{105-120}$-specific cytokine-producing cells, in the spleen of challenged mice, were enumerated by ELISpot. Each data point represents the number of specific ELISpot-forming cells in the spleen of a single mouse. These are pooled results from three independent experiments with three mice per group (n = 9).

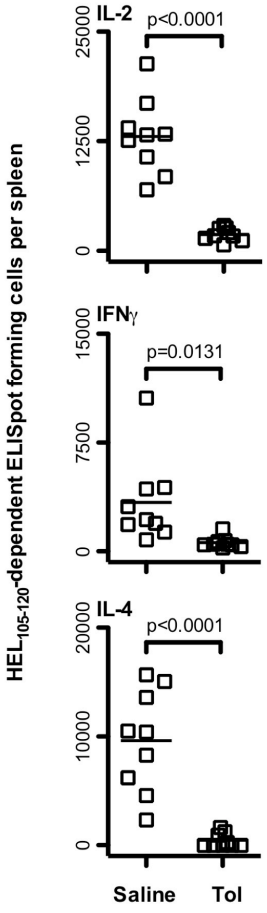

IL-2
p<0.0001
25000
12500
0
IFNγ
p=0.0131
15000
7500
0
IL-4
p<0.0001
20000
10000
0
HEL105-120-dependent ELISpot forming cells per spleen
Saline
Tol

Figure 5.3.1

It was possible that inactivation of effector CD4 T cells at the peak of the primary response was facilitated by the normal contraction of effector cells following activation. To address whether activated CD4 T cells can be inactivated by peptide during the priming phase, we repeated the experiment shown in Figure 5.3.1, giving a single injection of 300μg of $HEL_{105-120}$ on the day of CFA immunization or four days later. Figure 5.3.2. shows the results of this experiment. We observed a decrease in the numbers of effector cells, as assessed by measuring levels of cytokine producing cells, particularly in the draining lymph node of $HEL_{105-120}$ treated mice, when assessed on day seven of the primary response. We interpret these results as evidence that effector cells were inactivated following the administration of 300μg $HEL_{105-120}$. Thus, we conclude that effector CD4 T cells are susceptible to inactivation during the priming phase. Because we obtained clear results that were largely in line with the observations of others, and for ethical reasons concerning the administration of CFA into the footpad of mice, we did not pursue further experiments in this system.

5.4 Tolerance Induction in Activated DO11.10 CD4 T Cells: I. *In Vitro* Cultures

Although others have published observations detailing the inactivation of activated T cells, we believed that our own findings were worth further exploration. Given our unique perspectives on the activation of CD4 T cells, and our findings of CD4 T cell cooperation, we thought it plausible that we would make advances in understanding the mechanisms controlling the inactivation of activated CD4 T cells. Christopher Rudulier, in our laboratory, had been employing very efficient in vitro cultures for assessing how precursor frequency affects the Th1/Th2 phenotype of the CD4 T cells activated in culture. We thought that this system could be adapted to study the inactivation of CD4 T cells. In his cultures, CD4 T cells from DO11.10 mice, bearing transgene-encoded TCRs that recognize $OVA_{323-339}$ in the context of $I\text{-}A^d$ are activated by APC presenting $OVA_{323-339}$. One particular advantage that a culture system of this type has over an *in vivo* system, is the ability to readily analyze the CD4 T cells by flow-cytometry, due to the availability of the clonotype-specific anti-TCR antibody KJ1-26.

Figure 5.3.2. *Inactivation of activated $HEL_{105-120}$-specific CD4 T cells can be induced by high-dose peptide given early during the priming phase*. All BALB/c mice were injected with 10μg $HEL_{105-120}$ in CFA, subcutaneously in the hind footpad. At the time of injection, or four days later, mice were treated intravenously with saline alone or with 300μg of $HEL_{105-120}$ in saline (T d0 or T d4). Seven days after the initial injection of CFA, the $HEL_{105-120}$-specific cytokine-producing cells, in the draining lymph nodes (DLN) and spleen, were enumerated by ELISpot. This is the result of a single experiment with three mice per group (n = 3).

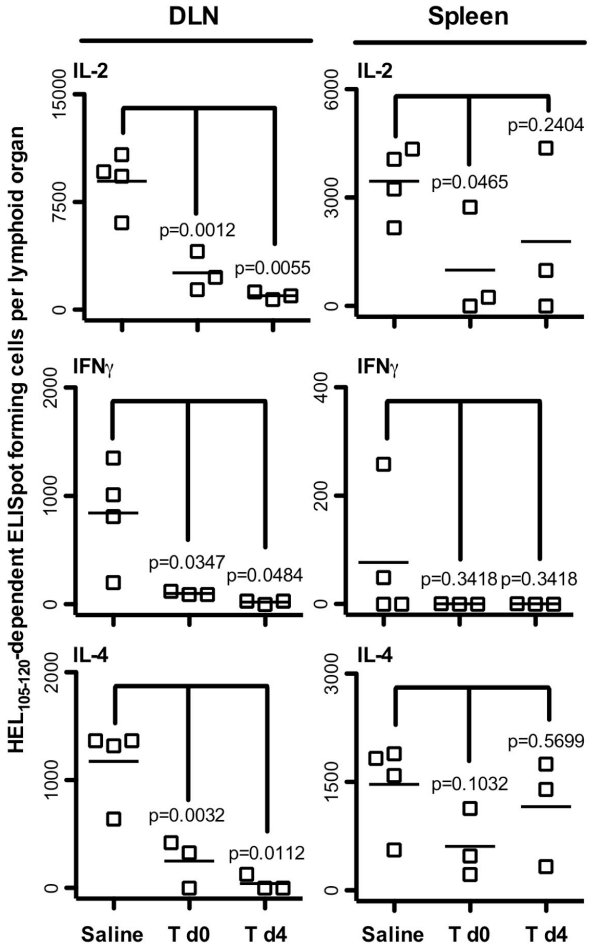

DLN
Spleen
IL-2
IL-2
IFNγ
IFNγ
IL-4
IL-4
HEL105-120-dependent ELISpot forming cells per lymphoid organ
p=0.0012
p=0.0055
p=0.0465
p=0.2404
p=0.0347
p=0.0484
p=0.3418
p=0.3418
p=0.0032
p=0.0112
p=0.1032
p=0.5699
Saline
T d0
T d4
Saline
T d0
T d4

Figure 5.3.2

The finding that CD4 T cells can be inactivated in vivo suggests that excess signal one, in the absence of signal two, or, more TCR signaling than co-stimulation, leads to their inactivation. The reasoning behind this conclusion is discussed in section 1.2. Briefly, administration of high dose peptide to mice, by systemic routes, is thought to enable presentation of this peptide by virtually all MHCII-bearing cells, since the majority of these cells are normally in an inactive, resting state, the majority of peptide-MHCII complexes available for triggering TCR will not be associated with co-stimulation. This is the basis for the steady-state model for the maintenance of T cell tolerance, a point to which we will return at the end of this chapter. The B-7 co-stimulatory molecules, CD80 and CD86 are well characterized and potentially the most important second-signal mediating molecules. These are upregulated on activated DC and B cells leading to their ability to potently stimulate T cells. We hypothesized that, due to their major role in T cell activation, limiting their signaling may tip the balance in favor of excess signal one, leading to inactivation and possibly apoptosis, in culture.

To test whether limiting signaling through B-7 molecules could drive inactivation of activated T cells when exposed to cognate peptide, we sought to develop a culture system wherein we could manipulate the availability of either of these signals to CD4 T cells. In order to establish this system, we first needed a way to generate uniformly activated CD4 T cells. Since we planned to employ DO11.10 cells, we explored ways to activate these cells in culture. As Figure 5.4.1 shows, MACS purified CD4+ DO11.10 cells from day 3 of culture produced effector cytokines (panel A) and displayed surface characteristics (high CD44; low CD62L; high CD86; high GITR) of activated T cells (panel B).

We thus proceeded to culture these purified activated CD4 T cells under three different conditions. We included, as a negative control, activated CD4 T cells, with T cell depleted splenocytes as APC, in the absence of antigen. The experimental culture conditions included activated CD4 T cells with APC and $OVA_{323-339}$ alone, or APC and $OVA_{323-339}$ with CTLA-4-Ig, to block B-7 molecules. We enumerated the $OVA_{323-339}$-specific cytokine producing CD4 T cells in these three cultures on the second and fourth day after plating. The pooled results of three of these experiments are displayed in Figure 5.4.2A.

Figure 5.4.1. *CD4+ DO11.10 T cells are highly activated following three days of culture with 0.3μM $OVA_{323-339}$ and syngeneic T cell depleted splenocytes*. CD4+ DO11.10 T cells were isolated from the spleen of transgenic mice by MACS negative selection. 1.5 x 10^5 per well of these cells were seeded into 24-well tissue culture trays containing 3 x 10^6 T cell depleted BALB/c splenocytes and 0.3μM $OVA_{323-339}$ peptide. After three days of culture, the CD4+ T cells were re-isolated by MACS negative selection. (A) The $OVA_{323-339}$-dependent cytokine secretion of these three day activated DO11.10 cells was assessed by ELISpot. This is pooled data from three independent experiments. (B) The cell surface phenotypes of day three activated DO11.10 T cells, and freshly isolated naive DO11.10 CD4 T cells, were assessed by flow cytometry. MACS isolated CD4+ T cells were greater than 95% as assessed by staining with an anti-clonotype TCR antibody (not shown) and thus histograms shown in B were ungated. Grey, dotted, histograms show the level of fluorescence of cells stained with a matched fluorophore-conjugated isotype-matched control antibody.

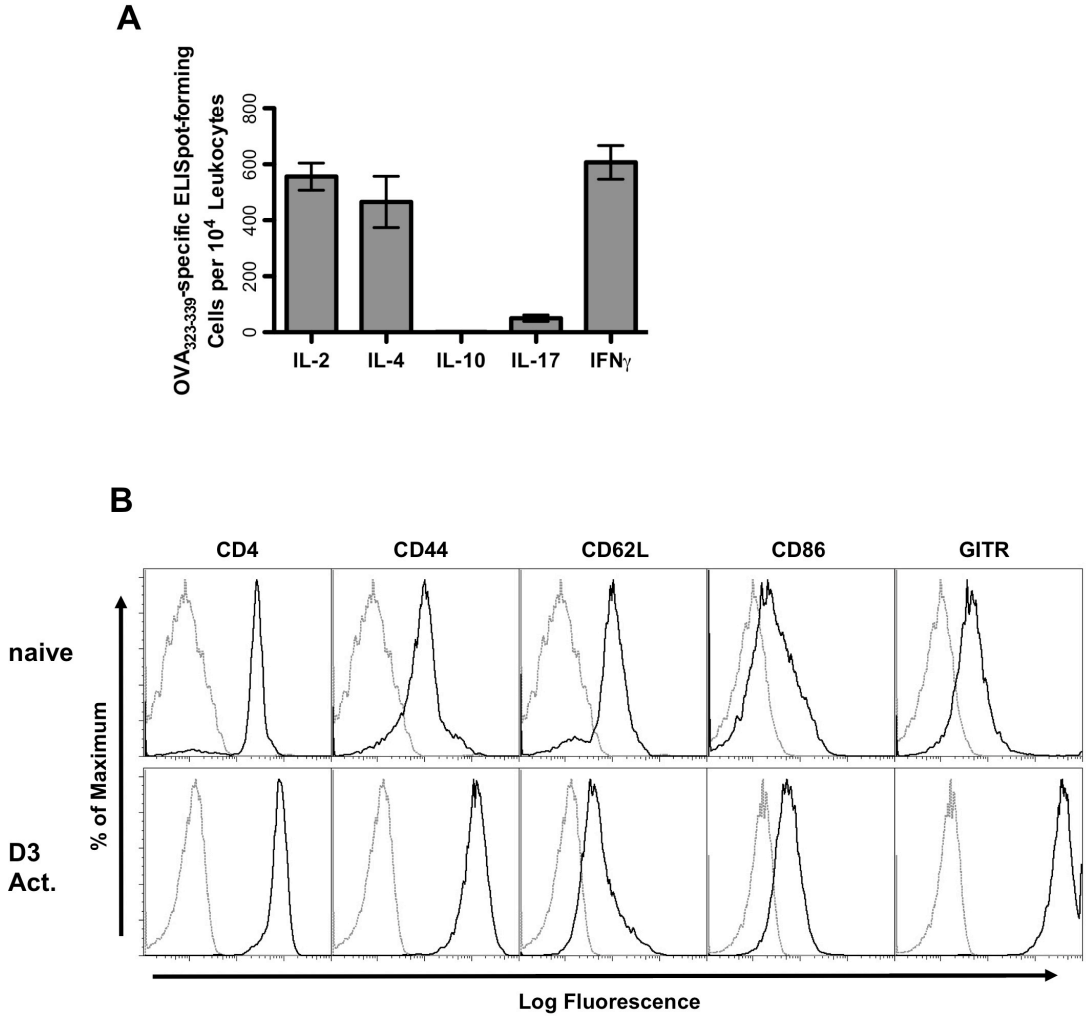

A
OVA323-339-specific ELISpot-forming Cells per 10^4 Leukocytes
0
200
400
600
800
IL-2
IL-4
IL-10
IL-17
IFNγ
B
CD4
CD44
CD62L
CD86
GITR
naive
D3 Act.
% of Maximum
Log Fluorescence

Figure 5.4.1

We observed a sharp increase in the number of cytokine producing cells, in both cultures containing peptide, and to a lesser extent in cultures without peptide, until day two. This somewhat unexpected finding indicates that many of the DO11.10 CD4 T cells likely acquired the ability to produce cytokine after plating into secondary cultures. This is, therefore, a caveat to our findings, as all cells were not potently producing cytokine. However, given the fairly uniform expression of T cell activation markers, like CD44 and GITR, we judge that virtually all of these cells were at least initially stimulated before harvest. It is clear, from theses results, that optimal expansion, or acquisition of cytokine production, requires both TCR ligation and B-7 co-stimulation in this culture system. Cultures containing CTLA-4-Ig with $OVA_{323-339}$ failed to support optimal expansion of cytokine-producing cells, while those cultures without antigen appeared to allow very slow expansion, possibly due to residual priming from the initial culture. By day four of culture, there was clear evidence that blocking B-7 co-stimulation influenced the cytokine secretion of the activated CD4 T cells in culture. We observed consistent declines in the numbers of cytokine-producing cells in both cultures containing peptide, possibly due to activation induced death of the effector CD4 T cells. However, the drop in numbers of $OVA_{323-339}$-specific effector CD4 T cells in cultures containing CTLA-4-Ig was greater proportionally; for all cytokines investigated, the number of cytokine producing cells in cultures containing $OVA_{323-339}$ and CTLA-4-Ig was found to be significantly lower or equal to the numbers in cultures containing no peptide. Conversely the numbers of CD4 T cells that produce cytokine in an $OVA_{323-339}$-dependent fashion remained significantly higher, in cultures supplemented with $OVA_{323-339}$ alone, compared with the other two culture conditions (Figure 5.4.2B).

Our findings in this *in vitro* culture system were suggestive of tolerance induction of activated CD4 T cells. However, it was possible that the effect of CTLA-4-Ig was mainly on the ability of these cells to produce cytokine, and not an influence on cell survival. We therefore decided to assess the expression of certain proteins, known to be involved in the activation and inactivation of CD4 T cells, in the remaining D011.10 CD4 T cells, after four days, in each culture condition.

Figure 5.4.2 *Loss of cytokine production in activated DO11.10 CD4 T cells cultured in the presence of $OVA_{323-339}$ and a CTLA-4-Ig*. Day three activated DO11.10 cells were isolated as described in Figure 5.4.1. 10^4 of these cells per well were seeded into 96-well V-bottom plates. 3 x 10^4 T cell depleted splenocytes per well were plated as APC. Wells were either cultured without antigen (no peptide), with 0.3μM $OVA_{323-339}$ alone ($OVA_{323-339}$), or with 0.3μM $OVA_{323-339}$ and 10μg/mL CTLA-4-Ig. (A) On the day of isolation and on days two and four of culture, the $OVA_{323-339}$ specific cytokine producing cells were enumerated by ELISpot. (B) The day four data in panel A is redisplayed for statistical analysis. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. These are pooled results from three independent experiments.

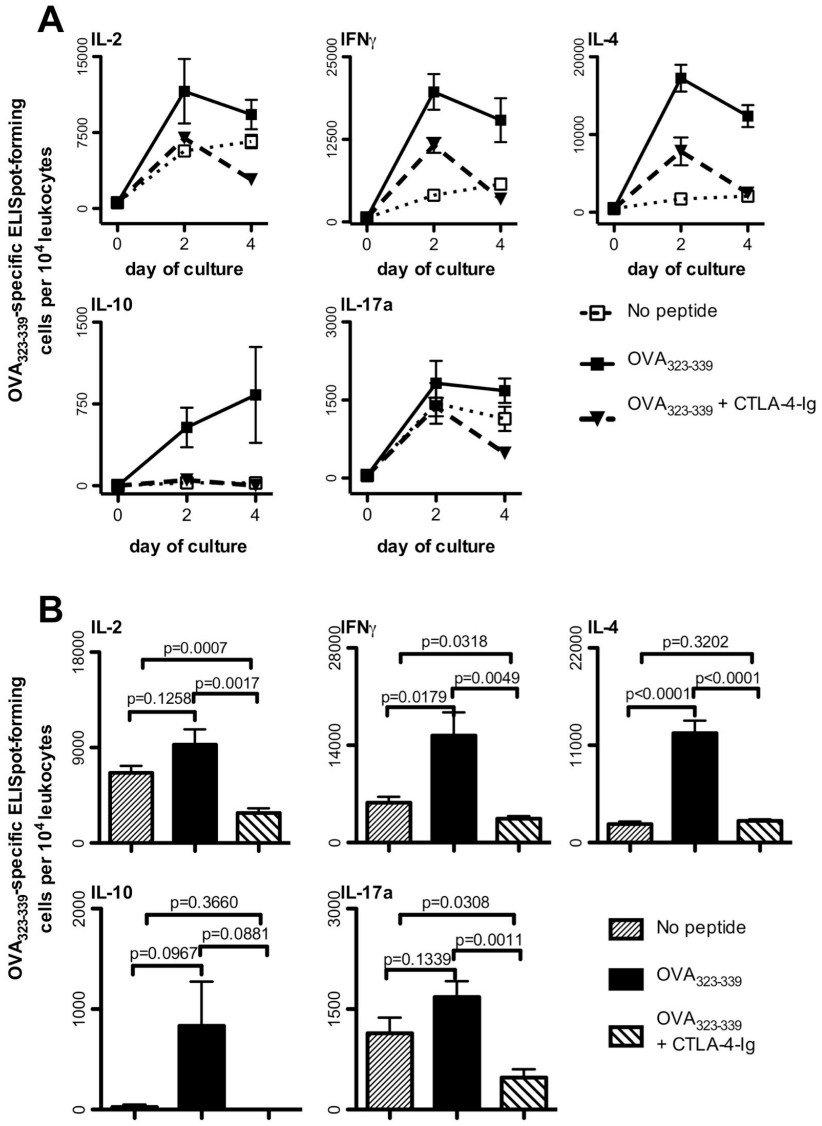

A
IL-2
IFNγ
IL-4
IL-10
IL-17a
day of culture
OVA323-339-specific ELISpot-forming cells per 10^4 leukocytes
No peptide
OVA323-339
OVA323-339 + CTLA-4-Ig
B
p=0.0007
p=0.0017
p=0.1258
p=0.0318
p=0.0049
p=0.0179
p=0.3202
p<0.0001
p<0.0001
p=0.3660
p=0.0881
p=0.0967
p=0.0308
p=0.0011
p=0.1339

Figure 5.4.2

We observed evidence of increased apoptosis in cells cultured with peptide and CTLA-4-Ig. Levels of Annexin V staining were higher, indicating that these cells had a higher amount of phosphotidylserine on their surface, a marker that precedes apoptosis (Vermes, Haanen et al. 1995). DO11.10 cells that were cultured with peptide and CTLA-4-Ig had also down-regulated the anti-apoptotic protein BCL-2. These cells also showed evidence of decreased activation, as indicated by lower levels of GITR on the surface and lower levels of intracellular BLIMP-1, a transcriptional regulator that is highly expressed in effector-phase CD4 T cells (Fazilleau, McHeyzer-Williams et al. 2009), compared with DO11.10 cells from cultures without peptide or with peptide alone. Additionally we observed similar levels of PD-1 expression, on the surface of DO11.10 cells from cultures with peptide alone or with peptide with CTLA-4-Ig, indicating that PD-L-mediated regulation did not play a major role in this system. Moreover, we did not find expression of FoxP3 in any of the DO11.10 cells analyzed, making it unlikely that a transition to Treg occurred as a result of any of the culture conditions. Additionally, the numbers of DO11.10 CD4 T cells in cultures treated with peptide alone, or with peptide with CTLA-4-Ig, did not differ by more than two-fold (not shown), suggesting that inactivation, rather than a difference in proliferation, was mainly responsible for the difference in numbers of cytokine-producing T cells between these two cultures.

Taken together, the decreased ability to produce cytokine and the expression of cellular markers of inactivation, in previously activated DO11.10 cells cultured with $OVA_{323-339}$ and CTLA-4-Ig, lead us to suggest that these conditions do indeed inactivate activated CD4 T cells. Thus both our *in vivo* evidence, and our evidence from this culture system, pointed to a mechanism of tolerance induction, in activated CD4 T cells, that is very similar to that of naive CD4 T cells. In order to take our analysis further, we decided to combine elements of both of these systems, the obvious relevance of *in vivo* observations, and the ready activation of TCR-transgenic CD4 T cells *in vitro*, in our subsequent studies.

Figure 5.4.3 *Activated DO11.10 T cells cultured with $OVA_{323-339}$ and CTLA-4-Ig show signs of inactivation*. CD4+ DO11.10 T cells were cultured as described in Figure 4.4.2. On day four of culture, cells were harvested and analyzed for their expression of the indicated surface markers by flow-cytometry. All histograms are gated on cells expressing the DO11.10 TCR. Grey histograms show the level of fluorescence of cells stained with a matched fluorophore-conjugated isotype-matched control antibody. Dotted histograms show the expression of marker on DO11.10 cells cultured without $OVA_{323-339}$; solid black histograms show expression on those cells cultured with 0.3μM $OVA_{323-339}$ alone; Dashed histograms are show expression of the indicated markers on DO11.10 T cells cultured with 0.3μM $OVA_{323-339}$ and 10μg/mL CTLA-4-Ig. These are the results of a single representative experiment of three.

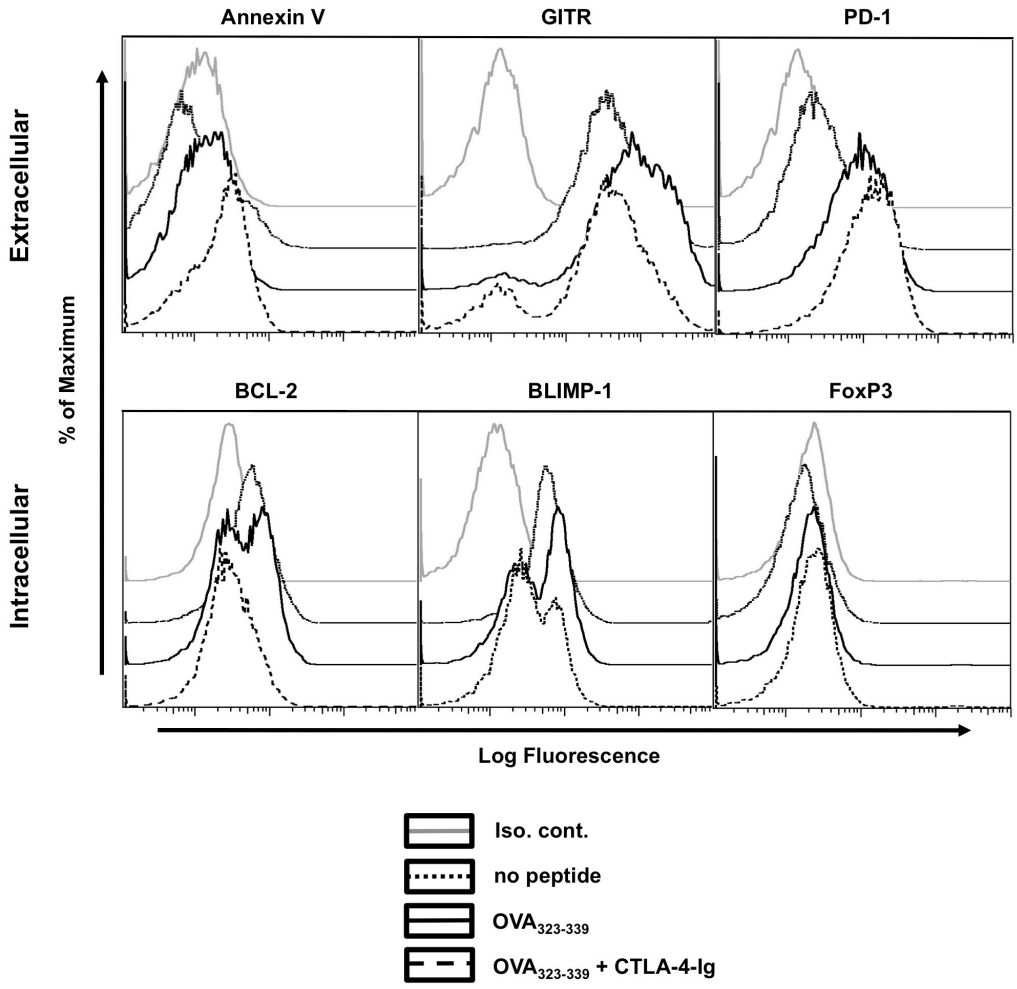

Annexin V
GITR
PD-1
BCL-2
BLIMP-1
FoxP3
Extracellular
Intracellular
% of Maximum
Log Fluorescence
Iso. cont.
no peptide
OVA323-339
OVA323-339 + CTLA-4-Ig

Figure 5.4.3

5.5 Tolerance Induction in Activated DO11.10 CD4 T Cells: II. Adoptive Transfer

Having established that activated CD4 T cells are rapidly inactivated upon exposure to antigen in the absence of appropriate second signals, *in vivo* and *in vitro*, we wished to see whether we could determine the nature of the critical second signal that would prevent inactivation of activated CD4 T cells *in vivo*. We decided to use a system in which we inactivate (through high-dose peptide injection), adoptively transferred, *in vitro* activated, DO11.10 CD4 T cells. Figure 5.5.1 displays pooled results from three independent experiments demonstrating that day three in vitro activated DO11.10 CD4 T cells are readily inactivated following adoptive transfer and administration of high-dose (300μg) $OVA_{323-339}$. As expected from our culture studies, the number of $OVA_{323-339}$-specific cytokine-producing CD4 T cells in the spleen of mice given 10^6 activated DO11.10 cells and $OVA_{323-339}$, intravenously, is decreased dramatically after only four days. Having established a protocol wherein activated CD4 T cells could be readily inactivated *in vivo*, we attempted to investigate whether we could rescue these cells from inactivation.

We hypothesized that systemic activation of APC, by anti-CD40 administration, would result in the prevention of inactivation in mice adoptively transferred with activated DO11.10 T cells and given high-dose peptide. We make this hypothesis because in a previous study administration of an agonistic antibody to CD40 prevented inactivation of CD4 T cells (Hawiger, Inaba et al. 2001). In addition, both B cells and DC express CD40 constitutively and it is known that stimulation through CD40 results in activation of these cells to express high-levels of co-stimulatory molecules such as B-7. We hypothesized that following administration of the agonistic antibody virtually all MHCII-bearing cells would present $OVA_{323-339}$ with co-stimulation and thus provide both signal one and signal two together and thus rescue CD4 T cell inactivation . Figure 5.5.2 shows the results of such an experiment.

Figure 5.5.1. *Adoptively transferred activated CD4+ DO11.10 T cells are inactivated upon administration of high-dose $OVA_{323-339}$.* CD4+ DO11.10 T cells were activated and isolated as in Figure 5.4.1. 10^6 of these cells were then adoptively transferred by intravenous injection into normal BALB/c mice. Following adoptive transfer, mice were either treated with saline or were given a single dose of 300μg $OVA_{323-339}$ intravenously. Four days later the $OVA_{323-339}$ specific cytokine producing cells in the spleen were enumerated by ELISpot. Each data point represents the number of specific ELISpot-forming cells in the spleen of a single mouse. P-values indicate the probability that the means of the indicated samples are not different as assessed by unpaired, two-tailed T-tests. These are pooled results from three independent experiments with three mice/group (n = 9).

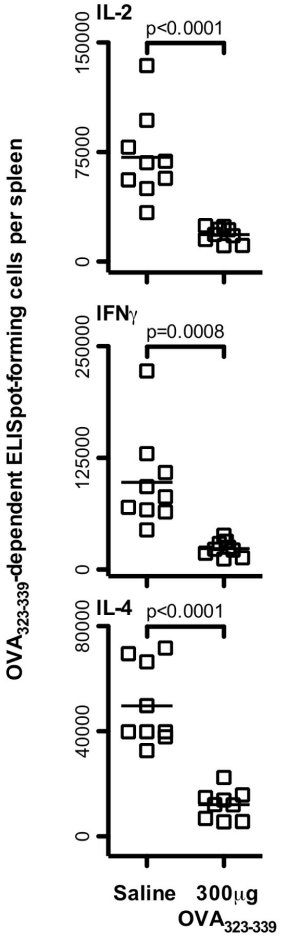

IL-2
p<0.0001
150000
75000
0
IFNγ
p=0.0008
250000
125000
0
IL-4
p<0.0001
80000
40000
0
OVA323-339-dependent ELISpot-forming cells per spleen
Saline
300μg
OVA323-339

Figure 5.5.1

We found that administration of 100μg FGK45 (a CD40 agonist) to mice given both activated DO11.10 cells, and high-dose peptide, completely prevented the inactivation of these activated CD4 T cells. We also reasoned that we could restore inactivation when in mice given FGK45 by blocking B-7 molecules with 100μg CTLA-4-Ig because CD40 ligation is known to enhance the expression of B-7 molecules on APC. Surprisingly we observed only a partial restoration of tolerance induction in mice treated this way and the IL-2 and IFNγ-producing $OVA_{323-339}$-specific cells in the spleen were not affected by administration of CTLA-4-Ig. We observed a partial decrease in the numbers of IL-4 producing cells in the spleen of mice treated with FGK45 and CTLA-4-Ig, suggesting that B-7 interactions may be more heavily involved in IL-4 production, a conclusion consistent with findings of Rudulier et al. (Rudulier, Kroeger et al. 2012), and others (Andres, Howland et al. 2004). We did not attempt to increase the level of CTLA-4-Ig given to these mice, however, blocking of B-7 molecules with even 100-fold less CTLA-4-Ig has been shown to substantially decrease primary CD4 T cell activation *in vivo* (Linsley, Wallace et al. 1992). This finding indicated to us that second signals, other than B-7 molecules alone, could be induced on APC, in the mice treated with FGK45.

Since others (see Chapter 4), have shown that CD40 stimulation of B cells leads to their expression of OX40L, we decided to test whether blocking OX40 signaling would restore tolerance induction in mice treated with FGK45. Significantly, we observed a nearly complete restoration in inactivation of activated CD4 T cells in mice treated with both FGK45 and a blocking antibody to OX40L. IL-4 producing cells appeared to be the least affected by the administration of this blocking antibody. We also addressed whether administration of an agonistic antibody to OX40 could reverse tolerance induction. We found that the administration of an OX40 agonistic antibody completely inhibited inactivation of activated DO11.10 CD4 T cells, thus confirming the plausibility of the interpretation that the major effect of FGK45 is to up-regulate OX40L expression. Taken together our results seem to indicate that both B-7 molecules and OX40L play a central role in maintaining activation of CD4 T cells exposed to peptide-MHCII. In other words, it appears that these two molecules are critical in prevention of tolerance upon TCR ligation. Moreover, we speculate that IL-4 production depends more

heavily on B7-CD28 interactions, while overall persistence of effector function depends more heavily on OX40-OX40L interactions.

Given our findings regarding the role of OX40-OX40L interactions in T cell cooperation, the results shown in Figure 5.5.2 are very interesting. The natural means of stimulation of APC through CD40 is by provision of CD40L by activated T cells. Thus our data lead us to suggest that cooperative interactions, mediated through CD4 T cell interaction with antigen-bearing APC, possibly lead to sustained CD4 T cell activation, resulting in the prevention of inactivation. However, we have not been able to directly test this hypothesis at the time of writing this thesis.

5.6 Conclusions

Here, we present data detailing the inactivation of CD4 T cells by the administration of high doses of soluble peptide. We found that tolerance induction, even in the face of stimulation with the potent adjuvant, CFA, results from such administration. We further demonstrate that CD4 T cells, deliberately activated, can be inactivated by the systemic administration of peptides that stimulate these cells through the TCR. We demonstrate that limiting B-7-CD28 interactions *in vitro*, when activated CD4 T cells are exposed to peptide-bearing APC results in their inactivation as assessed by cytokine production and by the expression of certain molecular markers of T cell inactivation. We demonstrate that activated CD4 T cells can be rescued from inactivation, *in vivo*, by the simultaneous activation of APC by anti-CD40 antibodies. This rescue depends on the expression of B-7 and, importantly, of OX40L, presumably by APC. We also propose that cooperative interactions, through the induction of OX40L on APC such as B cells, might prevent inactivation of CD4 T cells, which could lead to sustained activation of these cells. We conclude that effector CD4 T cells can be readily inactivated under appropriate circumstances.

Figure 5.5.2. *Agonist antibodies to CD40 prevent inactivation in adoptively transferred activated DO11.10 T cells and this prevention depends on the induction of OX40L and B-7 molecules*. Mice were seeded with day three activated CD4+ DO11.10 cells and treated with 300μg of $OVA_{323-339}$ as in the experiment whose results are recorded in Figure 5.5.1. The results from figure 5.5.1 are redrawn as bar graphs proximal to the Y-axis for comparative purposes. At the time of injection some $OVA_{323-399}$-treated mice received 100μg of the CD40 agonistic antibody, FGK45 (CD40 agonist), or 100μg of the agonistic antibody to OX40, OX86 (OX40 agonist). We did not observe any effect of administration of 100μg of isotype-matched antibodies with irrelevant specificity so responses in these mice were excluded for clarity. Some of the mice treated with FGK45 were also treated with either saline or 100μg isotype-matched control antibodies (iso. cont.), 100μg of the OX40L blocking antibody, RM134L (OX40L blocker), or with a non-cytolytic CTLA-4-Ig fusion protein (B7 blocker). On the fourth day after injection the $OVA_{323-339}$ specific cytokine-producing cells in the spleen were enumerated by ELISpot. Each data point represents the number of specific ELISpot-forming cells in the spleen of a single mouse. These are pooled results from between one and four independent experiments with three mice/group (n = 3-12).

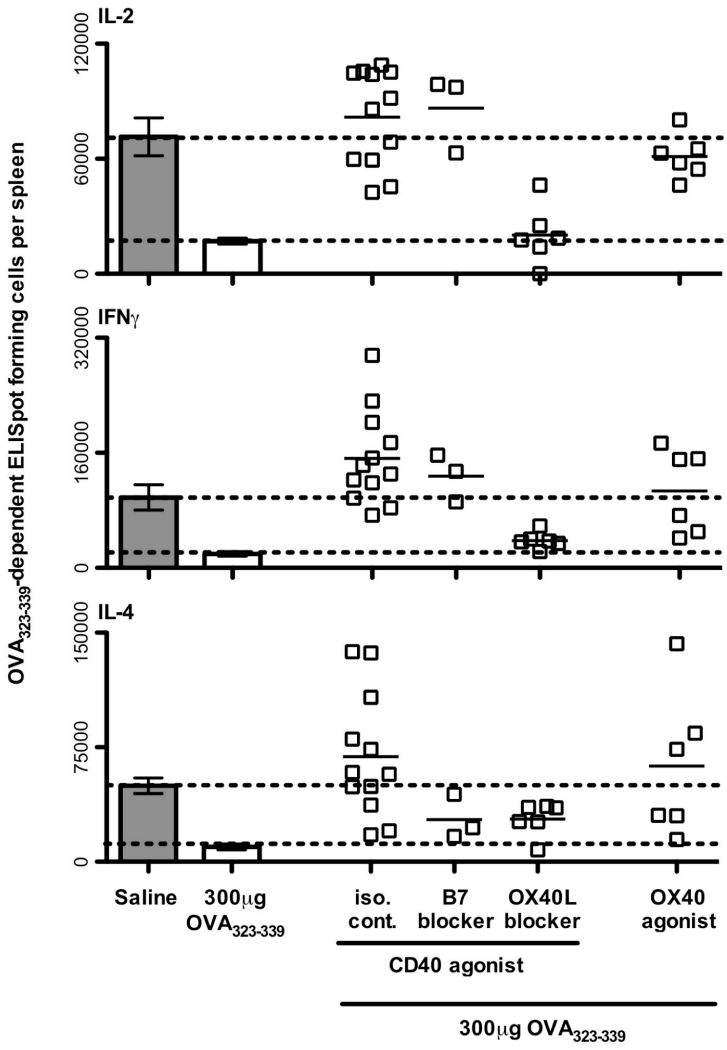

IL-2
120000
60000
0
IFNγ
320000
160000
0
IL-4
150000
75000
0
OVA323-339-dependent ELISpot forming cells per spleen
Saline
300μg OVA323-339
iso. cont.
B7 blocker
OX40L blocker
OX40 agonist
CD40 agonist
300μg OVA323-339

Figure 5.5.2

This conclusion is somewhat inconsistent with our previous working model of CD4 activation and inactivation. Both the two-step, two-signal model and the conventional Lafferty and Cunningham-style models for the initial activation of CD4 T cells into effector cells lead to the assumption that once activated, effector CD4 T cells are not subject to further regulation by inactivation. This assumption has led to extensive debate, as it would seem to also apply to aberrantly activated autoreactive T cells which would lead to prevalent autoimmunity. Due to considerations such as these, some have proposed that the activation of CD4 T cells requires additional priming steps that involve antigen-specific cooperation of CD4 T cells (Bretscher 1999). While our results do not support this model in its original form, they do suggest that cooperative interactions between CD4 T cells could be important in sustaining the activation of such cells under conditions that would normally result in their inactivation. Explicitly, these conditions would be normally met by the presentation of self-peptides by resting APC.

As will be further discussed in Chapter 6, our findings have important implications for the generation of autoimmunity. Since normally the majority of APC that present self-peptides will be in a constitutively inactive state, if autoreactive CD4 T cells are activated by APC presenting self-peptides in association with appropriate signals, then they will be continually inactivated by other APC that present self-peptides without the appropriate signals. Though autoimmunity is rare, it does occur, and is associated with prolonged inappropriate activation of autoreactive CD4 T cells, and epitope spreading (see Chapter 1). We hypothesize that, under certain circumstances, cooperative interactions between CD4 T cells may lead to their sustained activation even in situations where the autoreactive cells would normally be inactivated. This could occur when a sufficient number of APC, presenting self-peptides, are activated by T cells, shifting the balance of tolerance/activation towards activation by the expression of B-7 and OX40L.

The results obtained through the experiments shown in this chapter led us to a dramatic rethinking of our working model for CD4 T cell activation/inactivation. Our current view encompasses elements of both conventional models and the two-step two-signal model. A very brief outline of this model is given here.

Naive CD4 T cells can be activated by an appropriately activated APC, such as DCs, through peptide presentation in association with appropriate co-stimulation. These

activated effector CD4 T cells, may then cooperate with one another in antigen-mediated interactions with antigen-specific B cells. These cooperative interactions lead to enhanced activation, effector function, and survival of CD4 T cells. Autoreactive CD4 T cells will normally encounter autopeptides presented by resting APC, leading to the inactivation of these autoreactive CD4 T cells. In the event that autoreactive CD4 T cells first encounter peptides presented by activated APC, the CD4 T cells are activated. However, the majority of autoantigen-presenting cells will be resting, so activated autoreactive CD4 T cells will normally be quickly inactivated as they circulate or upon removal of inflammatory stimuli. Persistent autoimmunity may occasionally arise when sufficient numbers of autoreactive CD4 T cells are activated so that their cooperative activation with autoantigen-bearing B cells, and one another, results in sustained effector function. The relatively few autoreactive CD4 cells that exist in the periphery, combined with the overall tendency to remove autoreactive CD4 T cells and autoreactive B cells will mean that these circumstances will rarely be achieved. This model is consistent with the vast majority of current literature as well as our own observations. Moreover, it makes physiological sense in that it allows rapid responses to foreign antigens, particularly infectious agents, and at the same time minimizes the sustained generation of autoreactive CD4 T cells.

6.0 CHAPTER 6 – DISCUSSION

6.1 General Summary

The findings presented in this thesis support the hypothesis that CD4 T cells, following initial activation, cooperate with one another to enhance their own activation. I have detailed the results of experiments that attempt to determine the cellular and some of the molecular interactions involved in this cooperation. It appears that, in order for cooperative interactions between CD4 T cells to occur, the peptides for which they are specific must be presented by the same APC. This requirement for linked recognition suggests that specific uptake of antigen by the APC could be involved in influencing CD4 T cell cooperation. Indeed, I have presented evidence that B cells, which normally acquire antigen through the BCR, are efficient mediators of the cooperative interaction between CD4 T cells. It appears that DCs, or DC enriched populations, while potent in activating effector CD4 T cells, do not mediate cooperation between CD4 T cells. Artificial stimulation of B cells, *in vitro*, through an agonist antibody to CD40, leads to enhanced generation of peptide-specific cytokine producing CD4 T cells following adoptive transfer of these peptide loaded B cells. Furthermore, blocking OX40L impairs cooperative generation of CD4 T effector cells when B cells alone present peptides. The interaction between B cells and CD4 T cells appears to depend heavily on OX40-OX40L interactions, although we did not find evidence that OX40L was specifically induced on B cells following their interaction with CD4 T cells. Taken together, these findings lead to a model of CD4 T cell interaction whereby CD4 T cells, interacting via the provision of CD40L, enhance the ability of B cells to further stimulate CD4 T cells. This model is depicted in Figure 6.1.1.

Figure 6.1.1. *Model of proposed cellular interactions involved in CD4 T cell cooperation*. Cooperation between CD4 T cells may involve an initial priming stage (I) that is dependent on antigen presentation by DC bearing peptide:MHCII complexes and B-7 molecules. A secondary cooperation stage (II) could involve the activation of antigen presenting B cells by activated CD4 T cells through CD40-CD40L interactions which leads to upregulated OX40L on the surface of the B cells. OX40-OX40L interactions between CD4 T cells and B cells and between CD4 T cells may serve to further activate the CD4 T cells leading to enhanced proliferation, cytokine production, Th2 differentiation and survival.

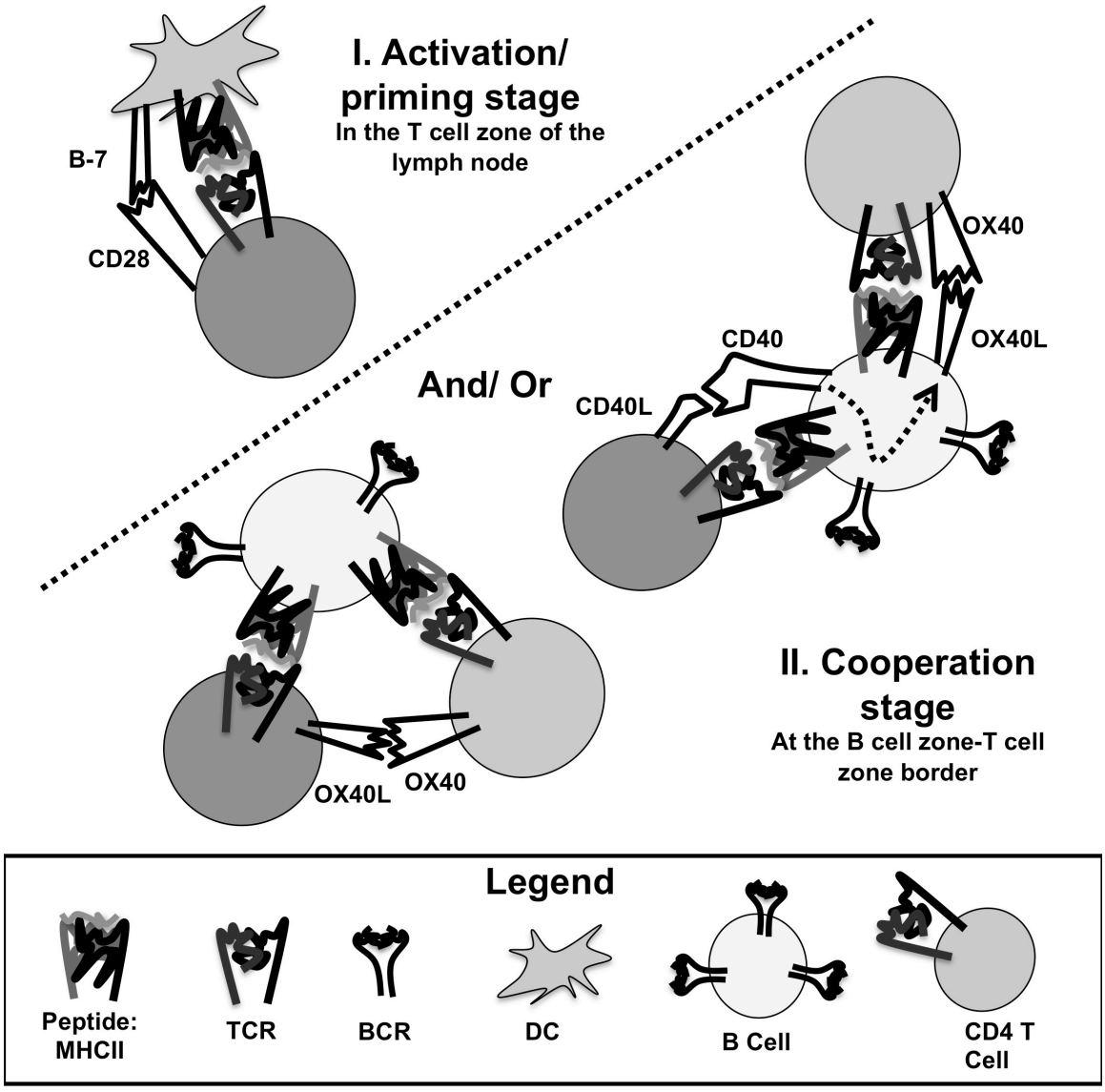

I. Activation/
priming stage
In the T cell zone of the
lymph node
B-7
CD28
And/ Or
OX40
CD40
OX40L
CD40L
II. Cooperation
stage
At the B cell zone-T cell
zone border
OX40L
OX40
Legend
Peptide:
MHCII
TCR
BCR
DC
B Cell
CD4 T
Cell

Figure 6.1.1

Given the roles of the CD40-CD40L and the OX40-OX40L interactions in cooperation between CD4 T cells and different APC, it is tempting to consider whether the observations detailed in the final experimental chapter can be viewed in the light of CD4 T cell cooperation. We show that inactivation of activated, cytokine producing CD4 T effector cells can be achieved in the periphery, by the administration of high doses of the peptide to which they respond. Inactivation can be prevented by simultaneous administration of agonist antibodies to CD40, and the prevention of inactivation by CD40 ligation appears to depend on the induction of B7 and OX40L expression. These findings lead us to connect the phenomena of CD4 T cell cooperation and the development of autoimmunity. Thus, cooperation between CD4 T cells can potentially lead to sustained survival of autoreactive T and B cells. This suggestion has the merit of accounting in a natural fashion for the phenomenon of epitope spreading.

The results presented here, as a whole, shed light on questions surrounding the involvement of CD4 T cells in the determination of their own fate. Whereas conventional models for the activation of CD4 T cells place emphasis on the DC-T cell interaction, these models cannot account for our findings. We show that CD4 T cells influence one another via their interaction with B cells, likely following initial priming by DCs. The interaction of primed CD4 T cells with B cells, leading to antibody production, is a well-described phenomenon. Our data lead us to suggest that the interaction may be more heavily involved in the generation of effector CD4 T cells than is currently believed.

6.2 Discussion

6.2.1 Administration of Peptides in IFA

Over the course of my thesis work, two separate experimental systems were developed to observe cooperation between CD4 T cells. In the first system, detailed in Chapter 3, we employed synthetic peptides, known to be recognized by BALB/c CD4 T cells and co-administered them in IFA. In employing such a methodology, we were uncertain about the populations of cells that would present these peptides. Given the

properties of water-in-oil emulsion adjuvants, such as IFA, it is not surprising that multiple soluble peptides could be delivered to the same APC, if incorporated into the same micelle. However, in light of our finding that B cells efficiently mediate cooperation between CD4 T cells, peptides, incorporated into our IFA emulsion, should have been available in solution as well. It is difficult to envisage that B cells, with affinity for components of IFA, preferentially took up micelles containing peptide. Thus, we are left to suppose that B cells, present in lymph nodes that receive afferent lymph from the site of the IFA depot, acquire peptides from that lymph, that bind directly to the empty MHCII molecules on the cell surface. In order for efficient cooperation to occur, both peptides should interact with MHCII molecules on the surface of the same B cell. Given these requirements, it is remarkable that cooperation was observed under these experimental conditions.

Our observations were not the first to show cooperation between T cells that respond to multiple peptides given in IFA. Valmori et al. have reported very similar findings, showing a cooperative interaction between CD4 and CD8 T cells, in the induction of CTL. This was achieved when MHCII- and MHCI-binding peptides were administered together in IFA (Valmori, Romero et al. 1994). However, our findings that blocking OX40L inhibits only the cooperation-dependent increase in the generation of effector CD4 T cells specific for $HEL_{105-120}$ when this peptide is given with others in IFA, and that this antibody inhibits the generation of all effector CD4 T cells when B cells are the sole APC, leads us to suggest that presentation by both non-B APC as well as B cells must occur when these peptides are administered in IFA. Moreover, it appears that the presentation of peptides by these non-B cell APC, likely DCs, does not depend on the OX40-OX40L interaction. That is, upon immunization with peptides in IFA, the peptides are presented by both DCs and B cells. DCs may initiate priming of CD4 T cells, while B cells mediate cooperation between them, and the latter process depends on OX40L.

It can be seen that, for the most part, cooperative interactions between CD4 T cells, specific for administered peptides, resulted in the increased generation of only $HEL_{105-120}$-specific cytokine producing cells. To what extent the results reported in this thesis can be generalized is therefore unclear. Since the development of $OVA_{323-339}$-specific CD4 T effector cells did not benefit from cooperation from $HEL_{105-120}$-specific

CD4 T cells, it is possible that different populations of CD4 T cells differ in their susceptibility to cooperative enhancement. Though we have no reason to believe that $HEL_{105-120}$-specific CD4 T cells are unique in their requirements for activation, we have no evidence that these cells are not different than the CD4 T cells specific for $OVA_{323-339}$. Conversely, $OVA_{323-339}$-specific CD4 T cells may not require cooperation from other peptide-specific CD4 T cells because they themselves are unique. $OVA_{323-339}$-specific CD4 T cells could be previously activated (potentially due to cross reaction with other environmental antigens) in our BALB/c mice. Previous activation may abrogate the need for cooperative interactions in the generation of optimal responses. Alternatively, the precursor frequency of $OVA_{323-339}$-specific CD4 T cells may be higher than that of $HEL_{105-120}$ specific CD4 T cells. This increased precursor frequency may lead to intraclonal cooperation, which would mask the contribution of CD4 T cell cooperation in the generation of $OVA_{323-339}$-specific effector cells. CD4 T effector cells specific for $LACK_{158-173}$ also appeared to be generated well in the absence of explicit CD4 T cell cooperation. It is interesting to note that both $OVA_{323-339}$ and $LACK_{158-173}$ are I-A^d binding peptides, while $HEL_{105-120}$ binds I-E^d. Therefore it is possible that the cells specific for $OVA_{323-339}$ and $LACK_{158-173}$ are more efficiently generated, either by enhanced positive selection by I-A^d or preferential TCR rearrangement, leading to enhanced precursor frequency. On the other hand, HEL_{11-25} is an I-A^d-binding peptide which does not appear to result in efficient generation of CD4 T effector cells, when given alone in IFA. Though we did not formally test whether cooperation can occur between naive $HEL_{105-120}$ and HEL_{11-25}-specific CD4 T cells, our observations (see Figure 3.3.3) suggest that this interaction would result in enhanced generation of effector cells specific for both peptides. Thus, as we have no reason to suspect that the CD4 T cells specific for $HEL_{105-120}$ are in some way unique in their requirement for cooperation, for their optimal transition to effector cells, we also have no direct evidence to the contrary. Further work will be required to demonstrate that the CD4 T cell cooperation observed here is a general phenomenon.

Oil emulsion adjuvants are currently in use for human vaccination and have been shown to elicit the transcription of a number of inflammation-associated transcripts (Mosca, Tritto et al. 2008). Our results, indicating that supplementing IFA with LPS, or

giving an agonistic antibody to CD40, does not result in enhanced production of peptide-specific cytokine producing CD4 T cells, when peptide alone is incorporated into the vaccine, are consistent with this finding. In other words, it appears that the IFA immunization itself results in sufficient inflammation to enable the activation of innate APC. Our findings are direct evidence that the inefficient activation of $HEL_{105-120}$ specific CD4 T cells, when mice are immunized with the peptide alone in IFA, is not due to a lack of stimulation of APC by inflammatory mediators. However, the findings that systemic administration of FGK45 does not enhance the generation of $HEL_{105-120}$-specific cytokine producing cells, whereas stimulation of B cells *in vitro* with FGK45 allows more efficient activation of $HEL_{105-120}$-specific CD4 T cells, upon their adoptive transfer, seem to be at odds with one another. As I have outlined above, giving peptides in IFA, should lead to their presentation by B cells. Therefore, administration of FGK45 to mice immunized with $HEL_{105-120}$ alone in IFA should result in the presence of B cells with increased ability to enhance the generation if $HEL_{105-120}$-specific effector cells. However, we did not observe such enhancement. In order to explain this discrepancy, I speculate that, in the case of systemic FGK45 administration, a vast excess of APC are activated to express co-stimulatory molecules, chemokines, and adhesion molecules that increase the frequency and duration of APC-T cell interactions. Many of these interactions will be unproductive as they will be between either peptide-specific CD4 T cells and APC that are not presenting $HEL_{105-120}$, non-specific CD4 T cells and APC that do present peptide, and between non-specific CD4 T cells that interact with APC that are not presenting peptide. Normally, the interaction between APC and T cells is made more efficient by the expression of molecular mediators and homing receptors that bring activated peptide-bearing APC in contact with T cells. In situations where many APC are activated but do not present the appropriate peptide, i.e. following systemic administration of FGK45, the generation of peptide-specific CD4 T cells may actually be inhibited. The generation of cytokine producing CD4 T cells was enhanced when only a limited number of pulsed, FGK45 stimulated, B cells were given because all the these APC were presenting peptide. Thus, the discrepancy between our two seemingly contradictory observations made with FGK45 may be resolved. However, FGK45 administration, when HEL protein was given in IFA, enhanced the generation of HEL-specific CD4 T effector cells. It is

possible that, by virtue of their efficient uptake of protein antigen, by way of the BCR, that HEL-specific B cells were able to focus interactions between HEL-peptide-specific CD4 T cells, thereby bypassing the inhibition seen when peptide was given in IFA. However, these possibilities will remain in the realm of speculation.

The most significant finding made in the IFA administration system is that CD4 T cells, specific for $OVA_{323-339}$, had to be present so that co-administration of $OVA_{323-339}$ could enhance the generation of $HEL_{105-120}$-specific cytokine producing CD4 T cells. Given the results shown in Figure 3.5.3, that administration of OX86 restored the effector function of inactivated $HEL_{105-120}$-specific CD4 T cells, we assume that high-dose administration of $OVA_{323-339}$ resulted in the induction of anergy of endogenous $OVA_{323-339}$-specific CD4 T cells. Therefore, our conclusions can be extended. Functional, non-anergic, CD4 T cells, specific for $OVA_{323-339}$ are required for the cooperative enhancement of CD4 T cells specific for $HEL_{105-120}$ when both peptides are given together in IFA. This evidence led us to conclude that the observed cooperation, when peptides were given together in IFA, was due to genuine interactions between CD4 T cells and not simply to non-specific effects of our experimental immunization. In order to further explore the mechanisms underlying the cooperation of CD4 T cell populations we developed a further experimental system.

6.2.2 The Peptide-Pulsed APC System

The development of a "pulsing-system" wherein the adoptive transfer of APC, loaded with peptide in culture, could be employed to elicit cooperation between CD4 T cell populations, was a major advance in our methodology. This system allowed us to inquire into the nature of the APC that mediates CD4 T cell cooperation, as well as into the molecular signaling that is involved in this interaction. In this system mice were seeded with 10^5 purified $CD4^+$ DO11.10 $OVA_{323-339}$-specific TCR transgenic T cells. The generation of $HEL_{105-120}$-specific effector cells, in mice given APC pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$, depends on the number of DO11.10 cells given (Figures 4.2.6 and 4.2.7). This data in itself is evidence that DO11.10 T cells can cooperate with HEL_{105-}

$_{120}$-specific CD4 T cells upon the administration of pulsed APC, enhancing the generation of $HEL_{105-120}$-specific effector cells.

Our investigations on the nature of the APC responsible for mediating cooperation between populations of CD4 T cells identified the B cell as this mediator. Two lines of evidence support this conclusion.

Firstly, as shown in Figure 4.3.1, cooperative interactions between $HEL_{105-120}$ and $OVA_{323-339}$-specific CD4 T cells require the peptides to be presented by the same cell. As discussed in sections 1.3 and 1.4, presentation by the antigen-specific B cell is viewed as the most efficient way that such "linked" interactions can be mediated. Normally, antigen-specific B cells endocytose antigen after having encountered it through the BCR. Therefore, only antigens that are physically associated with the antigen, recognized by the BCR, will be presented by the B cell. In the context of CD4 T cell cooperation and infection, this mechanism would ensure that only CD4 T cells specific for peptides derived from the same pathogen would cooperate with one another, while CD4 T cells specific for peptides derived from other pathogens, self molecules, or allergens etc. would not be affected. This type of mechanism for ensuring independence of immune responses is potentially important in avoiding the influence of CD4 T cell cooperation on the immune phenotype of responses to simultaneous infection with other pathogens or on the survival of autoreactive T cells that are present in the periphery. It is difficult to envisage how this type of independence could be accomplished by non-specific APC, like DCs.

The second line of evidence, supporting the role of the B cell in mediating CD4 T cell cooperation, is more direct. We isolated B cells from cultures of pulsed spleen APC. These B cells, untouched by our depletion protocol, were found to be at least 95% pure. B cells, pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$ mediated cooperation between CD4 T cells that recognize these peptides in the context of MHCII. Thus, positive evidence, in a reductionist model, indicates that B cells can directly mediate cooperation between DO11.10 T cells and endogenous $HEL_{105-120}$-specific CD4 T cells.

We attempted to address whether potent, non-antigen-specific APC, the conventional DCs and plasmacytoid DCs from the spleen, could mediate cooperation between CD4 T cells. Although our isolation protocol resulted in very poor purity, we,

nevertheless, observed substantial activation of CD4 T cells after injection of only 2 x 10^5 peptide-pulsed DC-enriched cells. According to our flow-cytometry results this corresponds to a number of injected DC on the order of 5-10 x 10^4. These cells elicited robust CD4 effector T cell activation, equivalent to that elicited from the injection of 3.5 x 10^6 B cells, but failed to elicit cooperation between $HEL_{105-120}$ and $OVA_{323-339}$-specific CD4 T cells. The potency of the DCs, in activating CD4 T cells, suggests that these cells are functional. However, though we think it unlikely, we cannot rule out the possibility that our culture or isolation protocol affected the spleen DCs in a manner that left their ability to activate CD4 T cells intact, while disrupting their ability to mediate cooperation between CD4 T cells.

We attempted to investigate the molecular mechanisms involved in this CD4 T cell cooperation mediated by B cells. Since CD4 T cells have the capacity to stimulate B cells through provision of CD40L, we attempted to mimic the effects of this stimulation by administration of the agonistic FGK45 antibody in culture. We found that ligation of CD40 in this artificial way enhanced the ability of single-peptide-pulsed B cells to activate $HEL_{105-120}$-specific CD4 T cells. B cells cultured with DO11.10 CD4 T cells in the presence of $OVA_{323-339}$ did not appear to enhance the activation of $HEL_{105-120}$-specific CD4 T cells. However, we observed a similar trend as was observed for FGK4-simulated cells. Given the complexity of this system, it is likely that optimal conditions for B cell activation are not achieved by this protocol. Therefore further optimization of this protocol may be beneficial in further developing this system.

Since we had observed a role for OX40L in cooperative CD4 T cell priming, we hypothesized that the ligation of CD40, or activation by cognate B cell-T cell interaction, would lead to the upregulation of OX40L on B cells. Surprisingly, we observed no difference in the level of expression of OX40L-encoding mRNA in B cells cultured either in the presence or absence of anti-CD40 or in the presence or absence of appropriate CD4 T cell "help". Rather, our data indicated that all B cells, pulsed and cultured under our standard conditions, express similar levels of OX40L. We cannot rule out the possibility that regulation of OX40L protein expression occurred post-transcriptionally; further optimization of the experimental protocol may lead to different results. However, this finding is reflected in the results of our functional study. When purified B cells are the

only APC presenting peptide antigen, the OX40-OX40L interaction appears to be critical for the induction of IFNγ and IL-4-producing CD4 T cell responses, in either a cooperative or conventional manner. Evidence for this conclusion is seen in Figure 4.4.7. Interestingly, blocking OX40L resulted in impaired $OVA_{323-339}$-specific CD4 effector generation, for which a role for CD4 T cell cooperation was not observed. Thus, B cells appear to deliver the OX40L as a co-stimulatory molecule during the priming of CD4 T cells. Given that others have shown that OX40L expression on B cells is regulated, our findings could be, at least in part, artificial; the culture protocol employed may cause expression of OX40L on its own. However, it is unclear how this complication could be avoided. Functional studies, employing cells from co-stimulatory molecule knockout mice as APC, might yield more incisive results regarding the molecular mechanisms of CD4 T cell cooperation in the future.

The expression of co-stimulatory ligands is not restricted to APC. Both B-7.2 and OX40L can be expressed by CD4 T cells. Such findings have led to the suggestion that CD4 cells may stimulate one another via direct cell-to-cell contact (Soroosh, Ine et al. 2006; Rudulier, Kroeger et al. 2012). Though our experimental systems were not designed to differentiate APC-T cell interactions from T cell-T cell interactions, it appears that T cell-T cell interactions do not play a direct role in the cooperativity observed. The finding that DC-enriched APC appear not to mediate cooperation between CD4 T cells, while B cells do, suggests that cooperation is APC-specific. If the APC simply acts as a "dock", around which antigen-specific CD4 T cells can interact, then no APC preference should be observed. B cells could provide critical signals to CD4 T cells that then enable them to cooperate via T cell-T cell interaction. However, in the absence of direct evidence for this, Occam's razor would lead us, at least until such evidence is found, to disregard this possibility.

A potentially important and relevant observation, made during our pulsing studies, was that cooperation between CD4 T cells specific for $HEL_{105-120}$ and DO11.10 cells specific for $OVA_{323-339}$ generates enhanced $HEL_{105-120}$-specific memory. We observed greater numbers of $HEL_{105-120}$-specific cytokine secreting CD4 T cells, following a secondary challenge with HEL protein, in mice previously immunized with B cells pulsed with $HEL_{105-120}$ compared to mice that received B cells pulsed with the $OVA_{323-339}$.

However, we observed a significantly greater number of $HEL_{105-120}$-specific secondary effector cells following challenge in mice that received injections of B cells pulsed with both $HEL_{105-120}$ and $OVA_{323-339}$. Whether this striking result is due to enhanced survival of $HEL_{105-120}$-specific CD4 T cells, or it is due to enhanced potency of the memory cells produced, is unknown. However it is remarkable that cooperation between CD4 T cells specific for one peptide and CD4 T cells specific for another single peptide can lead to such dramatic differences in the secondary immune response. These data indicate that CD4 T cell cooperation, leading to enhanced generation of effector CD4 T cells, may also lead to enhanced memory T cell formation. This possibility has implications for vaccine design. Based on these results, it would appear that immunization with preparations containing few immunogenic epitopes would result in poorer memory than immunization with more epitopes. However, consideration should also be given to the ability of CD4 T cell cooperation to affect the Th1/Th2 nature of immune responses, if CD4 T cell cooperation is to be harnessed for vaccination purposes.

As outlined in section 1.3, CD4 T cell cooperation has been associated with Th1/Th2 immune deviation. Though this phenomenon was not addressed directly in my experiments, the results of experiments seem to demonstrate it. In virtually every case, the production of IL-4 is effected the most by cooperative interactions. That is, the number of IL-4 producing effector CD4 T cells is enhanced more than IL-2 or IFNγ-producing cells upon CD4 T cell cooperation. This finding is very much in line with previous investigations on the functional consequences of CD4 T cell cooperation, and with more current findings within our laboratory. We intend to investigate this more fully in a future study.

6.2.3 The Inactivation of Activated CD4 T cells

The third experimental chapter of this thesis details my investigations on the inactivation of activated CD4 T cells. Much of this work was done in collaboration with another member of the lab, Christopher Rudulier. As is shown in Chapter 5, we observed that activated CD4 T cells during their initial priming, or at the peak of their proliferation, could be inactivated by the intravenous administration of high doses of the peptide, for

which their TCRs have affinity. Three sequential doses of $HEL_{105-120}$, given to mice primed to $HEL_{105-120}$ in CFA, resulted in the inability to generate CD4 T cells specific for this peptide upon challenge with HEL protein.

We decided to determine whether this unresponsiveness was the result of enhanced activation induced cell death, after the peak of a normal CD4 T cell response, or was due to the active tolerogenic effects that directly inactivated $HEL_{105-120}$-specific effector cells. In mice that were immunized with $HEL_{105-120}$ in CFA, peptide-specific effector cells were inactivated within 3 days of a single intravenous injection of 300μg $HEL_{105-120}$. Similar results were obtained in a different experimental system in which activated CD4 T cells were adoptively transferred. Inactivation of activated DO11.10 CD4 T cells occurred within a four-day period. Though we did not attempt to track these cells by flow cytometry, this could be easily accomplished. Our functional readout was the number of peptide-dependent ELISpot forming cells in the spleen or lymph nodes. Therefore, our data that addressed whether the primary effector function of these cells, cytokine secretion, remained intact. Our data lead us to believe that activated CD4 T cells can be readily inactivated by the administration of high dose peptide. These findings, in line with the observations of others, suggest that the provision of excess signal 1 in the absence of co-stimulation results in inactivation of T cells at any time in their life cycle. However, in this system, we cannot rule out the possibility that "tolerized" effector cells migrated out of the spleen (or popliteal lymph nodes) and were therefore not detected. This possibility seems unlikely given the findings of Kearney et al. (Kearney, Pape et al. 1994) and our findings *in vitro*.

A culture system was developed to potentially address the mechanism by which activated CD4 T cells are inactivated *in vivo*. We demonstrate that blocking B-7 co-stimulation, by provision of a high concentration of CTLA-4-Ig effectively limits the cytokine production by activated CD4 T cells. Moreover, we show that CD4 T cells, cultured with antigen and CTLA-4-Ig express markers of cells undergoing apoptosis. This is in contrast to those cultured without antigen or with peptide alone. Given these results, we concluded that limiting signal 2, in the presence of excess signal 1, could potentially result in inactivation of effector CD4 T cells *in vivo*. We therefore chose to address this possibility, directly, *in vivo*.

Returning to our adoptive transfer system, we showed that the inactivation of activated DO11.10 effector CD4 T cells by the administration of high dose peptide can be blocked by systemic APC activation via the administration of FGK45. We hypothesized that this was due to enhanced expression of co-stimulation on the cells that present $OVA_{323-339}$. We attempted to demonstrate this by blocking co-stimulatory ligands. We determined that both B-7 and OX40L co-stimulation were responsible for the prevention of inactivation of activated CD4 T cells in our system. Interestingly, blocking B-7 molecules, following administration of high dose peptide and FGK45, appeared to severely limit only the production of IL-4 by activated DO11.10 cells. In contrast, blocking OX40L appeared to nearly completely prevent the ability of the DO11.10 cells to make IL-2 and IFNγ while leaving IL-4 production almost intact. These findings lead us to suggest that B-7 co-stimulation may be more important for IL-4 production, while OX40L appears to sustain production of IL-2 and IFNγ. These findings are consistent with some observations (Andres, Howland et al. 2004) and inconsistent with others (So, Song et al. 2006). At most, we conclude that B-7 and OX40L have a role in preventing the CD40-stimulation-mediated reversal of the inactivation of activated CD4 T cells by the administration of high dose peptide.

As our results are still preliminary, we are hesitant to draw firm conclusions. However, the results obtained in this experimental system suggest a mechanism by which activated CD4 effector T cells can be sustained *in vivo*, possibly leading to sustained autoimmunity. Stimulation of B cells via CD40 is normally achieved by activated CD4 T cells bearing CD40L. We speculate that if a sufficient number of activated autoreactive CD4 T cells were generated, then these cells could sustain their own activation though CD4 T cell cooperation. As discussed in section 1.3, the few autoreactive CD4 T cells that are activated following infection should normally be rapidly inactivated by subsequent interactions with self-peptide bearing resting APC. However, if sufficient numbers of activated autoreactive CD4 T cells exist in the periphery, or if these cells accumulate due to less efficient silencing, then B cells presenting the peptides for which they are specific, may be continually activated, leading to sustained levels of autoreactive T cells in the periphery. As suggested by Mamula, this initial stage of autoimmunity may

lead to a secondary stage wherein autoreactive B cell-T cell interactions may lead to epitope spreading and chronic disease (Mamula 1998).

As a whole, the data reported in this thesis support the idea that CD4 T cells play an active role in self- non-self discrimination. It appears that if sufficient numbers of CD4 T cells are activated, under appropriate conditions, optimal effector function will be achieved. By this mechanism, CD4 T cells require a level of agreement amongst clones, on whether to respond to an antigen or not. That is, in order for effective immune responses to be initiated, a threshold level of CD4 T cell activation must be crossed. In some cases, it appears that this agreement may lead to induction of autoimmunity. Therefore, for CD4 T cells, as for those who study them, rule by consensus can be dangerous.

7.0 CHAPTER 7 - REFERENCES

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