AN ABSTRACT OF THE THESIS OF

Amy R. Weatherill for the degree of Doctor of Philosophy in Microbiology presented on April 25, 2002.
Title: Investigations of T cell Costimulation and Autoimmunity in Mice, and Development of Flow Cytometric Methods to Assess Lymphocyte Stimulation in Dogs.

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Abstract Approved: _____
Jean A. Hall

Proper immune function is indispensable, as failure to mount an immune response against a pathogen can lead to serious complications or even death. T cells act by enhancing the activation of phagocytic cells as well as the activation of B cells. Their widespread influence on an immune response makes optimal T cell activation vital. Maximal T cell proliferation and survival is accomplished by stimulation with antigen, a costimulatory signal, and an adjuvant. However, excessive T cell activation can lead to chronic B cell activation and the production of autoantibodies, a hallmark of autoimmune disease.

In this thesis, optimal T cell stimulation was studied using an in vivo adoptive transfer model. Results showed that antigen stimulation of T cells along with ligation of the costimulatory molecule OX40 led to an accumulation of antigen-specific cells. OX40 ligation allowed the antigen specific cells to proceed through more cell cycles than cells stimulated with antigen alone. The addition of

the adjuvant lipopolysaccharide (LPS) to this system allowed for increased cell survival.

However, the continual presence of an adjuvant may also have injurious effects. This was highlighted with the appearance of "Toxic Oil Syndrome" (TOS) in which an adulterated rapeseed oil, an oil with known adjuvant activity, was sold for human consumption. People developed an autoimmune condition characterized by polyclonal B cell activation and autoantibody production. A genetic predisposition was implicated with TOS and was further investigated in this thesis. Although the A.SW mouse has the genetically susceptible genotype, these mice did not develop TOS following exposure to "toxic oil" indicating that other factors may be important in TOS susceptibility.

Extending the techniques used in these studies and applying them to the canine immune system was the final topic investigated in this series of studies. Understanding immune pathways of the mammalian immune system is particularly important for comparative studies when dogs are used as models to investigate human immune system disorders. These studies combined will allow for a better understanding of the balance between an optimal immune response and an imbalance leading to hypersensitivity or immunosuppression, as well as interspecies relationships.

Investigations of T cell Costimulation and Autoimmunity in Mice, and Development of Flow Cytometric Methods to Assess Lymphocyte Stimulation in Dogs

By
Amy R. Weatherill

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Amy R. Weatherill, Author

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CONTRIBUTION OF AUTHORS

Chikara Takahashi and Joseph R. Maxwell performed many of the preliminary experiments and perfected the techniques used in chapter 2. Andrew D. Weinberg supplied the anti-OX40 monoclonal antibody that was used in all of the experiments in chapter 2. Anthony T. Vella was the primary investigator for the project presented in chapter 2. Kathleen O'Hara dosed all of the mice used in chapter 3 and was involved with the collection of blood for the studies in chapter 4. Bernadette Stang was involved with the anti-nuclear antibody assays described in chapter 3. Jean A. Hall and Loren D. Koller were the primary investigators for the project presented in chapter 3. Jean A. Hall was also the primary investigator for the studies performed in chapter 4.

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DEDICATION

This is dedicated to my family for their unwavering support and encouragement throughout the years and for instilling in me the joy of learning.

INVESTIGATIONS OF T CELL COSTIMULATION AND AUTOIMMUNITY IN MICE, AND DEVELOPMENT OF FLOW CYTOMETRIC METHODS TO ASSESS LYMPHOCYTE STIMULATION IN DOGS

CHAPTER 1
INTRODUCTION AND BACKGROUND

Everyday, we are bombarded by microscopic invaders. While some of these organisms are harmless, others are able to cause great bodily harm. It is therefore imperative that we have the means to defend ourselves. In higher organisms, this is accomplished by an immune system. While the complexity of this system may vary by species, the main purpose is the same: to defend the body against pathogens. My studies will focus on the mammalian immune system and its ability to confer protection to infectious agents.

BRANCHES OF THE IMMUNE SYSTEM

The mammalian immune system is composed of two main branches: the innate immune system and the adaptive immune system. The innate immune system has several components of which I will discuss three. This innate immune system is always present in higher mammals. It is not specific for a particular pathogen, and it treats all pathogens in the same manner.

The first component of innate immunity is the surface epithelia. This covering forms the skin as well as the linings of our mucous membranes, including the gastrointestinal tract, the genito-urinary tract, and the respiratory tract. It functions by forming a protective barrier that separates the internal environment from the external environment. This protective covering equally blocks a variety of pathogens, and performs this function independent of the type of pathogen. Only if a pathogen is able to penetrate this covering, or if the covering is damaged, as in the case of burns or wounds, will colonization occur.

The second type of innate immunity is phagocytosis. Phagocytosis is a process in which particles or even whole bacteria are engulfed and destroyed. Macrophages and neutrophils are the main phagocytic cells in mammals. Macrophages are found throughout tissues and within organs including the lungs, gastrointestinal tract, liver, and spleen. Neutrophils are found in the blood. When an invader crosses the protective barrier it is immediately recognized as foreign by circulating neutrophils and/or macrophages located in the sub-epithelial layers. These phagocytic cells will then engulf the pathogen, initiating a process that activates these phagocytic cells. This activation leads to the release of several factors designed to eradicate the pathogen; several of these secreted factors are called cytokines. These cytokines lead to the attraction and accumulation of more phagocytic cells and increase the size and permeability of blood vessels so that greater numbers of immune cells can reach and "leak" out into the infected tissue.

Jointly, this response is called inflammation. It is characterized by an increased blood flow, which causes heat and redness, and increased vascular permeability, which causes swelling and pain.

The third innate defense mechanism is the complement cascade. This is comprised of a network of proteins that ultimately leads to the formation of the membrane-attack complex. These proteins attach to the surface of a pathogen and proceed through a series of activation steps; ultimately, several components combine to form a pore in the membrane of the invader and thereby kills it.

Although these innate protection mechanisms are very effective, a second line of defense is available should an invader evade the innate immune response. This protection is conferred by the adaptive immune response.

The adaptive immune system is specific for each pathogen. This arm of the immune system is very complex and has many components. The key cellular players are T cells, B cells, and antigen presenting cells. When an antigen is detected by this arm of the immune system, immune cells are activated that are only able to fight the specific pathogen. Thus, the cells involved in the adaptive immune system are specific for an individual antigen. Only after a cell has seen its specific antigen will a response be mounted. This response will be directed toward and restricted to a particular antigen or a particular pathogen. Should a second pathogen invade the organism, a second adaptive immune response would have to be mounted. Following an adaptive immune response, a small number of antigen-

specific cells will "remember" the pathogen (1-3). Should a second infection with the same organism occur, these cells will be able to react and eradicate the pathogen more rapidly than the first time they were activated. This is called immunological memory.

Regulation of the adaptive immune system is accomplished by cytokines. These are soluble proteins that are produced by cells that in turn affect the behavior of surrounding cells. Cytokines interact with surrounding cells via cytokine receptors on the cells' surface. Cytokines produced by T or B lymphocytes and macrophages are commonly termed interleukins (IL). Cytokine signals are essential for a properly functioning adaptive immune response. Cytokine signaling is responsible for both the production (up regulation) of an immune reaction and the reduction (down regulation) of an immune response. Therefore, optimum immune function is cytokine dependent.

ANTIGEN PRESENTING CELLS

Antigen presenting cells (APCs) are cells that are able to detect antigens, engulf them, process then, and then present parts of them to the T cells. The main APCs for T cells are macrophages, dendritic cells, and B cells (4-11). Antigen presentation is effected via a surface molecule called the major histocompatibility complex (MHC) (12). This is a cell surface glycoprotein that presents antigen fragments to T cells. There is extreme variability in MHC molecules, which allows

great diversity and, therefore, the ability to recognize many antigens. This is possible because there are several genes with multiple loci involved in coding MHC molecules. These can be further differentiated by different alleles for each locus.

There are two different classes of MHC molecules, class I and class II (13, 14). MHC class I molecules are made up of an α-chain and a β_2-microglobulin chain (Figure 1.1). MHC class II molecules are made up of an α-chain and a β-chain (Figure 1.1). Antigens are presented to T cells by lying in a groove located in the MHC molecule (15, 16). Another difference between the two classes of MHC is that each class is capable of presenting antigens from different sources. Pathogens that are found in the cytosol, such as viruses and some bacteria, are digested and their antigens are presented in the MHC class I molecule. However, the MHC class II molecules present antigens derived from an extracellular source (17). Pathogens may be endocytosed by a phagocytic cell and then antigenic fragments may be presented by the MHC class II molecule. Alternatively, extracellular proteins may bind to cell surface receptors on the APC. This protein would then be internalized and fragments would be presented by the MHC class II molecule.

The distribution of these MHC molecules is related to the types of antigens they present. MHC class I molecules can be found on all cells in the body. Because MHC class I molecules present antigens from an intracellular source, this

distribution is critical for fighting viral and other intracellular pathogens. Therefore, if a virus affects a body cell, or any non-immune cell, the cell is able to present a viral antigen and signal the immune system. In contrast, MHC class II molecules are found only on professional APCs, such as B cells, macrophages, and

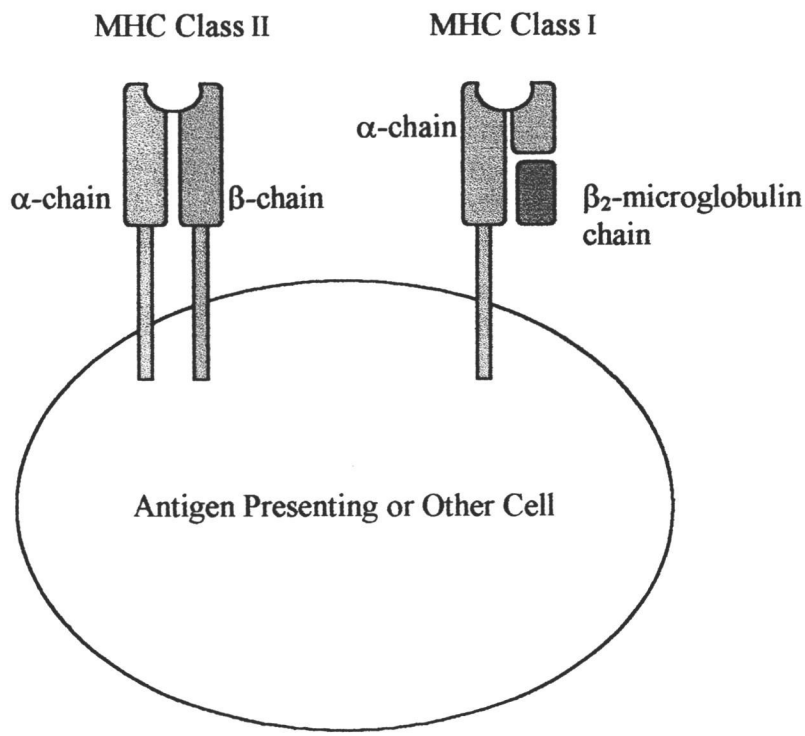

Figure 1.1: Structural composition of MHC class II and MHC class I molecules. MHC class II is composed of an α-chain and a β-chain. In contrast, MHC class I is composed of an α-chain and a β_2-microglobulin chain. At the top of each MHC molecule is a cleft that will display an antigen. Additionally, the MHC class II molecule has two transmembrane signaling chains, whereas the MHC class I molecule has only one transmembrane chain. Because these molecules are expressed on different types of cells, the cell to which these molecules are attached is just a representative cell.

dendritic cells. These cells are important for fighting extracellular or bacterial pathogens. These cells are capable of phagocytosis, and can, therefore, pick up antigens located extracellularly. These antigens are then presented to the adaptive immune cells and initiate a response. This is accomplished by the MHC class II molecules interacting with the other main cell type of the adaptive immune system, the T cell.

T CELLS

T cells are a key cellular component of the adaptive immune response. T cells are so named because following generation in the bone marrow, they migrate to the thymus to undergo maturation. There are two classes of T cells, CD8 and CD4, and both participate in the cell-mediated immune response. In addition, the CD4 T cell population can be further divided into Th1 and Th2 T cells. The first class of T cells that will be addressed are CD8 T cells.

CD8 T cells are also referred to as cytotoxic T cells because of their ability to kill target cells. These T cells are able to recognize antigens that are expressed in MHC class I molecules (18). Because MHC class I molecules express antigens that are found within the cytoplasm of cells, they are able to present antigens derived from viruses and intracellular bacteria. This makes them an integral player in fighting viral and bacterial infections.

Once a naïve CD8 T cell recognizes a presented antigen, it is activated to become a cytotoxic T cell, also known as an effector cell. The CD8 T cell releases cytotoxic effector proteins that are stored in lytic granules within the CD8 T cell. These proteins include perforin and granzymes (19). Perforins polymerize and form pores within the cell membrane of the infected cell, which results in its inability to regulate cell functions. Granzymes are proteases that degrade proteins and trigger the infected cell to undergo programmed cell death, or apoptosis. During this process a cell destroys itself from within. Several of the processes involved in apoptosis result in nuclear blebbing, cell morphology changes, and DNA fragmentation. Once a target cell has been triggered to die, the CD8 T cell can then move on to other infected cells, or it may stop its killing action. Because the effector function of CD8 T cells is so potent, it is imperative that it is precisely targeted.

A second action of CD8 T cells is the release of cytokines, primarily IFN-γ, TNF-α, and TNF-β. The main function of IFN-γ is to halt viral replication. IFN-γ is also able to induce expression of MHC class I, which would help target infected cells. IFN- γ is also able to activate macrophages. Macrophages are important antigen presenting cells and they also function as effector cells. The effector function of macrophages includes the production of potent antimicrobial products, including nitric oxide (NO) and oxygen radicals. The activation of macrophages also causes upregulation of MHC class II and the costimulatory molecule B7. This

upregulation helps to activate the other arms of the cell mediated immune response. TNF–α and TNF-β are able to synergize with IFN-γ to provide the second signal required for activation of macrophages.

The second class of T cells involved in the cell-mediated immune response is the CD4 T cell. This class of T cells can be further divided into two types: Th1 and Th2. Like the CD8 T cells discussed above, the Th1 CD4 T cells are involved in eradication of bacteria. However, the main action of these cells is recruitment and activation of macrophages. Several bacterial pathogens are able to infect macrophages and then survive inside them. These bacteria prevent the fusion of the phagosome and lysosome that is required for their destruction. As a result, an infection is able to develop. Th1 T cells have evolved to deal with this situation. A Th1 T cell is able to recognize an antigen from the invading bacteria that is presented on the surface of the macrophage in the context of MHC class II (20). Activation of the Th1 T cell also helps to activate the macrophage, and will make the macrophage more responsive to further activation. Th1 cell activation will stimulate the macrophage to produce NO and oxygen radicals. Activated macrophages also secrete the cytokine IL-12, which skews naïve CD4 T cells to develop into Th1 T cells. In addition, the interaction of the Th1 cell with the macrophage activates the Th1 cell. The interaction with the activated macrophage causes the Th1 cell to release several cytokines including IFN-γ, TNF-α, TNF-β, and IL-2 (21, 22). As discussed previously, IFN-γ, TNF-α, and TNF-β cause

further activation of the macrophage. Additionally, TNF-α and TNF-β recruit phagocytic cells to the site of infection. IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) produced by the Th1 cells increase the production of macrophages in the bone marrow. This activation is self-feeding and there is a strong need for regulation. This is provided by the second class of CD4 T cells, the Th2 T cells. They are able to produce IL-10 which inhibits macrophage activation. Without such regulation, chronic macrophage activation would cause massive tissue damage to the host.

The second category of CD4T cells are the Th2 T cells. Unlike the CD8 and Th1cell types previously discussed, these cells are not involved with cell-mediated immunity, but rather they play a role in humoral, or antibody-mediated immunity (23, 24). These cells are responsible for activating B cells to produce antibody. B cells pick up antigens via their cell surface receptors. The antigens are internalized and then fragments are presented on the cell surface in the context of MHC class II (20). The Th2 cells specific for this antigen are then able to recognize the antigen via the interaction of their T cell receptor (TCR) and the antigen/MHC class II complex. The interaction leads to the activation of both the B cell and the Th2 T cell. Following activation, the Th2 cell produces cytokines that stimulate the B cell to secrete antibody. Two key cytokines secreted by the Th2 cells are IL-4 and IL-5 (21, 22). IL-4 is able to influence non-differentiated

CD4 T cells into becoming Th2 T cells. These cytokines also drive the B cell to proliferate and secrete antibody.

ANTIBODIES

Antibodies are proteins that are specific for a particular antigen. They have multiple functions depending on their class. They are composed of two heavy chains and two light chains held together by disulfide bonds (Figure 1.2). Although they all have the same basic structure, the particular type of heavychain determines

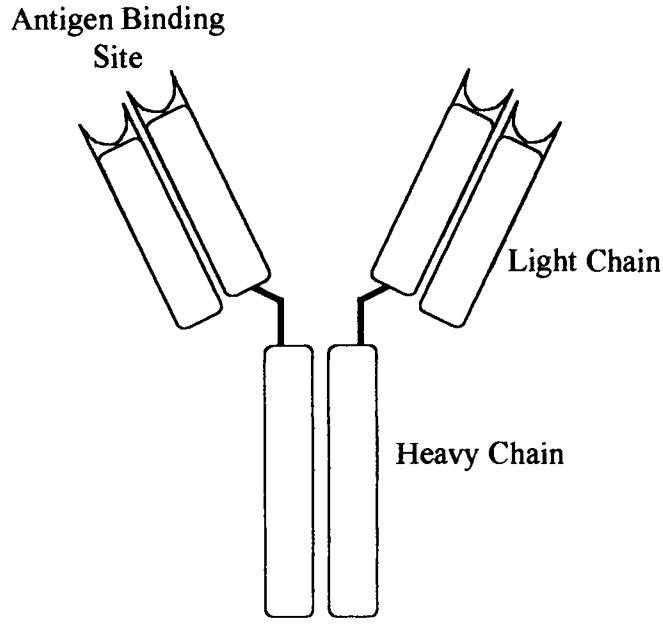

Figure 1.2: General structure of an antibody. Antibodies are composed of two heavy chains and two light chains. The antigen binding site is located at the top and is composed of sections of both the heavy and light chains.

the isotype of the antibody. There are five isotypes: IgD, IgM, IgG, IgE and IgA. Each isotype has a particular function. IgD is found only on naïve B cells and little is produced at any time. IgM is the first antibody produced after B cell activation. Its main function is to activate the complement cascade (25). IgG is capable of neutralization and opsonization (26, 27). This isotype is able to be further divided into subclasses: IgG1, IgG2, IgG3, and IgG4. IgE is responsible for the sensitization of mast cells, which is important in immediate type hypersensitivity reactions such as allergies. IgA is present at the epithelial surfaces of the body. It is thought that IgA functions by protecting the epithelial surfaces from pathogens. IgA is also present in breast milk and is important in conferring protection to babies.

The particular cytokine milieu present determines which antibody isotype is produced. The presence of IL-4 leads to the production of IgG1 and IgE, the presence of IL-5 helps IgA production, and TGF-β induces IgG2b and IgA. Even the cytokine IFN-γ secreted by Th1 T cells, which are not usually associated with antibody production, is able to affect antibody production. Although Th1 cells are poor activators of B cells, IFN-γ induces the production of IgG2a and IgG3. Just as chronic activation of CD8 T cells and Th1 T cells is not desirable, neither is chronic activation of Th2 or B cells. In order to dampen this response, Th1 cells secrete IFN-γ, which inhibits B cells. This regulation shows that each arm of the immune response is responsible for regulating the other.

ADAPTIVE IMMUNE SYSTEM SIGNALING

The interactions that occur between an APC and a T cell are the driving force for an adaptive immune response. These interactions occur between several cell surface receptors, which in turn send activating signals to the cell. It is now common knowledge that two signals are required for immune cell activation (28, 29). The first signal required is antigen. The second signal is collectively called the costimulation signal. If both signals are present, an immune cell will become activated and will become an effector cell. However, if only one signal is present, the immune cell is unable to mount a response. This is termed anergy. The cells are present, but are unable to respond and eradicate the pathogen.

The first cell that must become activated is the APC. The first signal in APC activation is the recognition of an antigen. Upon recognition, the APC will internalize the pathogen and present antigen fragments in its MHC class II molecule. In the case of macrophages, which interact with the Th1 T cells, the second activation signal is given by the T cells themselves. Upon recognition of the antigen by the Th1 cell, the Th1 cell is induced to express CD40 ligand and releases the macrophage activator IFN-γ. CD40 ligand will bind CD40 found on the surface of the macrophage (Figure 1.3). These two signals will lead to full activation of the macrophage and will cause the macrophage to express increased MHC class II, CD40, and TNF receptor on its surface. The increased expression of

these molecules will lead to continual macrophage activation, and therefore, continual Th1 T cell activation.

The complete activation of B cells is somewhat different. Similar to macrophages, B cells present antigen in the context of MHC class II. However, in

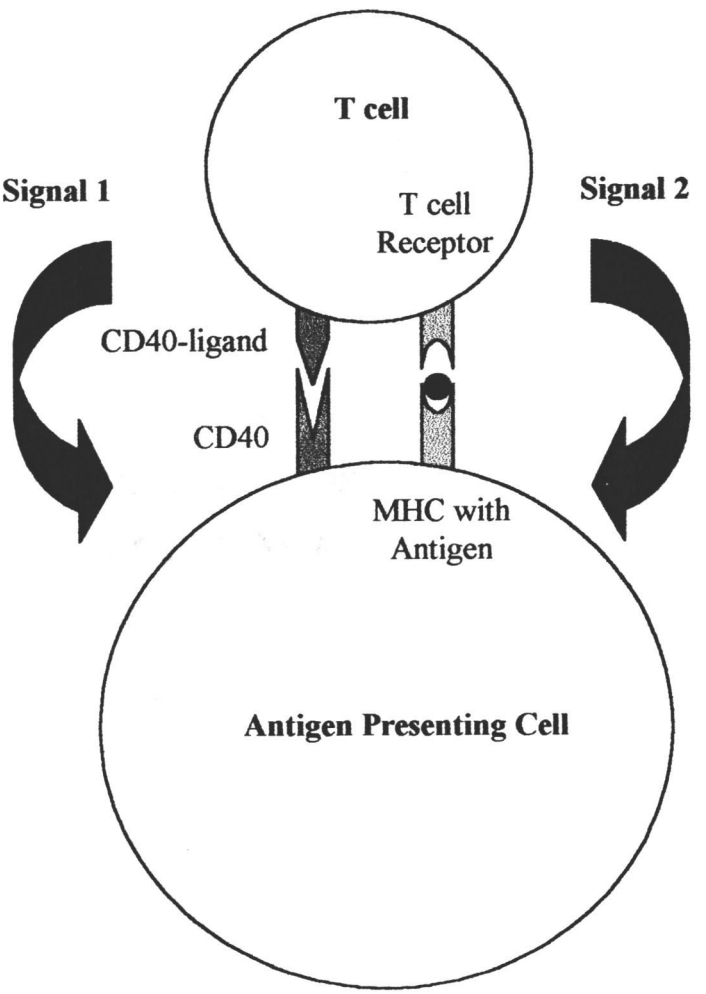

Figure 1.3: Two signals are required for full activation of an antigen presenting cell. The first signal is through the T cell receptor (TCR) and the antigen bound to MHC. The second, or costimulatory signal, is the interaction of CD40-ligand on the T cell with CD40 on the APC.

contrast to macrophages, B cells are the primary APC for Th2 T cells. B cells are able to internalize antigens that have been picked up by their surface receptors. This internalization of antigen is the first signal for B cell activation. These internalized antigens are then processed and presented to Th2 T cells in MHC class II molecules. The costimulatory, or second activation signal for B cells is the same as the one for macrophages: CD40 (30, 31). This cell surface molecule interacts

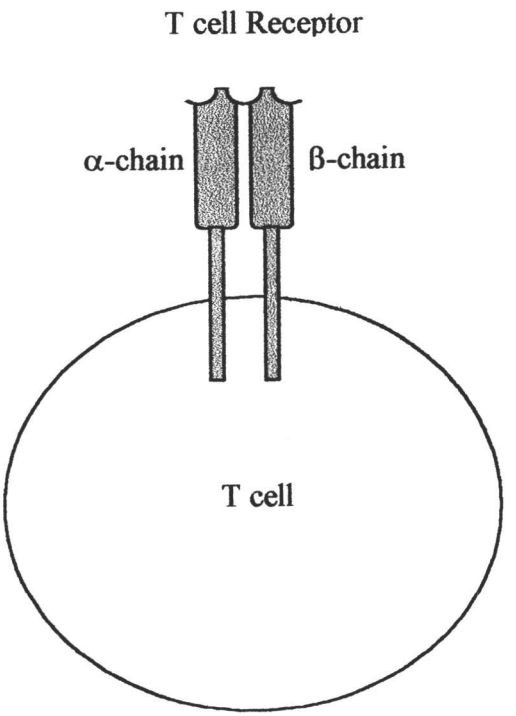

Figure 1.4: Structure of the T cell receptor (TCR). The TCR is composed of an α-chain and a β-chain. It also has two transmembrane chains that send signals to the T cell.

with its ligand, CD40 ligand, found on the surface of T cells. When a B cell receives both of these signals, it is able to further activate Th2 T cells, and it is able to secrete antibody (32).

Similar to APC activation, the activation of T cells also requires two signals. The first interaction that must occur is the recognition of an antigen by the T cell. In order for a T cell to identify an antigen, the antigen must be displayed in a MHC molecule. A T cell will then be able to identify an antigen through its T cell receptor (TCR). Similar to MHC molecules, this is a cell surface molecule with extreme specificity. Each TCR is specific for only one antigen. The TCR is composed of an α-chain and a β-chain similar to the MHC class II molecule (Figure 1.4) (33, 34). The TCR has two transmembrane chains that are able to signal the cell if the TCR has recognized an antigen. Following antigen recognition, the T cell becomes activated and proceeds to rapidly divide. This clonal expansion allows for the specificity of the immune response; only cells that have recognized the antigen will multiply.

Although the interaction between the MHC and the TCR is the first signal required for T cell activation, research has shown that, similar to APCs, two signals are required for the generation of a functional T cell response. The T cell costimulatory signal involves the interaction between two cell surface molecules: B7 and CD28 (35, 36). B7 is expressed on activated APCs and interacts with the T cell surface molecule CD28 (Figure 1.5) (37-39). When a T cell receives both of

these signals, it is able to undergo clonal expansion (40-43). During this stage, an antigen-specific T cell replicates itself many times so that there are a greater number of antigen-specific T cells. If any of these signals are missing, cell activation will not occur and anergy will result.

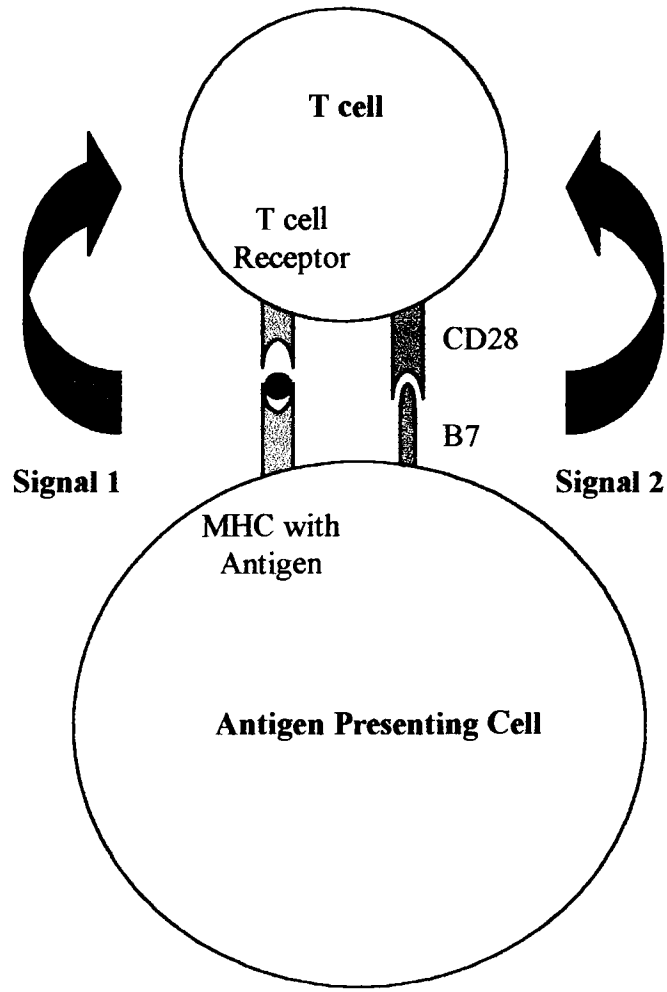

Figure 1.5: Two signals are required for activation of a T cell. The first signal is through the T cell receptor (TCR) and the antigen bound to MHC. The second signal is the interaction of CD28 on the T cell with the costimulatory molecule B7 on the APC.

In summary, there are two classes of T cells, and each has a unique way of being activated. The first class, CD8 T cells, is activated by antigen in the context of MHC class I (18). These cells are instrumental in fighting viral and intracellular bacterial infections. Because these pathogens are capable of infecting any cell in the body, all cells display MHC class I on their surface. Should a cell become infected, antigens from the pathogen will be presented to the CD8 T cells in the context of MHC class I. If the infected cell is also displaying B7, the CD8 T cell will be activated, which leads to the death of the infected cell. However, if the antigen is recognized, but there is little or no B7 costimulation, the CD8 T cell will not respond. In these instances, CD4 T cell help may be required (24).

The second class of cells, the CD4 T cells, are activated in much the same way as CD8 T cells except that they recognize antigen in the context of MHC class II on APCs (20). Because MHC class II molecules express antigens derived extracellularly, these cells are important in fighting extracellular or intravesicular pathogens. Again, the CD4 T cell recognizes antigen in MHC class II by its TCR. The costimulatory interaction of CD28 and B7 is also required. B7 is upregulated on APCs following ingestion of a pathogen. Interaction of a CD4 T cell TCR and MHC class II on the APC also induces increased B7 expression. This costimulation induces the CD4 T cell to produce IL-2, a cytokine essential for the proliferation of the T cell. This activity may also lead to the activation of CD8 T cells (24). If a CD4 T cell stimulates an APC to upregulate B7, this molecule is

able to send a costimulatory signal to a CD8 T cell. Alternatively, if a CD4 T cell becomes activated and produces IL-2, any CD8 T cell in the vicinity is able to utilize this IL-2 to stimulate its own proliferation. However, regardless of the activation route, the outcomes are all the same: increased numbers of antigen-specific T cells to fight the pathogen.

THE DIFFERENCE BETWEEN A PRIMARY RESPONSE AND A MEMORY RESPONSE

Now that immune cell activation has been discussed, it is important to note that not all immune responses are the same. There are two types of immune responses: a primary response and a secondary (memory) response. A primary response occurs when naïve T cells see an antigen for the first time. Upon infection, the immune cells are activated and are triggered to undergo clonal expansion. This process leads to many identical copies of the originally activated cell. This allows for the specificity of the immune response because only the antigen-specific cells are being replicated. However, peak proliferation and antibody production does not occur for about 4 to 5 days. Once the infection has been cleared, these expanded cells undergo deletion. During this process most of the antigen-specific cells undergo apoptosis and die. However, a few of the original cells persist and become what are termed memory cells. These cells are then able to respond quicker and more effectively upon re-infection (1-3). The

response generated by these memory cells peaks after only 1 or 2 days. It is this memory response that forms the basis for immunizations. Following an immunization, an immune response ensues. Specific immune cells are then primed to react should the person be exposed to the pathogen in nature. Further protection can be conferred in the form of booster shots. These shots function by exposing the person a second time to the antigen. This causes clonal expansion, deletion, and memory cell generation the same as the primary exposure did. However, because these cells have now been exposed twice, there are more memory cells left following deletion, and therefore, there is even more protection.

PERMUTATIONS OF THE ADAPTIVE IMMUNE RESPONSE

Although the basics of an immune response have been reviewed, there are many permutations of this response that can occur. Researchers have been investigating the possibility of generating an even greater immune response by stimulating the T cells with multiple costimulatory signals. It is also thought that if antigen-specific T cells did not undergo clonal deletion, then more memory cells would develop and greater immunity would be conferred. Several cell surface molecules, including CD40, 4-1BB, and OX40, have been investigated (44, 45). CD40 is a cell surface molecule that is expressed on APCs such as B cells, macrophages, and dendritic cells. Its ligand is CD40 ligand (CD40L) and it is found mainly on CD4 T cells (46, 47). Research has found that when an agonist

anti-CD40 antibody is given prior to antigen stimulation, there is increased clonal expansion of the antigen-specific T cells, and their clonal deletion is delayed. This is thought to be mediated by an increase in B7 expression on the APC (48, 49).

A second molecule, 4-1BB, is another cell surface molecule that has been shown to enhance an immune response. 4-1BB is expressed on activated T cells, although preferentially on CD8 T cells (50, 51). Its ligand is expressed on APCs. Following antigen stimulation and concomitant 4-1BB stimulation, there is an increase in clonal expansion of both CD4 and CD8 T cells. However, only CD8 T cells are rescued from antigen-induced apoptosis (52-55). These results verify that is it possible to prevent the death of antigen-stimulated cells.

A third molecule, OX40, has also been targeted as a potential costimulatory signal capable of rescuing antigen-activated T cells from death (56, 57). OX40 is another cell surface molecule that is expressed on activated CD4 T cells, while its ligand (OX40L) is expressed on APCs (58, 59). Research has shown that stimulation of T cells with both antigen and an anti-OX40 antibody leads to greater clonal expansion and increased survival (56, 57, 60). Although these molecules have shown promise in generating a more potent immune response, absolute success has been elusive. It has now become apparent that survival of antigen-activated T cells may hinge on a third signal.

Although increased clonal expansion and delayed, or inhibited, clonal deletion has been achieved by the use of two costimulatory signals, the eradication

of clonal deletion altogether has not, as yet, been accomplished. It is now hypothesized that a third signal is required to fully rescue antigen-activated T cells from death. This third signal has been called the "danger signal" (5, 61, 62). The danger signal theory proposes that a stimulus that causes some sort of biological injury is required for the generation of a long-lived immune response. Components of this danger signal would be able to trigger tissue damage or necrotic death of cells.

Tissue damage can occur in the wake of an inflammatory response. Inflammatory mediators are capable of not only hurting a pathogen, but also can act upon the host. The culmination of the complement cascade is the formation of the membrane attack complex. This complex is able to form holes in the extracellular membrane of a pathogen. However, this complex is also able to form holes in host cells. These holes cause the cells to disintegrate, a process known as necrosis. Contrary to apoptosis, necrosis is a chaotic event caused by physical or chemical injury.

Theoretically, many substances that are found naturally should be able to send the "danger signal." One such substance is bacterial lipopolysaccharide (LPS), a potent inflammatory mediator (63). LPS has been shown to have profound effects on an immune response (64-66). LPS has been shown to prevent the death of antigen-activated T cells. This prolonged survival is mediated by resistance to Fas-induced killing (66). Fas is a molecule found on the surface of

activated T cells that, when ligated, induces the cell to undergo apoptosis (44). It is thought that this is the mechanism by which clonal deletion occurs. When this signal is missing, the immune response is perpetuated because the antigen-activated cells persist. When LPS is given in conjunction with multiple costimulatory signals, survival is enhanced. When LPS is given with antigen and OX40 stimulation, clonal expansion is augmented and the number of antigen-specific T cells is increased (60). These antigen-specific cells are also able to survive and avoid clonal deletion. While this survival has several benefits, including increased memory cell production, there can be severe consequences leading to an interminable immune response.

AUTOIMMUNE RESPONSES

The term autoimmunity is usually used to describe an event in which an individual's immune system mounts an attack on the body rather than a pathogen. This occurs when the immune system recognizes a "self antigen" and mounts an immune response against it. The immune responses involved in autoimmunity are diverse. In some cases a self protein may be altered and an antibody response is mounted against that altered epitope. However, the problem lies in that those antibodies are also able to attack unaltered self proteins. This is the theory behind systemic lupus erythematosis. Alternatively, autoimmune disease may be mediated by the T cells directly. In rheumatoid arthritis, Th1 cells react to a self antigen

present in joints. Upon activation, these cells release inflammatory cytokines that lead to the accumulation of immune cells and consequent joint damage. In insulin-dependent diabetes mellitus, CD8 T cells mediate the response. These T cells are activated against a self protein found on the insulin-secreting β cells in the pancreas. As a result, these cells are trigged to die via the cytotoxic effector function of the CD8 T cell.

The exact mechanism by which this attack against "self" is triggered is not known. Some theories have implicated antigenic mimicry in which viral or bacterial antigens that resemble self-antigens can prompt a response against the analogous self antigen (67, 68). Other theories have suggested flawed apoptosis, whether increased or decreased (69, 70). However, one of the most accepted theories of autoimmunity is that it results from a genetic predisposition. This predisposition is linked to the MHC molecules of an individual. Regardless of the trigger, the results are often the same, i.e., damage to self tissues. Because the exact causes and progression of autoimmune disease remain unknown, it is unclear to what degree an immune response can be enhanced without induction of autoimmunity.

Although LPS is the main lipid known for enhancing an immune response, LPS is not the only lipid that is capable of augmenting an immune response. Many other exogenous lipids are also capable of triggering an immune response (71). One such lipid is rapeseed oil (71, 72). Other agents, such as aromatic anilides,

have also been shown capable of inducing cell proliferation (71, 73). These compounds became the subject of intense study after an autoimmune disease-like epidemic was diagnosed in Spain. "Toxic Oil Syndrome" (TOS) emerged in 1981 after industrial rapeseed oil denatured with aniline was sold illegally as cooking oil. Following consumption, people became ill with autoimmune-like symptoms. Researchers immediately began looking for the immune system trigger. It was found that these exogenous compounds were capable of inducing widespread immune cell activation and chronic inflammation. As explained by the "danger signal" theory discussed above, this persistent state of inflammation could lead to chronic immune cell activation. However, in this case, continuous immune cell stimulation led to autoantibody production and a loss of immune tolerance.

Heavy metals are also capable of inducing autoimmunity. Mercuric chloride has been thoroughly studied because of its ability to induce autoimmunity in genetically susceptible mice. It has been found that treatment with mercuric chloride leads to increased serum levels of IgG1 and IgE, and autoantibody production (74-76). This is hypothesized to result from the activation of T cells that are presented with self antigens altered by the mercury ion (77). These self-reacting T cells are then able to induce autoantibody production by the antigen presenting B cells. Similar to the lipids discussed above, mercury ions may induce apoptosis in a localized fashion that leads to an inflammatory response. This inflammatory response could act as the "danger signal" required for the prolonged

activation of immune cells. Similar to the TOS epidemic mentioned above, this chronic inflammation could lead to the onset of autoimmune disease.

EXTENSION TO OTHER SPECIES

The vast majority of immune system research is performed in mice, rats, or humans. There are many reagents available for these species (i.e., purified species-specific proteins, corresponding antibodies, and assay kits), and many techniques have already been perfected. Although it is of utmost importance to understand immune function and develop therapies for humans, there are other species (e.g. dog, cat, horse) for which treatments are needed also. In addition, these animal species often can serve as models for human disease.

Significant advances have been made in the treatment of human diseases because of animal research. The new treatments and drug therapies that have been developed using animal models for human diseases are invaluable. Chronic obstructive pulmonary disease has recently been studied in horses, and a dog model of Alzheimer's disease is also being investigated. Because most diseases, including the two mentioned above, have an immune component, immune assays for a variety of species need to be developed. Specifically, in vitro cell culture techniques need to be developed in the dog so that immune cell function and proliferation can be assessed under various experimental conditions. The

knowledge that can be gleaned from such assays may lead to even greater advances in human disease therapy.

SUMMARY

The topics discussed here demonstrate the complexity of the immune system. While certain activation of immune cells is desirable and beneficial, excess activation may be harmful. In the case of costimulation, increased T cell expansion leads to the generation of desirable memory cells. These memory cells are then able to protect the host upon re-infection. However, excessive T cell stimulation causes harm to the host via autoimmune disease. In the latter, immune cell activation leads to systemic autoimmune disease characterized by autoantibody production.

I have investigated the mechanism of OX40 costimulation both with and without concomitant LPS stimulation. I have also investigated the induction of toxic oil syndrome in mice. This investigation looked at autoantibody production following treatment with toxic oil containing anilides. In addition, I investigated autoantibody production in mice following mercuric chloride treatment. The last step in these investigations involved the development of assays to investigate the immune system response in another species, i.e., assays specific for the dog.

How can T cell stimulation lead to such varying outcomes? Is there a certain level of activation that must be achieved prior to autoimmune induction?

Are we constantly on the verge of autoimmune disease with every pathogen we fight? The intricacies of a normal immune response versus the onset of an autoimmune situation are still elusive. However, continued research will help to answer some of the fundamental questions about optimal immunity versus autoimmunity.

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CHAPTER 2

OX40 LIGATION ENHANCES CELL CYCLE TURNOVER OF AG-ACTIVATED CD4 T CELLS IN VIVO

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ABSTRACT FOR CHAPTER 2

OX40 costimulates T cells, increases activated T cell longevity, and promotes memory acquisition. T cells activated in vivo with agonist anti-OX40 and ovalbumin, have a unique pattern of survival and cell division as compared to control cells, but are able to respond to recall Ag equally well. BrdU incorporation shows that early cellular division rates of the anti-OX40 treated and the control group are similar. Nevertheless, more $BrdU^+$ Ag-specific T cells accumulate in lymphoid tissue upon anti-OX40 administration. Thus, OX40 ligation does not necessarily lead to increased cell cycle entry, but promotes the accumulation of dividing cells. However, CFSE staining shows that OX40 ligation allows cells to progress through more cellular division cycles, while control cells stall or die. Moreover, OX40 ligation leads to a proportional decrease of apoptotic Ag-specific T cells. Thus, OX40 costimulation functions by promoting cell cycle progression, thereby increasing the lifespan of Ag-activated CD4 T cells.

INTRODUCTION

The fundamental purpose of an immune response is to eradicate pathogens and to protect the body upon re-exposure. Primary effector T cells are involved in the initial clearance of the infectious agent, while memory T cells generate a response to secondary exposure. It is the generation of memory cells that is targeted by vaccines.

In order to generate a potent memory response, optimal T cell activation and expansion are necessary. Landmark studies have shown that at least two signals are required for clonal expansion and CD4 T cell activation (1, 2). The first of these two signals is TCR interaction with peptides in the context of MHC class II molecules. The second signal, a co-stimulatory signal, has been shown in many systems to provide T cells with the ability to produce IL-2. Of the costimulatory signals studied, CD28 has received the most attention (3). The ligands for CD28 are B7-1 and –2 which are expressed on activated APCs including dendritic cells (3-8). In vivo data has shown that indeed CD28 ligation is important for T cell clonal expansion but does not prevent clonal deletion (9, 10). However, previous experiments have shown that inflammatory stimuli can prevent the death of many of these activated T cells (11). Thus, it has been hypothesized that some factor(s) from inflammatory reactions provide a survival signal to the activated T cells and that costimulation helps provide an appropriate environment for clonal expansion (12).

In general, it is believed that expansion is controlled by the amount of growth factors that are present (13). For example, the presence of IL-2 enhances T cell growth in most circumstances. Therefore, it is logical to assume that costimulation increases IL-2 production, which leads to an increase in T cell clonal expansion (14). Nevertheless, there are data that have shown IL-2 increases apoptosis of activated T cells (15). Thus, it is likely that depending on the microenvironment, and the availability of other cytokines and cell surface markers, different responses can be obtained that will favor deletion or survival of Ag-specific T cells.

In this study, we wanted to uncover the mechanism used by T cell populations to achieve optimal clonal expansion. To complete this task we investigated the T cell costimulatory receptor OX40. OX40, a member of the TNF/NGF superfamily of receptors, has been shown to enhance CD4 T cell proliferation, cytokine production, and is expressed on activated CD4 T cells only (16). In vivo studies have shown that OX40 enhances clonal expansion and delays clonal deletion of Ag-activated T cells (12). In addition, OX40 knockout mice have weakened CD4 T cell responses to viral challenge, but maintain normal CTL and antibody responses (17). The OX40-ligand is mainly expressed on activated APCs (16). Mice deprived of OX40 ligation do not present Ag efficiently and have an impaired ability to mount immune responses to Leishmania major (18). This lack of

response is mainly attributed to poor Th2 cell development, but it is also well documented that OX40 contributes to IL-2 and IFN-γ synthesis (17, 19).

These responses help to explain why OX40 has been shown to be important in a variety of clinical models of disease. For example, inhibition of OX40 binding to OX40L in vivo at the onset of experimental autoimmune encephalomyelitis (EAE) inhibits clinical disease in mouse models (20). Deleting $OX40^+$ cells in rat EAE has also been shown to diminish the amount of Ag-specific cells leading to decreased clinical signs of EAE (21). In contrast, OX40 engagement in vivo has been shown to enhance anti-tumor immunity (22). Collectively, these studies suggest that costimulation through OX40 potently enhances CD4 T cell responsiveness to Ag.

Nevertheless, the mechanism by which OX40 promotes optimal clonal expansion remains unclear. In this study we have designed experiments to examine how OX40 ligation accomplishes this task. We hypothesized that OX40 stimulation promotes clonal outgrowth by either enhancing entry into the cell cycle or by facilitating completion of the cell cycle. Our system involved the use of DO11.10 TCR transgenic T cells which can be tracked over time in vivo using a mAb that is specific to the idiotype of the DO11.10 TCR. Thus, this model was used to follow the fate of ovalbumin-activated T cells in a transfer model previously described (23). Our data show that although OX40 may slightly enhance initial cell cycle entry of splenic Ag-specific T cells, it is definitely clear that OX40 provides a robust signal

that increases the numbers of cell cycles completed by the Ag-specific T cells. In the absence of OX40 ligation, the DO11.10 T cells appeared to have fewer rounds of division. The enhanced OX40 response is accompanied by an increase in the number of annexinV^- DO11.10 T cells. Additionally, it is clear that the surviving OX40-stimulated T cells are not anergic, but in fact are responsive to recall Ag.

These data show that OX40 costimulation contributes to optimal clonal expansion by enhancing cell cycle progression, which thereby increases the ability of these cells to resist Ag-induced cell death and accumulate in larger numbers as compared to priming with Ag alone.

MATERIALS AND METHODS

Mice

Female DO11.10 and BALB/c mice aged 6-12 weeks were used for all experiments. BALB/c mice were purchased from the National Cancer Institute (Bethesda, MD). DO11.10 mice (from Drs. Kerkvleit and Jenkins, Oregon State University, OR and University of Minnesota, MN; respectively) were bred by our laboratory. All mice were housed in our animal facility under specific pathogen free conditions according to federal guidelines.

Reagents and Antibodies

Chicken egg ovalbumin, OVA, was purchased from SIGMA Chemical Company (St. Louis, MO) and administered by intra-peritoneal (i.p.) injection at 500 μg per mouse in 200 μl balanced salt solution (BSS). The anti-OX40 mAb was injected i.p. at 50 μg per mouse (24). As a control for the anti-OX40 treatment, 50 μg of rat IgG (SIGMA) was injected i.p. into a separate group of control mice. Lipopolysaccharide (LPS) (SIGMA) was administered by i.p. injection at 40 μg per mouse. 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes (Eugene, OR) and was used to stain cells for cell cycling experiments. 5-bromo-2'-deoxyuridine, BrdU, (SIGMA) was injected i.p. at 3 mg per mouse in 200 μl PBS. Ovalbumin peptide, OVA 323-339, was used to stimulate in vitro cultures at 50 μg/ml. ^{3}H-thymidine (ICN, Costa Mesa, CA) was added to cell cultures to determine proliferation at a concentration of 1 μCi per well.

Anti-BrdU-FITC was purchased from Becton Dickinson (San Jose, CA). SA-APC, annexinV-PE, and anti-CD4-PE were purchased from PharMingen (San Diego, CA). Anti-DO11.10 TCR, KJ1-26 (25, 26), was purified over a protein G column from Pharmacia (Piscataway, NJ). This was then conjugated to FITC or biotin by our laboratory (27). Briefly, purified antibody was dialyzed against PBS. Protein concentration was adjusted to 1 mg/ml and incubated with N-hydroxysuccinimidobiotin or FITC-celite (SIGMA). For biotin conjugation incubation was for four hours and then salt was removed by dialysis against PBS.

For FITC conjugation, incubation was for 30 min and then the Ig was purified by centrifugation and passed through a Sephadex G25 column (BioRad, Hercules, CA).

Cell Processing, staining, and flow cytometry

Spleens and LN (axillary, bronchial, and inguinal) were teased through a nylon sieve purchased from Falcon (Santa Clara, CA). Red blood cells were lysed with ammonium chloride, and cells were washed two times. Lymphocytes were purified from the cell suspensions by nylon wool column purification (28). Briefly, 3 cc syringes were packed with 0.12-0.15 grams of washed and brushed nylon wool. 500 µl of the cell suspensions were added to the columns, topped with 5% fetal calf serum in BSS, and incubated for 30 min at 37^0C. Columns were partially drained and incubated for another 30 min. The columns were then eluted with 4 ml of 5% FCS in BSS.

For surface staining, 0.5-2x10^6 eluted cells were incubated with 10 µl of blocking solution. The blocking solution, used to prevent non-specific binding, consisted of 5% normal mouse serum, anti-mouse Fc receptor mAb supernatant (24.G2) (29), and 10 µg/ml human γ-globulin (SIGMA). Cells were incubated for 30 minutes on ice with primary mAb. Following incubation cells were washed twice with 200 µl of staining buffer, 3% FCS and 0.1% sodium azide in BSS. When biotinylated Abs were used, a second staining step was included. Cells were incubated with streptavidin-PE or -APC for 30 minutes on ice and then washed

twice with staining buffer. Following the wash, the cells were analyzed. Analysis was completed using a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer and data was analyzed using CELLQuest™ (Becton Dickinson).

For BrdU staining (30), purified T cells were first surface stained as described above. After washing, the cells were resuspended in 300 µl 0.15 M NaCl and then transferred to tubes containing 200 µl 0.15 M NaCl. To these tubes, 1.2 ml of 95% ethanol was added dropwise while vortexing, and afterwards the cells were incubated on ice for 25 min. The cells were then washed twice with staining buffer, and brought up in 1 ml of BSS with 1% paraformaldehyde (J. T. Baker, Phillipsburg, NJ) and 0.01% tween 20 (Fisher, Pittsburg, PA) for 25 minutes in the dark at room temperature. After spinning the sample, 1 ml of 0.15 M NaCl, 4.2 mM $MgCl_2$, and 10 µM HCl was added (all reagents purchased from SIGMA). To denature the DNA in the pellet, 50 Kunits units of DNase (SIGMA) were added. Following 20 minutes at 37^0C the cells were washed twice and then stained with 0.25 µg of anti-BrdU-FITC. Following a 25 minute incubation at room temperature, the cells were washed twice and then analyzed.

Experimental Protocols

For BrdU studies, DO11.10 mouse LN and spleen cells were isolated, washed, and then approximately $2x10^6$ KJ1-26$^+$ T cells were injected i.v. into BALB/c mice. The day after transfer (day 0) ovalbumin was injected. This was

followed by one of the experimental groups receiving anti-OX40, and the other rat IgG. On day 1, a subset of the anti-OX40 and rat IgG treated groups received an i.p. injection of LPS. Additionally, a second injection of anti-OX40 or rat IgG was administered to the experimental and control groups respectively. On day 2, all mice received a second injection of ovalbumin, the anti-OX40 group received a third injection of anti-OX40, and the control group received a third injection of rat IgG. I.p. injections containing 3 mg of BrdU in 200 µl PBS were given on days 2, 3, and 4. LN and spleen cells were isolated and analyzed on days 5 and 12.

For CFSE studies, DO11.10 mouse LN and spleen cells were isolated, washed, and then stained with CFSE (31). Briefly, following enumeration they were resuspended in 1 ml of BSS and CFSE was added to a final concentration of 1.5 µM. Following a10 minute incubation at 37^0C, the cells were washed twice with ice cold 5% fetal calf serum BSS. Approximately $2x10^6$ DO11.10 KJ1-26^+ T cells were then i.v. injected into BALB/c mice. Ovalbumin was i.p. injected the day after transfer, which is regarded as day 0. Immediately following the ovalbumin injection, anti-OX40 was i.p. injected into one of the experimental groups, and rat IgG was used for the control group. LN and spleen cells were isolated from the BALB/c mice on various days after ovalbumin injection.

Proliferation assays were conducted using the spleen cells from mice treated as in the CFSE studies described above. Briefly, spleen cells were brought up to a concentration of $5x10^6$ cells per ml in complete tumor media (CTM). CTM

consisted of minimal essential media combined with FCS, amino acids, salts, and antibiotics. Serial dilutions were plated starting at a concentration of $5x10^5$ cells/well, and proceeding to $0.061x10^5$ cells/well by 3 fold dilutions. All samples were done in triplicate. Cells were then stimulated with 50 μg/ml ovalbumin peptide 323-339, or media as a control. The plates were incubated at 37^0C for 72 hours. During last 8 hours, the cells were incubated with ^{3}H-thymidine at a concentration of 1μCi per well. ^{3}H-thymidine uptake was counted using a Wallac Trilux scintillation counter (Wallac, Turku, Finland).

RESULTS

Treatment with anti-OX40 leads to an early increase in number of Ag-specific T cells.

Our first study investigated the effect that OX40 ligation would have on increases or decreases in Ag-stimulated T cell numbers and percentages. DO11.10 mouse T cells were transferred into BALB/c mice as developed previously in the Jenkins laboratory (23). The day following transfer, day 0, one group of BALB/c mice received an injection of ovalbumin in BSS immediately followed by an anti-OX40 injection. As a control, a second group of BALB/c mice received an injection of ovalbumin followed by an injection of rat IgG. Cells from each experimental group were removed from the LN and spleen and analyzed for DO11.10 T cell content on days 1, 2, 3, 4, and 5.

Our results show that when mice received the anti-OX40 treatment in the presence of Ag, there was an increase in both the DO11.10 T cell number and percent (Figure 2.1). In the LN at 120 hours, there were 6.8% DO11.10 T cells in the group that

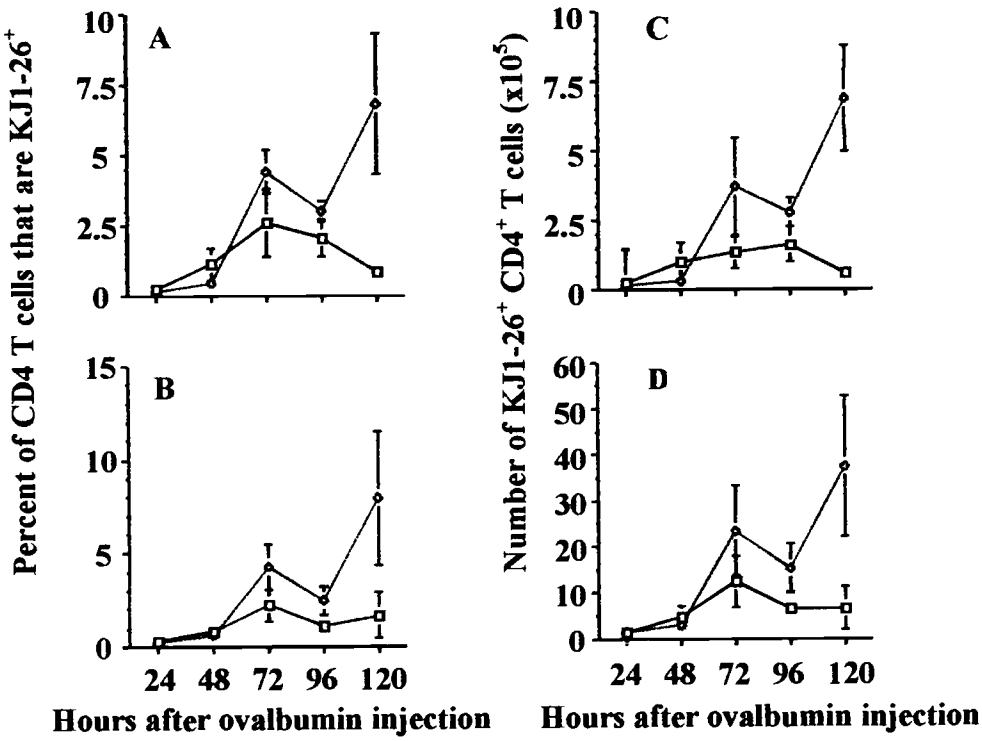

Figure 2.1: Ligation of OX40 leads to increased clonal expansion. Approximately $2x10^6$ DO11.10 T cells were injected i.v. into BALB/c mice. 500 μg OVA was injected i.p. the day after transfer, which is regarded as day 0. Immediately following the OVA injection, 50 μg of anti-OX40 was injected i.p. into one of the experimental groups, ◇ -OVA/anti-OX40. 50 μg of rat IgG was used for the control group, □ -OVA/rat IgG. LN (A and C) and spleen (B and D) cells were isolated from the BALB/c mice on days 1, 2, 3, 4 and 5. The cells were stained for the DO11.10 transgenic TCR using the anti-idiotypic mAb KJ1-26. A and B are the percent of KJ1-26$^+$ T cells present. C and D are the number of KJ1-26$^+$ T cells present. This graph was generated by combining data from four separate experiments and represents the mean ± SEM of 4 mice/group.

received anti-OX40 as compared to only 0.86% DO11.10 T cells in the control IgG group (Figure 2.1A). Data from the spleen were similar. For example, by 120 hours, 8.0% of the T cells in the spleen from anti-OX40 treated mice were DO11.10 T cells, whereas only 1.7% of the IgG treated splenic T cells were DO11.10$^+$ (Figure 2.1B). When DO11.10 T cell numbers are analyzed, the data confirm these results. For example, there was an 11 and 5.6 fold increase in DO11.10 T cell numbers between the anti-OX40 treated and control IgG groups in the LN and spleen respectively at 120 hours (Figure 2.1 C and D). These results extend previous data which showed that OX40 ligation increased the number of Ag-specific memory T cells compared to Ag without OX40 ligation (12). Thus, anti-OX40 treatment in the presence of Ag leads to increased Ag-specific T cell numbers, but a mechanism remains enigmatic. Several activities could lead to an increase in cell numbers such as a higher rate of cell division, the ability to progress through more cell cycles, an increase in cell longevity, or an increase in migration of DO11.10 T cells into the LN and spleen. We addressed these issues below with our in vivo model.

Anti-OX40 treatment has minimal effects on the initial division rate of Ag-stimulated T cells.

BrdU is a thymidine analog that can be incorporated into a cell's DNA in place of thymidine. Therefore, any cell that is undergoing S phase transition while the BrdU concentration is greater than the endogenous thymidine concentration will

have BrdU incorporated into its DNA. An anti-BrdU mAb can detect cells that have incorporated BrdU. This method was used to determine the effect of OX40 ligation on initial proliferation rates of Ag-stimulated T cells. DO11.10 mouse LN cells and splenocytes were transferred into BALB/c mice as described in the methods. These transferred cells were activated in the BALB/c mouse with either ovalbumin and anti-OX40 or ovalbumin and rat IgG as a control on day 0. In addition, the effect of a natural adjuvant, LPS, was also investigated. A subset of both the ovalbumin/anti-OX40 and the ovalbumin/rat IgG treated groups also received an LPS injection. Also, a control ovalbumin/rat IgG group was used as a no BrdU control to determine background staining, while the other groups received BrdU on days 2, 3 and 4. LN and spleen cells were removed and stained for BrdU content on days 5 and 12.

It was hypothesized that OX40 ligation would allow the Ag-activated DO11.10 T cells to enter the cell cycle faster than the control IgG treated DO11.10 cells. This would be evident by a higher percentage of cells incorporating BrdU in the ovalbumin/anti-OX40 and the ovalbumin/anti-OX40/LPS treated groups than the ovalbumin/rat IgG and ovalbumin/rat IgG/LPS control groups. However, the data did not uniformly support this hypothesis. When the percent of DO11.10 T cells incorporating BrdU in each of the four groups were compared at days 5 and 12, the rates of incorporation were all relatively equal in the LN samples (Figure 2.2 A and B). However, there were differences in the anti-OX40 treated spleen cells

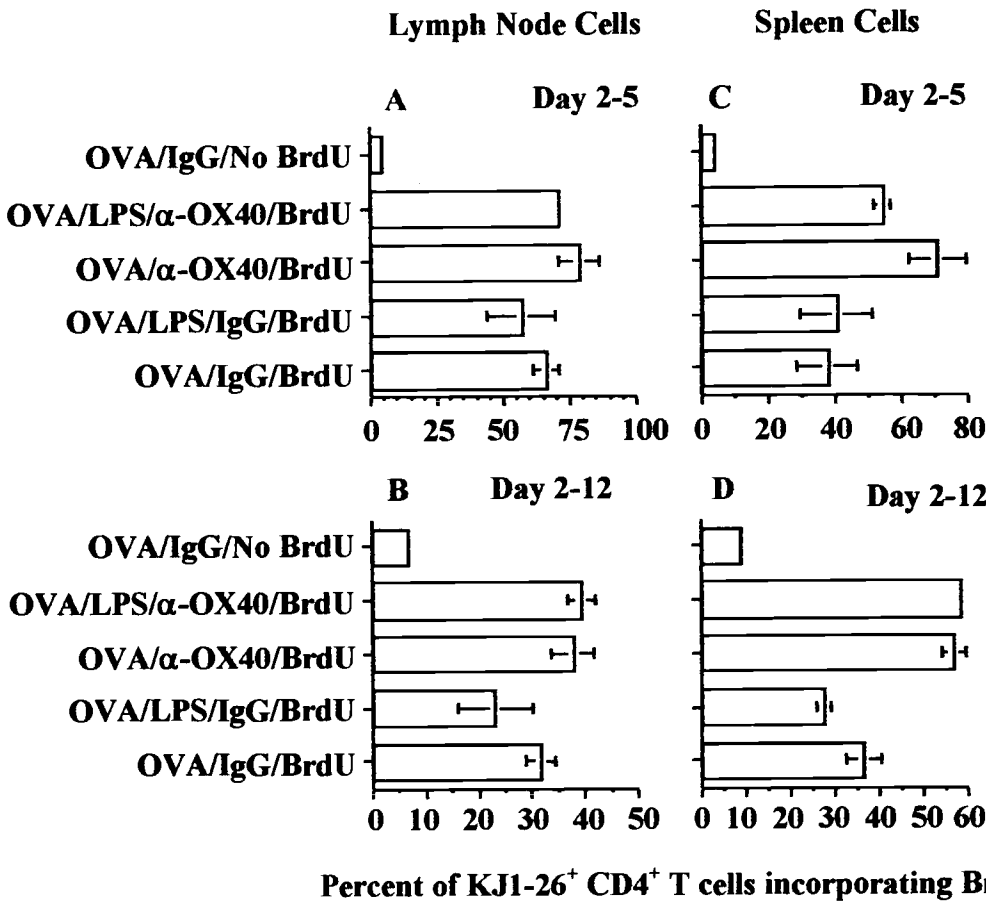

Figure 2.2: OX40 ligation has minimal effect on cell division rates.
On day 0, 500 μg of OVA was injected into BALB/c mice that had previously received approximately $2x10^6$ DO11.10 T cells. Immediately following the OVA injection, one of the experimental groups received 50 μg of anti-OX40, while the control group received 50 μg of rat IgG. On day 1, a subset of the anti-OX40 and rat IgG treated groups received an i.p. injection of LPS. Additionally, a second injection of anti-OX40 or rat IgG was administered to the experimental and control groups respectively. On day 2, all experimental mice received a third injection of anti-OX40 and the control group received a third injection of rat IgG. Injections of BrdU were given on days 2, 3 and 4. LN (A and B) and spleen (C and D) T cells were isolated and analyzed on days 5 and 12. The cells were stained for BrdU incorporation, and the percent of $KJ1\text{-}26^+$ T cells incorporating BrdU was calculated. Each experimental group contained 3 mice and the mean ± SEM are shown for each timepoint. These data are a representative experiment of two repeat experiments.

(Figure 2.2 C and D). Although groups that received the anti-OX40 treatment did show a slight increase in the percent of DO11.10 T cells incorporating BrdU, indicating a higher division rate, this difference is unlikely to be significant enough to account for the difference seen in LN DO11.10 T cell numbers (Figure 2.1).

To examine whether the cells incorporating BrdU had different survival rates, we analyzed absolute cell numbers of the treated groups. The groups that received anti-OX40 in addition to Ag had significantly higher DO11.10 T cell numbers incorporating BrdU at day 5 and 12 than those groups that had received the control antibody and Ag (Figure 2.3). In both the LN and spleen, there are approximately twice as many DO11.10 T cells in the anti-OX40 treated group as compared to the rat IgG treated group. Thus, anti-OX40 treatment minimally influences initial rates of cell division (Figure 2.2), but rather dramatically increases cell survival (Figure 2.3). However, upon the addition of LPS, the DO11.10 T cell numbers containing BrdU were slightly decreased at day 5 in both the anti-OX40 and control IgG treated groups as compared to the respective groups that did not receive LPS (Figure 2.3 A and C). Despite the initial negative effect of LPS, the long term effect was beneficial.

Our data indicate that the effects of LPS are observed later in the response, rather than in the beginning. The positive effects of LPS seem to be augmented in the presence of OX40 ligation. This is shown by a 7.1 and 6.5 fold increase at day 12 in the number of DO11.10 $BrdU^+$ T cells in the anti-OX40 and LPS treated

group compared to the IgG and LPS group in the LN and spleen respectively (Figure 2.3 B and D). The number of cells incorporating BrdU in the ovalbumin/anti-OX40 versus the ovalbumin/anti-OX40/ LPS group demonstrates the beneficial effects of LPS. In the LN, there is a 15.37 fold decrease in the

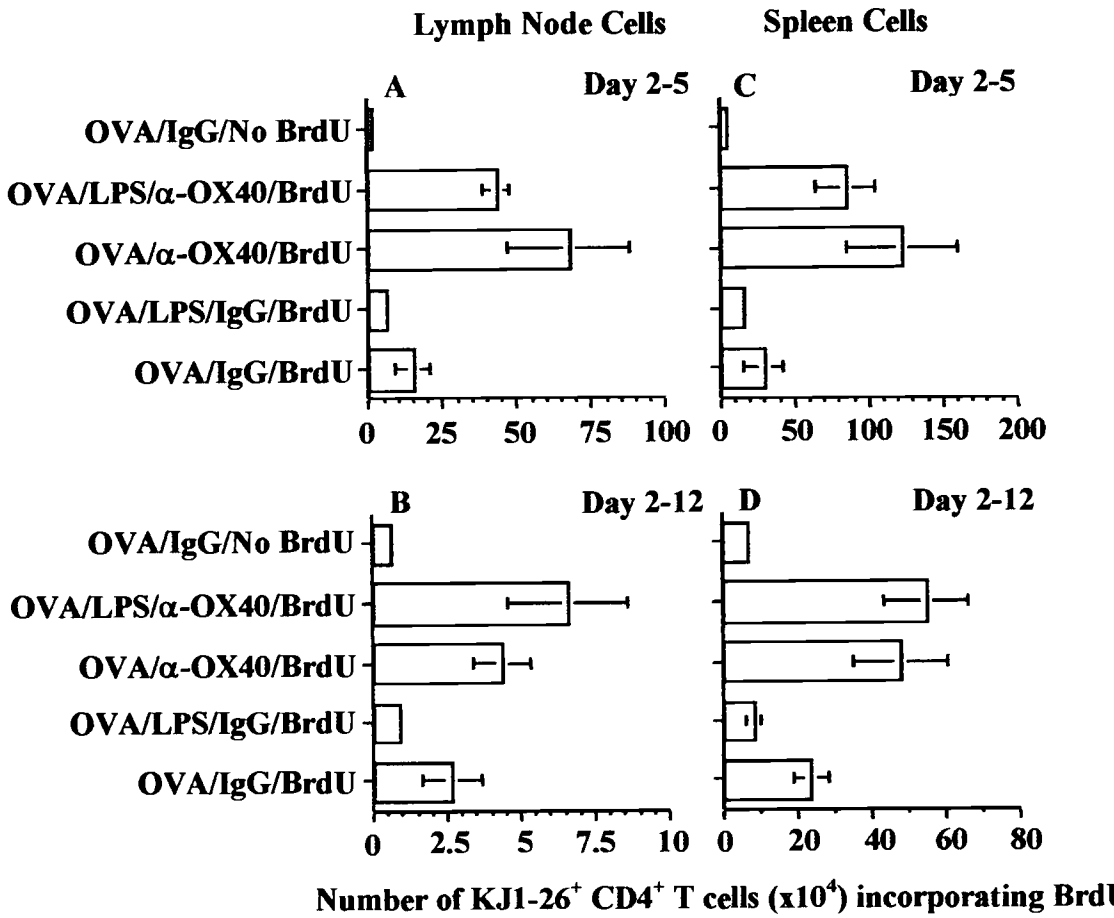

Figure 2.3: Ligation of OX40 enhances Ag-specific T cell survival.
These are data taken from Figure 2.2. The numbers for KJ1-26$^+$ T cells ± SEM are shown here.

number of cells that have incorporated BrdU in the ovalbumin and anti-OX40 group between days 5 and 12 (Figure 2.3 A and B). The group treated with ovalbumin, anti-OX40 and LPS had only a 6.55 fold decrease in T cell numbers that have incorporated BrdU (Figure 2.3 A and B). These data indicate that, of the cells treated with Ag and anti-OX40 alive at day 5, only 6.5% of them survived until day 12. This is compared to 15.2% of the cells surviving in the Ag, anti-OX40, and LPS treated group. The data is similar in the spleen (Figure 2.3 C and D). Only 39.18% of the cells alive at day 5 in the ovalbumin and anti-OX40 group survived until day 12. The survival increases to 65.66% when LPS is added in addition to ovalbumin and anti-OX40. The data presented here indicate that although LPS may not positively affect cell division rates, it does have an effect on Ag-specific T cell longevity.

Treatment of Ag-activated T cells with anti-OX40 enhances cell cycle turnover.

CFSE is a stain that enters into cells and becomes covalently attached to cytoplasmic proteins. Cellular esterases then cleave the molecule and render it fluorescent. Because the stain is permanently bound within the cytoplasm, when a cell undergoes one division, the stain is halved in each of the daughter cells. This decrease in fluorescence can then be detected by flow cytometry.

For our experiments, cells from DO11.10 mice were removed from LN and spleen, stained with CFSE, and adoptively transferred into BALB/c mice as

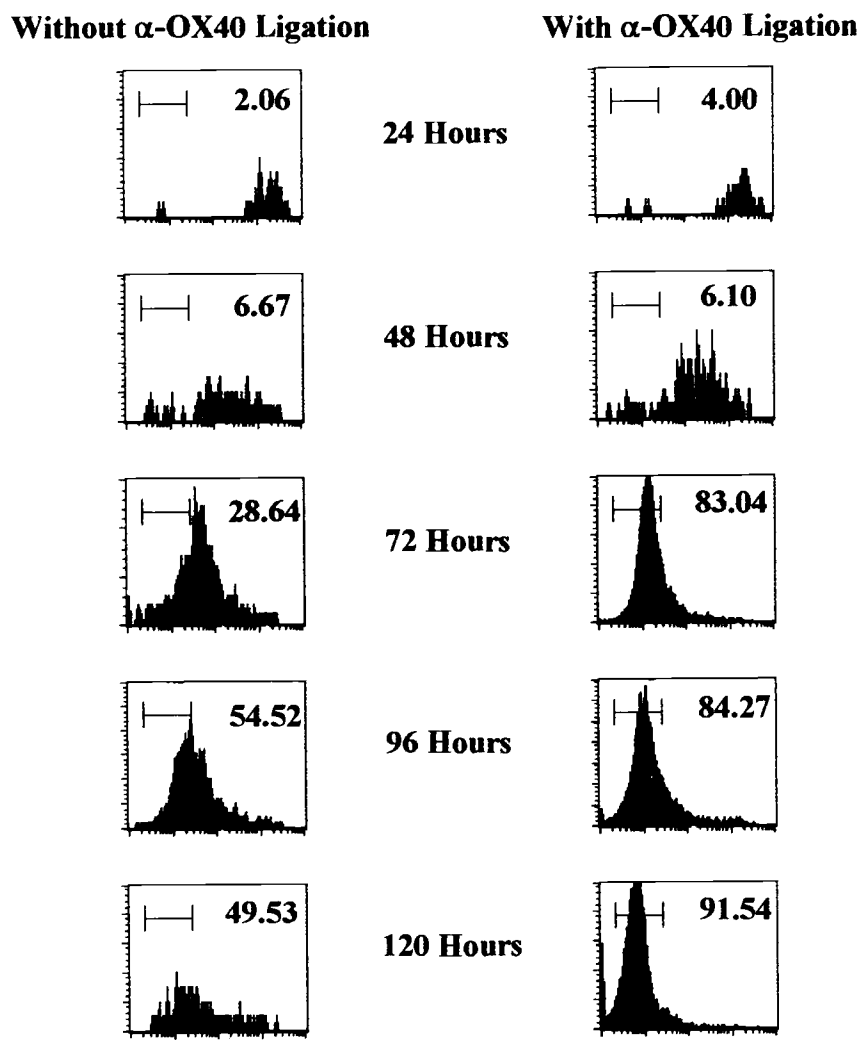

Figure 2.4: Treatment with anti-OX40 leads to enhanced cell cycle turnover in the LN. For CFSE studies, DO11.10 mouse LN and spleen cells were stained with CFSE and then injected i.v. into BALB/c mice. 500 μg OVA was injected i.p. the day after transfer, which is regarded as day 0. Immediately following the OVA injection, 50 μg of anti-OX40 was injected i.p. into one of the experimental groups (right hand column), and 50 μg of rat IgG was used for the control group (left hand column). LN cells were isolated from the BALB/c mice 24, 48, 72, 96 and 120 hours after ovalbumin injection. The number in the upper right hand corner denotes the percentage of cells in the indicated analysis region, which we found to be the limit of detection. These data are from one experiment representative of 12 separate experiments with a total of 7-17 mice analyzed for each time point.

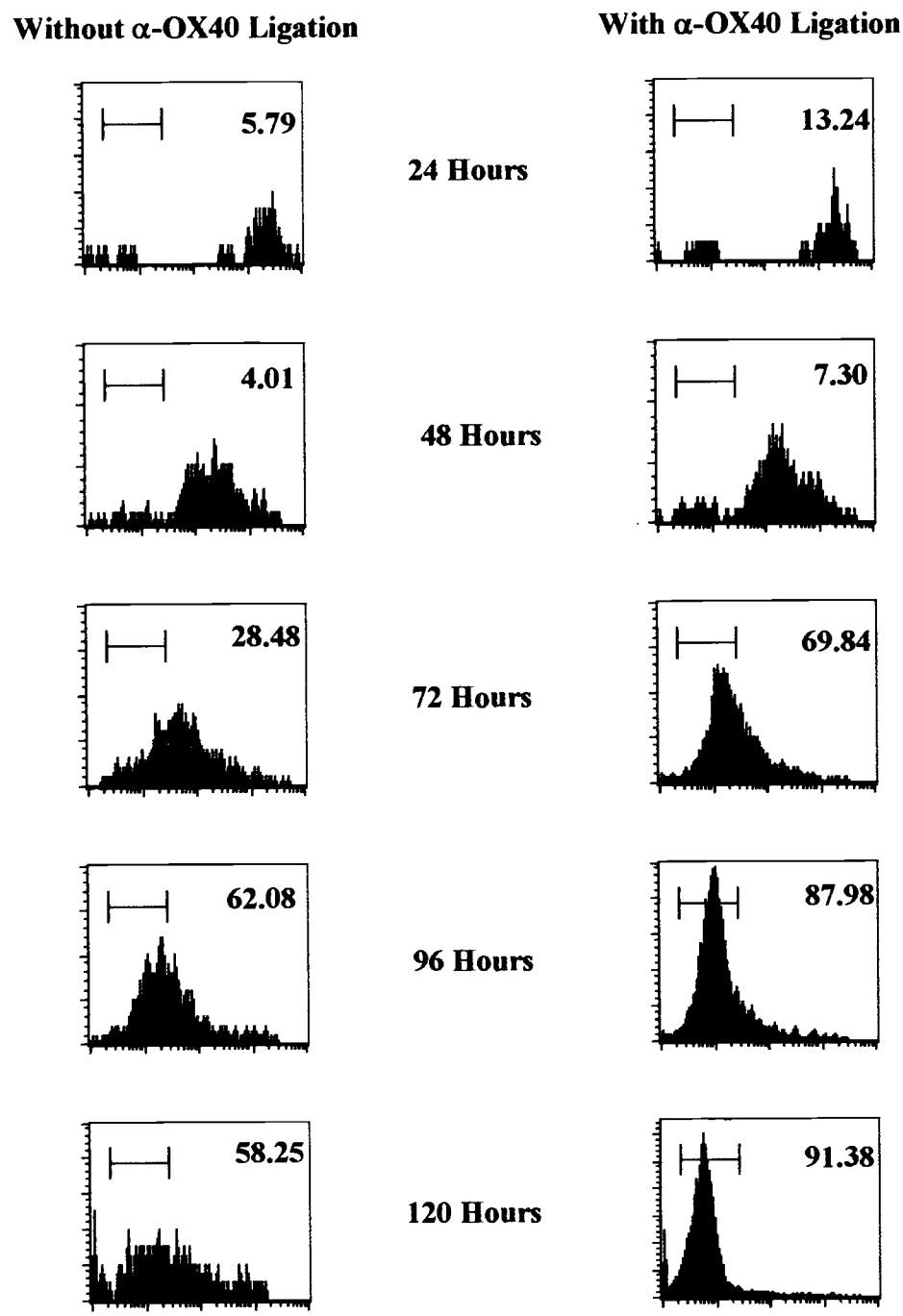

Figure 2.5: OX40 ligation enhances cell cycle progression in the spleen. These data are taken from Figure 2.4 and show the results from the spleen.

described in the methods. The mice received either ovalbumin and anti-OX40, or ovalbumin and a control rat IgG antibody at time 0, one day after transfer. DO11.10 T cells from the LN and spleen of the BALB/c mice were analyzed for CFSE intensity at 24, 48, 72, 96 and 120 hours after activation. This allowed us to directly track cell cycle progression for each treatment group. At 24 and 48 hours after activation, there was no significant difference in division between the anti-OX40 treated group and the control IgG group (Figures 2.4 and 2.5). However, by 72 hours in the LN, the anti-OX40 treated group had undergone more cell divisions, 83.04% of the cells are in the limit of detection, than the control group, 28.6% of cells are in the limit of detection (Figure 2.4). In fact, the control IgG group appeared to stall and was never able to catch up to the anti-OX40 group. Alternatively, the cells may have died and therefore never survive to 120 hours. Even by 120 hours in the LN only 49.5% of the control IgG treated DO11.10 T cells reached the limit of detection. This is compared to 91.54% of the anti-OX40 treated group (Figure 2.4). These data are consistent with analyses completed in the spleen (Figure 2.5). Therefore, we conclude that OX40 ligation on Ag-activated T cells induces these cells to proceed through more cell cycles as compared to Ag-activation in the absence of OX40 mAb stimulation.

Treatment of Ag-activated T cells with anti-OX40 proportionally generates fewer apoptotic cells in vivo.

A trademark of apoptotic cells is the flipping of phosphatidylserine, a molecule usually found on the inner leaflet of the cell membrane, to the outer leaflet where it is exposed on the cell surface. AnnexinV is a phospholipid-binding proteinthat is able to bind phosphatidylserine (32, 33). Therefore, when an apoptotic cell has phosphatidylserine exposed, annexinV can bind to it. This system allows the use of a fluorescently labeled annexinV molecule to detect the first signs of apoptotic cell death. The transferred DO11.10 cells were activated in the BALB/c mice with either ovalbumin and anti-OX40 or ovalbumin and rat IgG at time 0. LN and spleen cells from the BALB/c mice were stained with KJ1-26 mAb and annexinV at 96, 108, and 120 hours after activation. Previous experiments performed in our laboratory had shown that most of the Ag-specific T cells delete during this time period (data not shown). In addition, unactivated DO11.10 cells were analyzed 24 hours after transfer in order to establish normal levels of KJ1-26$^+$ annexinV$^+$ T cells.

For all time points examined (96, 108, and 120 hours) the groups treated with ovalbumin and anti-OX40 had a lower percentage of apoptotic DO11.10 T cells in both the LN and spleen as indicated by annexinV binding (Figure 2.6). By 120 hours, in the ovalbumin and anti-OX40 treated LN, only 10.5% of the DO11.10 T cells were annexinV positive, whereas 17.1% of the DO11.10 T cells were annexinV positive in the ovalbumin and rat IgG group (Figure 2.6A). Data for the

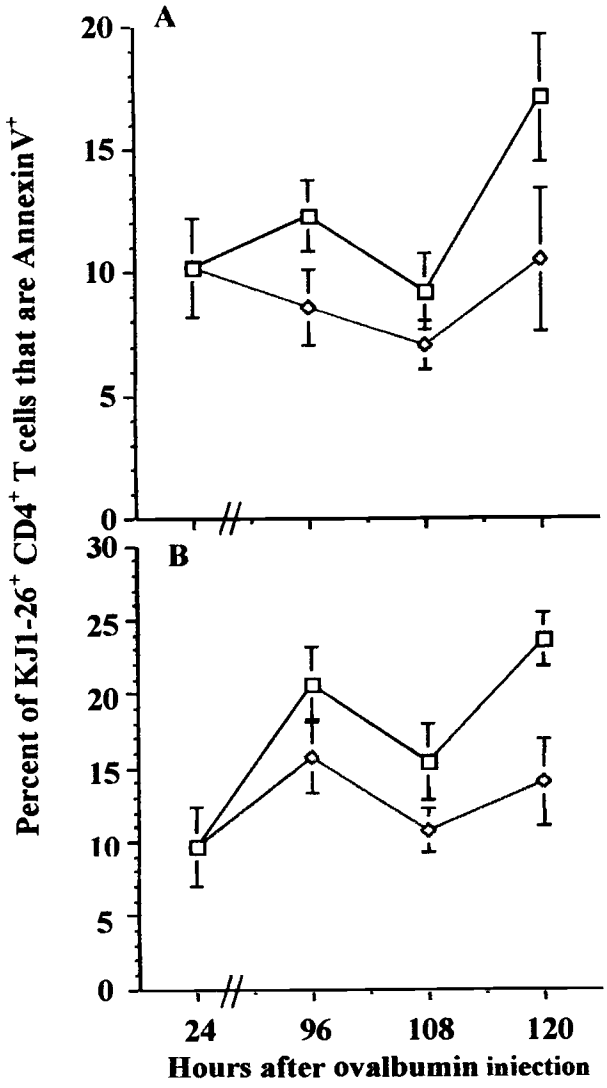

Figure 2.6: Treatment with anti-OX40 reduces the percent of Ag-specific T cells that are apoptotic. On day -1, approximately $2x10^6$ DO11.10 T cells were injected i.v. into BALB/c mice. The following day, 500 μg OVA was injected. This was directly followed by an injection of 50 μg of anti-OX40 into one of the experimental groups, ◇ -OVA/anti-OX40. 50 μg of rat IgG was used for the control group, □ -OVA/rat IgG. LN (A) and spleen (B) cells were isolated from the BALB/c mice at 96, 108, and 120 hours. Unactivated cells were analyzed 24 hours after transfer in order to establish normal levels. The cells were stained with the KJ1-26 mAb and with annexinV in order to detect apoptotic cells. The percent of double positive cells was calculated. Each data set represents the mean ± SEM from 8 mice/group. This is combined data from four separate experiments.

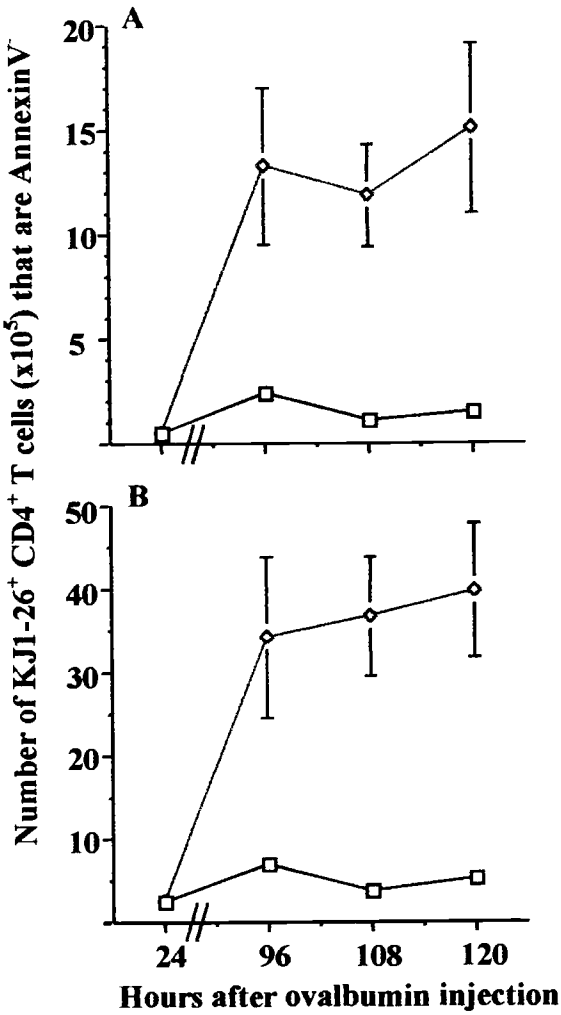

Figure 2.7: OX40 engagement increases the number of viable Ag-specific T cells. Two groups of BALB/c mice received DO11.10 LN and spleen cells and were treated with ovalbumin and either anti-OX40 (◇), or a control IgG (□). LN (A) and spleen (B) cells were isolated at 96, 108, and 120 hours, and stained with annexinV as indicated in Figure 2.6. The data shown are KJ1-26$^+$ T cells that are annexinV negative. These data are the combined results of four separate experiments. Each data point is the mean ± SEM from 8 mice just as in Figure 2.6.

spleen at 120 hours were comparable: 14.0% of the ovalbumin and anti-OX40 treated group were annexinV positive as compared to 23.6% of the ovalbumin and rat IgG group (Figure 2.6B).

The number of annexinV negative DO11.10 T cells was also tabulated. Again, at all timepoints, the ovalbumin and anti-OX40 treated group had higher numbers of annexinV negative DO11.10 T cells compared to the ovalbumin and rat IgG treated group (Figure 2.7). By 120 hours, the group treated with ovalbumin and anti-OX40 had a 9.6 and 7.4 fold increase in annexinV negative DO11.10 T cells over the ovalbumin and control IgG treated group in the LN and spleen respectively (Figure 2.7). These results allowed us to directly examine programmed cell death. At all timepoints the ovalbumin and anti-OX40 treated group had proportionally fewer apoptotic DO11.10 T cells.

Treatment with anti-OX40 yields T cells capable of responding to the immunizing Ag.

We next tested whether anti-OX40 treated cells could respond to antigen upon restimulation. The ability of effector T cells to respond to Ag is a fundamental property of cell-mediated immunity. If anti-OX40 treatment generated anergic T cells, efficacy of this treatment in vaccines would be nullified. To test this possibility, DO11.10 cells were transferred into BALB/c recipients. These cells were activated with ovalbumin and the experimental group received an injection of anti-OX40, while the control group received an injection of rat IgG. Spleen cells

were removed 4 days post-activation and were cultured in vitro. These cells were re-stimulated with ovalbumin peptide, or nothing as a control. The measurement of ^{3}H-thymidine incorporation allowed us to determine the relative amount of cell division by the bulk population. These spleen cells were also stained with the KJ1-26 mAb to determine the percent of DO11.10-derived T cells, which allowed us to calculate the amount of proliferation by bulk Ag-specific cells.

When all cells were examined, we found that proliferation of total anti-OX40 treated cells was greater than proliferation of total rat IgG treated cells. There was a 5.4 fold increase in the amount of ^{3}H-thymidine uptake in the cells that received ovalbumin and anti-OX40 compared to ovalbumin and rat IgG (Figure 2.8A). When the CPM were evaluated based on the actual number of KJ1-26^{+} cells in the culture, it was clear that anti-OX40 treated cells did not respond any better than the IgG treated group. For example, 12,400 KJ1-26^{+} T cells from the control IgG group contained 12,285 CPM versus 33,800 KJ1-26^{+} T cells from the anti-OX40 treated group producing 28,791 CPM. Clearly, these data show that OX40 ligation generates effectors that are responsive to recall Ag, but are not more responsive than their rat IgG counterparts at the timepoint tested.

DISCUSSION

OX40 is a membrane bound protein belonging to the TNF/NGF receptor family. It is a potent co-stimulator of T cells since, in the presence of Ag, OX40

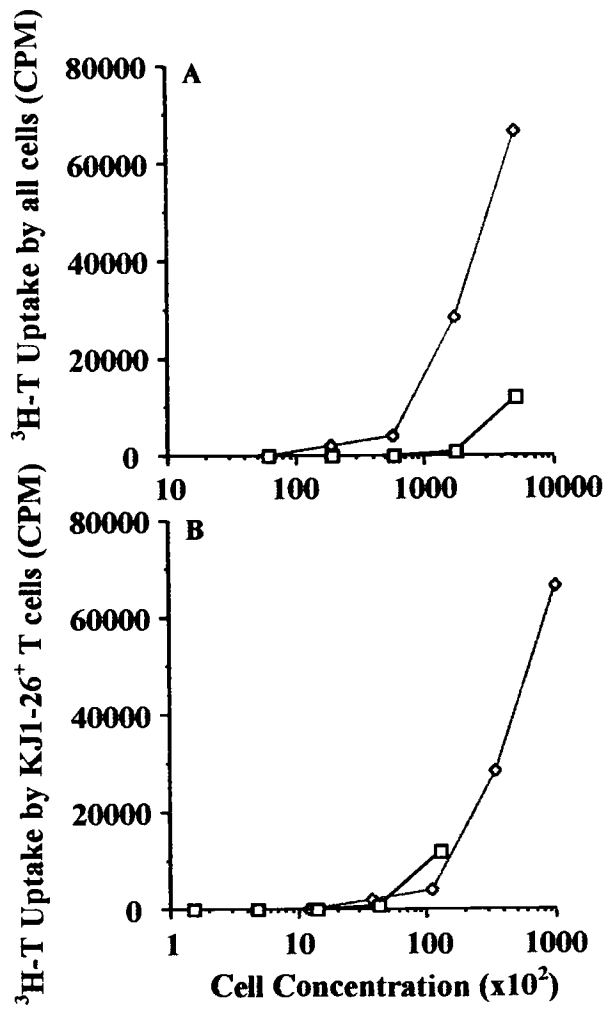

Figure 2.8: Treatment with anti-OX40 does not generate anergic T cells. BALB/c mice received DO11.10 T cells and a day later, day 0, the mice were injected with ovalbumin. Immediately following the Ag injection, the mice were injected with anti-OX40 (◇) or a control IgG (□). Four days later, spleen cells were stimulated *in vitro* with or without ovalbumin 323-339 peptide. Serial dilutions of each culture were plated in triplicate in 96-well plates. The plates were incubated at 37°C for 72 hours. ^{3}H-thymidine was added the last eight hours of culture at a concentration of 1 μCi per well. ^{3}H-thymidine incorporation was counted and used to determine proliferation of all cells (A), and of KJ1-26^{+} cells (B). Each point is from triplicate samples from 3 mice with each point being the mean ± SEM. This is a representative sample of two separate experiments.

ligation leads to increased Ag-specific T cell clonal expansion in vivo (12). The details and mechanism of OX40 enhanced Ag-specific T cell costimulation remain unclear. We examined the in vivo effects of OX40 ligation on Ag-stimulated cells and found that OX40 has a specific mode of action that leads to an accumulation of surviving T cells.

Several additional members of the TNF/NGF receptor family have been shown to influence T cell responses such as CD40 and 4-1BB (34). Two of these molecules, 4-1BB and OX40 are found on activated T cells, with OX40 only found on CD4 T cells ex vivo, but both molecules activate NFκB via TRAF proteins (34, 35). The third molecule, CD40, is found on APCs. Upon ligation, these molecules transduce a signal that profoundly affects T cell responsiveness. When CD40 is ligated on APCs, and T cells are offered Ag, the result is a large increase in clonal expansion of both Ag-specific $CD4^+$ and $CD8^+$ T cells (9). In addition, the concomitant clonal deletion of both T cell subsets is delayed with CD40 ligation. Furthermore, it was shown that the mechanism of delayed deletion and enhanced expansion is through CD28 ligation.

The action of 4-1BB is somewhat different than OX40. 4-1BB enhances clonal expansion of Ag-activated $CD4^+$ and $CD8^+$ T cells, however, only the $CD8^+$ cells are inhibited from deletion (36, 37). Here we show that the ligation of OX40 also leads to clonal expansion of Ag-activated cells (Figure 2.1). In addition, OX40 ligation decreases the clonal deletion of the Ag-activated T cells (12). However, the

means by which there is an Ag-specific increase in T cell clonal expansion is unknown. Experiments with OX40 knockout mice have shown OX40 ligation to be integral in the generation of a $CD4^+$ T cell response (17). These experiments demonstrate that the additional stimulation T cells receive through OX40 ligation potentiates a T cell response. Because OX40 ligation leads to enhanced T cell responses, we set out to elucidate the intricacies of the response. Initially, we reasoned that an increase in cell number, as seen with OX40 treatment, would most easily be obtained by the Ag-specific cells proliferating faster. To test this hypothesis, we examined the rate of initial cell division under circumstances of T cell activation in the presence or absence of OX40 stimulation. BrdU was used as an indicator because it would only be detected in cells undergoing DNA synthesis. Contrary to our initial expectation, we found the rate of division to be relatively similar, although there were some differences in the spleen (Figure 2.2). In addition, the use of an adjuvant, LPS, did not affect the division rate, but did increase the longevity of the $BrdU^+$ cells when LPS was given with the anti-OX40 mAb (Figure 2.3). Thus, the elevated T cell numbers observed in Ag-activated, anti-OX40 treated mice are a result of a mechanism not likely involving an increase in the initial cell division rate. This is also related to the fact that OX40 is not upregulated on T cells until 24 hours after activation (12, 16). These results implicate the role of OX40 to be later, rather than immediate, in the immune response because early division of Ag-specific T cells was similar. Thus, rather than peaking in activation

immediately, the ligation of OX40 may permit the Ag-activated cells to peak during the effector stage, which has been postulated by previous data (16, 38).

Because of our experiments with BrdU, we knew the rate of cell division could not explain OX40-enhanced T cell activation so a staining method was employed that directly detected cell division. CFSE staining and fluorescence allows direct detection of cell division by determining the number of cell cycles completed. A decrease in the fluorescence of the stain occurs with each cell cycle completed. This detection system was used to directly examine the effect of OX40 ligation on Ag-activated T cells. These experiments showed that Ag-stimulated cells that received anti-OX40 underwent more cell divisions, whereas control IgG treated cells halted division or died, while the cells receiving the OX40 stimulation kept on proliferating and survived (Figures 2.4 and 2.5). Our data may explain why $OX40^{-/-}$ T cells are less proliferative than wild type T cells (17). These data suggest that in the absence of OX40 ligation, T cells do not complete as many cycles of division. Additionally, these data are consistent with the fact that T cells from $OX40L^{-/-}$ mice produce less IL-2 and IL-4 than wild type T cells (19).

A second means by which this prolonged cell division could take place suggests that expression mechanics of OX40 and its ligand could influence cell division. Peak expression of OX40 occurs between 24 and 48 hours after activation (39). The expression of OX40L on B cells also occurs 24 to 48 hours after stimulation (19). Thus, maximal interaction of OX40 with its ligand does not occur

until 48 hours after T cell stimulation. Therefore, any influence exerted by the OX40/ OX40L interaction would not be evident before this time point. As our data show, there is a significant increase in Ag-stimulated cell division between 48 and 72 hours (Figures 2.4 and 2.5). The cells that do not receive OX40 ligation are unable to continue their division cycles and are effectively halted or deleted (Figures 2.4 and 2.5). It is possible that the cells are signaled to die, and therefore do not accumulate because they are eliminated before they finish traversing the cell cycle. Also, this delayed response may result from the CD28 dependence of OX40 signaling (40). This notion is consistent with our data because ovalbumin was given without adjuvant and therefore, theoretically speaking, little upregulation of B7 on APCs would be apparent.

Because anti-OX40 treatment enhances the numbers of cell cycles completed, we hypothesized that a healthy cell would undergo division, while an apoptotic cell would not divide. Therefore, anti-OX40 treatment should proportionally decrease the percent of apoptotic cells. To test this hypothesis we used annexinV staining. AnnexinV binds to the exposed phosphatidyl serine molecule on an apoptotic cell's surface (32, 33). AnnexinV staining allowed the comparison of the degree of Ag-induced death in the ovalbumin/rat IgG group compared to the ovalbumin/anti-OX40 treated group. As expected, we found that Ag-activated cells receiving anti-OX40 had proportionally fewer apoptotic cells than the group receiving control rat IgG (Figures 2.6 and 2.7). These findings support

previous data showing that there is an increase in Ag-specific memory T cells when activated T cells are treated with anti-OX40 (12). Together these results suggest that there is a greater development of memory because the Ag-stimulated cells treated with anti-OX40 are less susceptible to death. In contrast, Ag-activated cells that do not receive an exogenous anti-OX40 signal die early and do not lead to the enhancement of the memory response. One mechanism by which the anti-OX40 treated cells could survive longer is by down regulating Fas. Fas ligation can trigger apoptosis, and if this molecule was downregulated the "death" signal would be inhibited, which in turn would lead to increased survival. However, we found that Ag-activated cells treated with anti-OX40 did not down regulate Fas to any significant degree at the timepoints investigated (data not shown). Despite equal expression of Fas, other mechanisms exist by which anti-OX40 treated cells survive. One mechanism is increased cytokine production. Studies have shown that Ag-activated cells treated with anti-OX40 have increased cytokine production, primarily IL-2, IL-4 and IL-5 (39). This increase in cytokines may make the extracellular environment more conducive to cell survival. Without anti-OX40 treatment, this environment is not generated and growth factor withdrawl-induced death can occur.

IL-2 is a cytokine that has been shown to increase cell division and accumulation in vitro (41). However, in vivo studies with IL-2 have shown that it may not be required for cell division (42). In addition, experiments with memory cells have shown that IL-2 inhibits memory cell proliferation (43). We investigated

whether IL-2 addition would affect proliferation and cell accumulation in anti-OX40 treated mice. We reasoned that if the anti-OX40 treated cells remained effector cells, the IL-2 treatment might increase their proliferation and accumulation. We found that treatment of Ag-activated cells in vivo with anti-OX40 and IL-2 did not have an effect on proliferation or accumulation (data not shown). The number or percent of Ag-activated cells was not increased with IL-2 treatment. The dose used was equivalent or greated than that used in other studies (44). This suggests that the cells are not of a memory phenotype because the presence of IL-2 did not have an inhibitory effect. We also found that treatment with IL-4 did not lead to an increase in cell numbers or cell cycle progression (data not shown). These data indicate that cytokines other than IL-2 or IL-4 may be responsible for the Ag-specific responses observed with anti-OX40 treatment. It should also be noted that early experiments with a neutralizing anti-IL-2 mAb did not lead to an increase or decrease in the number or percent of Ag-specific cells (data not shown). This further implicates alternative cytokines in the function of OX40 mediated effects in vivo.

We also wanted to be sure that treatment of Ag-activated T cells with anti-OX40 did not lead to a large primary response, and then render the cells anergic. To determine this, Ag-specific proliferation assays were performed. In vivo stimulated cells were removed and then re-stimulated in vitro without any further anti-OX40 addition. We found that the anti-OX40 treated cells respond adequately

upon antigen re-exposure in vitro (Figure 2.8). Nevertheless, we did not test in these experiments whether OX40 ligation in vitro would enhance proliferation.

Our experiments indicate for the first time that Ag-activated T cells which receive OX40-ligation have enhanced cell cycle turnover. This increased proliferation leads to an accumulation of functional Ag-specific cells. It is this aspect of OX40 ligation that may be applicable to vaccine development. However, we next need to determine the environmental requirements for these effects. Several possibilities are that cytokines or other signals that we have not investigated are required. In addition, growth factors or chemokines that have not been examined may play a role in mediating the effects of OX40 ligation.

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CHAPTER 3

INVESTIGATING THE ONSET OF AUTOIMMUNITY IN A.SW MICE FOLLOWING "TOXIC OIL" TREATMENT

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ABSTRACT FOR CHAPTER 3

In 1981, over 20,000 people were struck with what would later be called toxic oil syndrome (TOS). It occurred in Spain when industrial rapeseed oil supplemented with 2% aniline was illegally refined and sold as olive oil. The exact culprit responsible for the disease still has not been identified. However, research is now beginning to look at genetic predisposition as a contributor to the disease. Mouse models have been extremely helpful in this search. It has been determined that the MHC genotype is extremely important in susceptibility to toxic oil induced autoimmunity. H-2s strains of mice have been shown to develop symptoms of TOS when they are exposed to toxic oil. However, experiments conducted with these mice were complicated by the early spontaneous induction of autoimmune disease. In this study, we examined the effects of toxic oil on the A.SW strain of mouse. This strain is also of the H-2s genotype but does not spontaneously develop autoimmunity. Therefore, any autoantibodies produced would result from exposure to the toxic oil. Mice were treated with three types of toxic oil: CO756 (case oil from Spain), RSD99 (rapeseed oil with no 3-(N-phenylamino)-1-2-propanediol (PAP) derivatives), and RSA99 (rapeseed oil supplemented with PAP derivatives). Each oil was administered undiluted (neat) or diluted 1:10 with canola oil. In addition, naïve and canola oil treated mice were used as controls. Mercuric chloride treated mice were used as a positive control. One-third of the mice in each group were killed at 2.5, 5, or 10 weeks after treatment. At each timepoint, body

weights, organ weights (liver, kidney, thymus, and spleen), antibody isotypes, and autoantibody formation were examined. We found that the RSD99 and CO756 treated mice had significantly increased body weights at 2.5 weeks of treatment. Interestingly, the canola oil treated mice had significantly decreased body weights when compared to naïve mice. These differences had disappeared by the 5 and 10 week timepoints. There were no consistent differences in organ weights at any timepoints. However, at 2.5 weeks there was a significant decrease in the liver weight as a percent of body weight in the RSA99 treated mice. At 2.5 weeks, canola oil treated mice had a significantly smaller spleen as a percent of body weight when compared to the naïve control. At 5 weeks, the CO756 neat treated mice had significantly smaller thymuses as a percent of body weight. By 10 weeks of treatment, there were no significant differences in the weights of any organs. We also found that treatment with toxic oil did not lead to increased serum levels of IgG1, IgG2a, or IgE at any timepoint. Treatment of A.SW mice with the toxic oils also did not induce autoantibody formation. However, treatment of mice with mercuric chloride led to significant effects. At 2.5, 5 and 10 weeks there were significant changes in organ weight as a percent of body weight. At 2.5 and 10 weeks all organ weights investigated (liver, kidney, thymus, and spleen) were significantly higher as a percent of body weight; only the kidneys and spleens were elevated at 5 weeks. Additionally, mercuric chloride treated mice all had elevated serum levels of IgG1, IgG2a, and IgE. These mice also had a noticeable production

of anti-nuclear antibodies. In summary, we were unable to generate TOS symptoms in the A.SW mouse model following treatment with toxic oils. However, treatment with mercuric chloride was able to induce an autoimmune state in the A.SW mouse.

INTRODUCTION

The toxic oil syndrome (TOS) affected over 20,000 people in Spain in 1981 (1-4). Symptoms began occurring after the consumption of an adulterated rapeseed oil. Industrial oil that was supplemented with 2% aniline was re-processed and then sold as a cheap olive oil (5-8). Following consumption, disease symptoms began to manifest themselves in three distinct phases. The acute phase began at the time of consumption and lasted from one to two months. It was characterized by fever, rash, eosinophilia, pulmonary edema, increased IgE levels, and myalgia (4, 9-14). Following the acute phase, 59% of people progressed into an intermediate phase. This was characterized by pulmonary hypertension, thromoboembolism, persistent myalgia, eosinophilia, skin edema, alopecia, and sicca syndrome (4, 10-14). Ten to fifteen percent of people with acute symptoms progressed to the chronic stage. The chronic phase consisted of pulmonary hypertension, scleroderma, peripheral neuropathy, and liver disease (4, 10-14). In addition, the chronic phase was characterized by the production of autoantibodies including anti-nuclear and anti-nucleolar antibodies (2, 3, 9, 15-18). Because of the similarities of TOS to other autoimmune diseases including systemic lupus erythematosus (SLE) and systemic scleroderma, researchers began looking for a cause (19).

Because TOS resembled several well characterized autoimmune diseases, researchers began to look for similarities. It was discovered that 74% of TOS patients with chronic symptoms had IgG antibodies to C reactive protein (CRP)

(20). When these results were compared to SLE or systemic scleroderma, it was found that 78% of SLE patients had IgG antibodies to CRP (19). It was also discovered that 45% of patients with systemic scleroderma had antibodies to ceruloplasmin. Significant reactivities to other acute phase proteins were also found in TOS patients (20). It was hypothesized that a switch toward autoimmunity could stem from a chronic inflammatory process, as seen with systemic autoimmune diseases. This persistent inflammation would cause tissue damage, which could in turn cause an alteration of CRP epitopes. If the presentation of such epitopes was above the level required for T cell activation, an immune reaction would ensue (19). Activated T cells would produce interleukins, primarily IL-3, IL-5 and GM-CSF. These interleukins would then stimulate the production of eosinophils (21-23). The production of IgG1 and IgE could be promoted by the production of IL-4 (15, 21-23). However, the causative agent that could invoke this transition to autoimmunity remains elusive.

Because the oil was denatured with 2% aniline, this immediately became a suspect. Aniline has the ability to form conjugates with fatty acids in the oil including oleic acid (3, 15, 24, 25). A second agent found in the case oil that has been implicated in the disease process is 3-(N-phenylamino)-1-2-propanediol (PAP) (8, 22, 26, 27). These compounds may have become even more toxic following heating. Because the case oil was used for cooking, the high temperatures reached during frying could have led to the generation of oxidized

anilide derivatives (24, 28). Studies have shown that di-oleyl esters of PAP are cytotoxic (22, 29). This apoptotic induction may expose autoreactive lymphocytes to abnormal amounts of self material, and lead to the development of autoimmunity (22). In addition, oleyl and linoleoyl anilides have been shown to possibly induce the full spectrum of TOS (30). However, studies involving TOS have been inhibited by the inability to find a suitable animal model.

It has been well documented that there is a genetic link to TOS susceptibility. It has been shown that women under the age of 40 were at high risk for developing the disease (31). In addition, there was a prevalence of the MHC class II genes HLA-DR3 and HLA-DR4 in female TOS patients (9, 32). This apparent genetic link has made finding an animal model to study TOS difficult. Researchers have been unable to induce the full spectrum of TOS symptoms with reproducibility in any animal species including rabbits, rats, and chickens (15, 33-35). When mice are studied, there are extreme variations between strains. B10.S mice treated with oleic acid intra-peritoneally (i.p.) for 6 weeks showed increased serum IgE and IgM levels. They also had increased IgG1 and autoantibodies to histone, denatured DNA and DNP, as well as increased expression of IL-1β and IL-6 mRNA (15). These results indicated that anilide treatment was capable of breaking immune tolerance and could generate symptoms similar to the chronic phase of TOS (36). By contrast, when A/J mice were treated with anilide they developed acute wasting disease. Splenocytes in these mice also expressed IL-1α,

IL-10, and IFN-γ mRNA (37). Their symptoms resembled the acute symptoms of TOS (36). To further complicate things, following anilide treatment, C57BL/6 mice showed increased serum IgE levels. However, these mice displayed no symptoms of disease (37). The MRL/lpr mouse strain has also been used in the study of TOS. In this case, the researchers were able to stimulate the production of autoantibodies to collagen, dsDNA, and nuclear antigens following toxic oil treatment (38). However, this study found that toxic oil treatment did not lead to an increase in serum immunoglobulin levels. Although the latter study was able to implicate PAP in the disease process, there was large variability between individual mice because MRL/lpr mice spontaneously, but inconsistently, develop autoimmune disease early in life (38). Thus, it was difficult to determine if the changes resulted from the toxic oil treatment, or whether the mice were simply undergoing spontaneous development of autoimmune disease. These differing results have made uncovering the mechanism behind the TOS epidemic very difficult.

In the study reported here, we examined the effects of different oil treatments in A.SW mice in the hopes that a suitable animal model could be developed. Although this strain is susceptible to autoimmunity, it must be induced. Therefore, any changes in immunity that we observed could be attributed to the toxic oil treatments. Three different toxic oils were tested: CO756 (rapeseed oil that contained aniline and was further refined leading to the formation of PAP

derivatives), RSA99 (a rapeseed oil with aniline but no PAP derivatives), or RSD99 (a rapeseed oil with aniline as well as PAP derivatives). Separate groups of mice were gavaged with these oils diluted 1:10 in canola oil, a rapeseed oil. For controls, groups of mice were treated with canola oil, or nothing. Additionally, a group of mice was given mercuric chloride in their drinking water at a concentration of 50 ppm. These mice were used as positive controls. Following treatment, three timepoints were examined: 2.5 weeks, 5 weeks, and 10 weeks after initiating treatment.

Young animals are particularly susceptible to toxins and contaminants. Therefore, exposure that occurs at a young age can have profound effects. We wanted to investigate exposure at a young age along with the effect of continued exposure over a period of time. Previous research had also shown that earlier timepoints after initiating treatment showed the greatest effect on mouse weight and organ weights (38). However, these previous studies did not include a 2.5 week timepoint. Thus, in this experiment, we wanted to test an earlier timepoint to see if a greater difference in mouse and organ weights as compared to controls was produced. Additionally, it has been noted that TOS may affect the size of individual organs while not affecting total body weight. Notably, splenomegaly has been noted in B10.S mice treated with oleic acid anilide (15). Additionally, if there is intense T cell activation, differences in thymus weights may be seen. Altered liver function is another characteristic of TOS (4). We also wanted to

determine if the A.SW mice would exhibit the same symptoms as human TOS patients.

Increases in individual antibody isotypes have been well documented in TOS. Elevated IgE levels have been seen in numerous models of TOS (9, 15, 32, 37, 39-41). An increased level of serum IgE has been used to identify the acute stage of TOS (1, 3, 9, 30, 35). Additionally, increased levels of IgG antibodies have been seen in TOS patients and TOS models (9, 15, 19, 20, 39). This characteristic of TOS has been used to describe the chronic stage of the disease (9, 36). Autoantibody production was another indicator of chronic phase TOS (9, 15, 17, 18, 37). However, as with other symptoms of TOS, autoantibody production did not occur in all mouse models investigated (9, 15, 37). Autoantibody production is responsible for the persistent pain that TOS patients must endure. Therefore, it is of utmost importance to understand when and under what conditions these autoantibodies are produced. In this investigation, we wished to investigate at what timepoint these autoantibodies could be detected, and if treatment with toxic oil could induce autoantibody production in the A.SW mouse.

MATERIALS AND METHODS

Mice

Female A.SW mice aged 6 to 8 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). They were housed in the Laboratory Animal Resource Center according to federal guidelines.

Reagents and antibodies

Toxic oil mixtures were obtained from Dr. Manuel Posada in Spain. The oils were administered via oral gavage of 0.25 ml three times a week. Two doses of oil were used, undiluted and a dilution of 1:10. Canola oil was used as the diluent. Mercuric chloride, which was used as a positive control, was purchased from Sigma Chemical Company (St. Louis, MO). It was administered at 50 ppm in the drinking water.

The ELISA coating antibodies, i.e., mouse anti-IgG1 and mouse anti-IgG2a, were purchased from Sigma. They were diluted to 2000 ng/ml in coating buffer. Coating buffer consisted of 0.1 M sodium bicarbonate (Sigma), pH 8. ELISA plates were washed with wash buffer containing 0.14 M sodium chloride; 0.05 M sodium phosphate monobasic, monohydrate; 0.05 M sodium phosphate dibasic, anhydrous; and 0.05 % Tween 20 (all from Sigma). Serum samples were diluted in sample dilution buffer containing 0.14 M sodium chloride; 0.05 M sodium phosphate monobasic, monohydrate; 0.05 M sodium phosphate dibasic, anhydrous;

and 0.5 % bovine serum albumin (all from Sigma). Mouse IgG1 and mouse IgG2a standards were purchased from Sigma and plated in serial dilution starting from 100 ng/ml and 50 ng/ml, respectively. The secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG, was diluted in sample dilution buffer to 1:2000. The detector used was mixed in 0.1 M citrate buffer containing citric acid and sodium citrate (Sigma). Twenty µl of 1.6 µM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma) were added per 1 ml of citrate buffer. To this, 4 µl of 8 % hydrogen peroxide (Sigma) per 1 ml of citrate buffer was added. The stop solution used was 2 N sulfuric acid.

IgE antibody production was measured using the Pharmingen (San Diego, CA) OptEIA IgE Kit according to instructions. Anti-collagen antibodies were detected using the Mouse IgG Anti-type II Collagen Antibody Assay Kit from Chondrex (Redmond, WA). All procedures were performed according to instructions.

Calf thymus histones (CalBiochem, La Jolla, CA) were dissolved in double distilled water at a concentration of 1 mg/ml. They were then dialyzed against PBS (1.9 mM sodium phosphate monohydrate, 8.1 mM sodium phosphate anhydrous, and 154.0 mM sodium chloride all from Sigma) containing magnesium chloride (hexahydrate) and calcium chloride (dihydrate) from Sigma. Phenylmethysolfonyl fluoride (Sigma) was added to a concentration of 0.5 mM, and DNase I (Sigma) was added to a concentration of 100 U/ml. This mixture was incubated for 4 hours

at 37°C. It was then cooled on ice and 2 N sulfuric acid was added to a final concentration of 0.4 N. This was incubated at 0°C for 30 minutes. Following incubation, the solution was centrifuged for 15 minutes at 10,000 rpm. It was then dialyzed against 0.05 M acetic acid at 4°C and finally dialyzed against water. These histones were then used to coat ELISA plates at a concentration of 6 μg/ml in PBS. The gelatin postcoating solution was made of PBS with 1% gelatin added. Samples were diluted in sample dilution buffer: PBS with 0.05% Tween 20, 1 mg/ml gelatin, 0.75 mg/ml bovine gamma globulin and 1 mg/ml bovine serum albumin (all from Sigma). Plates were washed with wash buffer (described above). The secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG, was diluted in PBS with 0.05% Tween 20 with 1 mg/ml bovine gamma globulin and 5 mg/ml of bovine serum albumin. The detector used was the same as used in the IgG1 and IgG2a ELISAs (0.1 M citrate buffer to which 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid and 8 % hydrogen peroxide were added).

Fluorescent antibody slides were used for the detection of anti-nuclear and anti-native DNA antibodies. Samples were diluted from 1:10 to 1:3200 in PBS containing 0.14 M sodium chloride; 0.05 M sodium phosphate monobasic, monohydrate; 0.05 M sodium phosphate dibasic, anhydrous. These dilutions were then added to ImmnoGlo™ slides (IMMCO Diagnostics, Buffalo, NY). Slides used for detection of anti-nuclear antibodies contained HEp-2 cells, while slides used for detecting anti-native DNA antibodies contained Crithidia luciliae. Slides

were developed with FITC-goat anti-mouse IgG and IgM (Jackson ImmunoResearch, West Grove, PA) and counter stained with Evan's Blue.

Experimental Protocols

Ten treatment groups were used for these experiments. Mice treated with mercuric chloride were used as a positive control. Naïve, untreated mice were used as a control for the mercury treatment group. Canola oil treated mice were used as a control for the toxic oil treated mice. Three different toxic oils were administered. Each oil was used at an undiluted (neat) concentration and at a dilution of 1:10. Toxic oil treated mice were gavaged three time a week with 0.25 ml of the respective toxic oil, or canola oil, as a control. Mercury treatment groups were dosed by dissolving mercuric chloride in their drinking water at a concentration of 50 ppm. Mercury-treated water was changed once a week. Mice were weighed before treatment and then once a week during treatment.

One third of the mice from each treatment group (10 for neat treatment groups and 8 for diluted treatment groups) were sacrificed at each timepoint: 2.5 weeks, 5 weeks, and 10 weeks. Animals were fasted for 24 hours prior to necropsy, and then weighed just before sacrifice. Blood was collected via cardiac puncture under anesthesia immediately prior to death. Serum was separated by centrifugation and stored at -70°C until assayed. At necropsy, selected tissues and organs (liver, kidneys, heart, lungs, spleen, thymus, lymph nodes, pancreas,

stomach, intestine, and adrenal gland) were collected and fixed in 10% buffered formalin. The liver, kidneys, spleen, and thymus were all weighed before fixation. Blood smears were also prepared at this time. They were later stained with Wright's stain and examined for cellular content.

The quantity of IgG1 and IgG2a antibodies present in the mice was determined by an enzyme linked immunosorbant assay (ELISA). Briefly, plates were coated with 2000 ng/ml anti-IgG1 or anti-IgG2a antibodies in 0.1 M sodium bicarbonate buffer. One hundred µl was added to each well and the plates were sealed and allowed to incubate overnight at 4°C. The coating solution was removed after 24 hours, and 100 µl StabilCoat was added to each well. The plates were incubated at room temperature for 60 to 90 minutes. The StabilCoat was then removed and the plates were washed 4 times with ELISA wash buffer (described previously). Standards and serum samples were added to the wells at 100 µl each. The plates were then sealed and incubated overnight at 4°C. Following incubation, the plates were washed 8 times with ELISA wash buffer. HRP-goat anti-mouse IgG was then added to the plate. This antibody conjugate was diluted 1:2000 in sample dilution buffer (described previously). The plates were incubated for 30 minutes at room temperature before they were washed another 8 times with ELISA wash buffer. The working detector, composed of 0.1 M citrate buffer, 8% hydrogen peroxide, and ABTS, was then added to the plate at 100 µl/well. The plates were allowed to develop for 10 minutes before the reaction was halted by

adding 2% SDS. The absorbance was read at 410 nm shortly after stopping the reaction.

The presence of IgE antibodies was determined using the OptEIA kit (Pharmingen). All procedures were followed as directed by the kit instruction packet. Briefly, plates were coated with 100 µl of anti-mouse IgE diluted 1:250 in coating buffer (0.1M sodium carbonate, pH 9.5). The plates were sealed and incubated overnight at 4°C. Following incubation, the coating solution was removed and the plates were washed 3 times with IgE wash buffer (described previously). The plates were then blocked by adding 200 µl per well of assay diluent (described previously). The plates were incubated for 1 hour at room temperature after which the plates were washed 3 times with IgE wash buffer. The standards and serum samples were then added at 100 µl/well. The plates were sealed and incubated overnight at 4°C. Following the incubation, the plates were washed 5 times with IgE wash buffer and 100 µl/well of working detector (described previously) was added. The plates were incubated for 1 hour at room temperature before they were washed 7 times with IgE wash buffer. Following the wash, the TMB substrate solution was added to the plates at 100 µl per well. The plates were incubated for 30 minutes in the dark before the reaction was stopped with 50 µl/well of 2 N sulfuric acid. The absorbance was then read at 450 nm within 30 minutes of stopping the reaction.

Detection of anti-collagen type II antibodies was performed using the Mouse Anti-type II Collagen Antibody Assay Kit from Chondrex. Briefly, the pre-coated plates were washed three times with wash buffer. After washing, 100 µl of Solution A (blocking solution) was added to each well and the plates were allowed to incubate for 1 hour at room temperature. The plates were again washed three times and then 100 µl of either a serum sample or standard was added to each well. The plates were incubated overnight at 4°C. After the incubation the plates were washed 6 times and then 100 µl of secondary antibody solution was added to all wells. The plates were incubated for two hours at room temperature, and then washed 6 times. Following the washes, 100 µl of the detector was added to each well. The reaction was allowed to proceed for 30 minutes after which 50 µl of Solution D (stop solution) was added to each well. The absorbance was read at 490 nm immediately after the reaction was stopped.

Anti-histone antibodies were also detected using an ELISA. Plates were coated with histones at a concentration of 6 µg/ml at 100 µl per well. The plates were incubated overnight at 4°C. The coating solution was then removed and 150 µl of 1% gelatin postcoating solution was added to each well. Serum samples and positive control samples were plated at 100 µl per well and the plate was then incubated overnight at 4°C. Following the incubation, the plates were washed 4 times with wash buffer and the secondary antibody was added. The plates were

then incubated for 1.5 hours at room temperature and then washed 6 times with wash buffer. Working detector solution was added at 100 µl per well immediately after the washes. Plates were allowed to incubate at room temperature until a monitor well reached an optical density of 1.0 at which time the plate was read at 410 nm.

Antibodies for anti-nuclear and anti-native DNA were determined by ImmunoGlo™ slides. Serum samples were diluted from 1:10 to 1:3200 in 0.1 M PBS. These diluted samples were added to the slides. Slides were incubated at room temperature for 30 minutes. Following the incubation, the slides were washed in PBS and the FITC conjugated goat anti-mouse IgG and IgM was added at a 1:15 dilution. The slides were again incubated for 30 minutes at room temperature, after which they were again washed with PBS to remove any unbound conjugate. The slides were then counterstained with Evan's blue and read with a Zeiss epifluorescence microscope fitted with a FITC filter.

Statistics

Treatment groups were compared to control groups, both naïve and canola, using a student's t test, Mann-Whitney Rank Sum test or ANOVA (SigmaStat, SPSS Inc., Chicago, IL). Results were considered significant if the *p*-values were <0.05 between treatment and appropriate control groups.

RESULTS

Treatment with toxic oil affects whole mouse body weights at early timepoints.

To evaluate toxic effect of the oil treatments and controls, mice were weighed weekly during treatment. Upon necropsy, organ weights were measured and organ weight/body weight ratios were calculated. Differences in body weights were apparent early in the treatment period, but not at later timepoints. At 2.5 weeks, several groups of mice had significantly different body weights (Table 3.1). Our data showed that body weights in RSD99 1:10 and the CO756 neat groups were significantly increased relative to those in the canola control. The average RSD99 1:10 mouse weighed 19.24 ± 0.71g while the average CO756 neat treated mouse weighed 19.01 ± 0.67g compared to the average canola oil treated mouse that weighed 16.62 ± 0.82g (Table 3.1). It is also important to note that there was a significant difference between the naïve control and the canola control. The average naïve mouse weighed 19.53 ± 0.38g compared to the average canola treated mouse that weighed 16.62 ± 0.82g (Table 3.1). These results verify the hypothesis that treatment with toxic oils may have a significant effect early in the treatment period. At 5 and 10 weeks however, there were no significant differences between any of the treatment groups when body weights were examined (Tables 3.2 and 3.3). These results verify that treatment with these toxic oils has the greatest effect on body weights early in the dosing period, whereas prolonged treatment negates the effect.

Table 3.1: Body weight, organ weight and organ weight as a percentage of body weight (mean ± SEM) for 7 to 9 week-old female A.SW mice after **2.5 weeks** of treatment with canola oil, toxic oil compounds, or mercuric chloride (50 ppm). Statistical analysis was performed on body weight and organ weight as a percentage of body weight only, as compared to their respective controls: canola oil or untreated tap water (naïve).

		Organ Weight (grams)				Organ Weight as a Percent of Body Weight			
Group	Body Weight (grams)	Liver	Kidney	Thymus	Spleen	Liver	Kidney	Thymus	Spleen
Canola oil	**16.62 ± .82**	0.73 ± .03	0.12 ± .01	0.05 ± .01	0.06 ± .01	4.43 ± .06	0.73 ± .02	0.27 ± .04	**0.37 ± .01**
RSD 99 undiluted	18.53 ± .62	0.78 ± .02	0.14 ± .01	0.05 ± .01	0.07 ± .01	4.20 ± .08	0.75 ± .01	0.28 ± .02	0.36 ± .01
RSD 99 1:10	**19.24 ± .71**	0.81 ± .03	0.15 ± .01	0.05 ± .01	0.07 ± .01	4.24 ± .06	0.76 ± .02	0.29 ± .02	0.35 ± .02
RSA 99 undiluted	18.77 ± .37	0.78 ± .01	0.14 ± .01	0.05 ± .01	0.07 ± .01	**4.16 ± .03**	0.73 ± .01	0.28 ± .02	0.35 ± .02
RSA 99 1:10	18.51 ± .48	0.79 ± .03	0.14 ± .01	0.05 ± .01	0.07 ± .01	4.25 ± .05	0.76 ± .02	0.29 ± .01	0.36 ± .01
CO756 undiluted	**19.01 ± .67**	0.80 ± .03	0.14 ± .01	0.06 ± .01	0.07 ± .01	4.23 ± .04	0.74 ± .01	0.31 ± .02	0.36 ± .01
CO756 1:10	17.74 ± .46	0.75 ± .02	0.13 ± .01	0.05 ± .01	0.06 ± .01	4.23 ± .03	0.75 ± .02	0.28 ± .01	0.33 ± .01
Naïve	**19.53 ± .38**	0.84 ± .02	0.14 ± .01	0.05 ± .01	0.08 ± .01	4.30 ± .06	0.71 ± .01	0.28 ± .02	**0.42 ± .01**
$HgCl_2$ 50 ppm	18.03 ± .61	0.82 ± .04	0.17 ± .01	0.07 ± .01	0.09 ± .01	**4.56 ± .09**	**0.94 ± .02**	**0.38 ± .02**	**0.51 ± .01**

Bolded text indicates values significantly different from respective naïve or canola oil control treatments ($P < 0.05$).

Treatment with toxic oil does not affect organ weights, but does affect the organ to body weight ratio.

While toxic oil treatment may not ultimately affect whole body weights, there may be effects on individual organ weights. No significant differences in individual organ weights were seen in any of the treatment groups over time. At 2.5 weeks of treatment, there were no significant differences in individual liver, kidney, thymus, or spleen weights (Table 3.1). However, when organ weights were expressed as a percent of body weight, there were significant differences. At 2.5 weeks, the liver as a percent of body weight was significantly decreased in the RSA99 neat group (4.16 ± 0.03) compared to the canola control (4.43 ± 0.06). There was also a significant increase in the $HgCl_2$ group (4.56 ± 0.09) as compared to the naïve group (4.30 ± 0.06) (Table 3.1). The $HgCl_2$ group showed significant differences in several other organs as well. Both the kidney and thymus weights as a percent of body weight were significantly increased in the $HgCl_2$ group, 0.94 ± 0.02 and 0.38 ± 0.02, respectively, as compared to the naïve control, 0.71 ± 0.01 and 0.28 ± 0.02, respectively (Table 3.1). Several groups showed significant differences when the spleen as a percent of body weight was examined. The canola oil treated group (0.37 ± 0.01) as well as the $HgCl_2$ group (0.51 ± 0.01) were significantly increased from the naïve group (0.42 ± 0.01) (Table 3.1). Together, these results showed that there were minimal effects on the organ weights individually or as a percent of body weight in the oil treated groups at 2.5

Table 3.2: Body weight, organ weight and organ weight as a percentage of body weight (mean ± SEM) for 7 to 9 week-old female A.SW mice after **5 weeks** of treatment with canola oil, toxic oil compounds, or mercuric chloride (50 ppm). Statistical analysis was performed on body weight and organ weight as a percentage of body weight only, as compared to their respective controls: canola oil or untreated tap water (naïve).

Group	Body Weight (grams)	Organ Weight (grams)				Organ Weight as a Percent of Body Weight			
		Liver	Kidney	Thymus	Spleen	Liver	Kidney	Thymus	Spleen
Canola	19.31 ± .39	0.76 ± .01	0.14 ± .01	0.05 ± .01	0.06 ± .01	3.92 ± .08	0.73 ± .02	0.24 ± .01	0.33 ± .02
RSD 99 undiluted	19.50 ± .46	0.77 ± .02	0.14 ± .01	0.05 ± .01	0.06 ± .01	3.96 ± .07	0.72 ± .01	0.25 ± .02	0.32 ± .02
RSD 99 1:10	18.28 ± .75	0.74 ± .03	0.14 ± .01	0.05 ± .01	0.06 ± .01	4.05 ± .07	0.75 ± .01	0.27 ± .01	0.33 ± .01
RSA 99 undiluted	20.33 ± .77	0.82 ± .03	0.14 ± .01	0.05 ± .01	0.07 ± .01	4.02 ± .06	0.71 ± .01	0.27 ± .02	0.33 ± .02
RSA 99 1:10	18.09 ± .60	0.76 ± .03	0.13 ± .01	0.05 ± .00	0.06 ± .01	4.19 ± .06	0.71 ± .01	0.25 ± .01	0.32 ± .01
CO756 undiluted	18.98 ± .80	0.75 ± .03	0.14 ± .01	0.06 ± .01	0.06 ± .01	3.97 ± .06	0.71 ± .02	**0.30 ± .01**	0.31 ± .02
CO756 1:10	18.01 ± .61	0.74 ± .03	0.13 ± .01	0.04 ± .01	0.06 ± .01	4.13 ± .08	0.73 ± .01	0.20 ± .02	0.34 ± .02
Naïve	19.48 ± .73	0.80 ± .03	0.14 ± .01	0.05 ± .01	0.07 ± .01	4.19 ± .30	0.73 ± .05	0.25 ± .03	0.36 ± .03
$HgCl_2$ 50 ppm	19.67 ± .46	0.85 ± .02	0.18 ± .01	0.06 ± .01	0.08 ± .01	4.33 ± .07	**0.89 ± .02**	0.28 ± .01	**0.43 ± .01**

Bolded text indicates values significantly different from respective naïve or canola oil control treatments ($P < 0.05$).

weeks. However, these results confirmed that differences exist in organ to body weight ratios in the mercury treated mice.

Significant differences declined as time progressed. At 5 weeks there were few treatments that yielded a significant difference in either organ weight alone, or organ weight as a percent of body weight. When organ weights alone were examined, none of the treatment groups were significantly different from the controls (Table 3.2). Similarly, when the liver weight as a percentage of body weight was examined, it was found that there were no significant differences between any treatment groups, including the controls. The $HgCl_2$ positive control group showed a significant difference in the kidney and spleen weights as a percent of body weights, 0.89 ± 0.02 and 0.43 ± 0.01 respectively, when compared to the naïve controls, 0.73 ± 0.05 and 0.36 ± 0.03, respectively (Table 3.2). When the thymus as a percent of body weight was assessed, the only significant difference appeared in the CO756 neat group, 0.30 ± 0.01 as compared to the canola control at 0.24 ± 0.01 (Table 3.2).

After 10 weeks of treatment with the toxic oils or the control treatment, minimal effects were seen in organ weights or organ weights as a percentage of body weight. When individual organ weights were examined, none of the treatment groups showed a significant difference, including the positive controls (Table 3.3). These results were similar to those that were seen at 5 weeks. Additionally, there were no significant differences in the treatment groups when

Table 3.3: Body weight, organ weight and organ weight as a percentage of body weight (mean ± SEM) for 7 to 9 week-old female A.SW mice after **10 weeks** of treatment with canola oil, toxic oil compounds, or mercuric chloride (50 ppm). Statistical analysis was performed on body weight and organ weight as a percentage of body weight only, as compared to their respective controls: canola oil or untreated tap water (naïve).

		Organ Weight (grams)				Organ Weight as a Percent of Body Weight			
Group	Body Weight (grams)	Liver	Kidney	Thymus	Spleen	Liver	Kidney	Thymus	Spleen
Canola oil	20.11 ± .40	0.77 ± .02	0.13 ± .01	0.03 ± .01	0.06 ± .01	3.84 ± .04	0.66 ± .01	0.18 ± .01	0.30 ± .01
RSD 99 undiluted	20.29 ± .70	0.73 ± .02	0.14 ± .01	0.04 ± .01	0.06 ± .01	3.62 ± .05	0.70 ± .02	0.17 ± .01	0.31 ± .01
RSD 99 1:10	19.65 ± .57	0.74 ± .03	0.14 ± .01	0.04 ± .01	0.06 ± .01	3.76 ± .04	0.71 ± .01	0.21 ± .01	0.31 ± .01
RSA 99 undiluted	20.29 ± .47	0.85 ± .07	0.14 ± .01	0.04 ± .01	0.10 ± .03	4.20 ± .33	0.71 ± .02	0.22 ± .02	0.36 ± .07
RSA 99 1:10	21.19 ± .73	0.79 ± .03	0.15 ± .01	0.04 ± .01	0.06 ± .01	3.74 ± .03	0.69 ± .01	0.19 ± .01	0.29 ± .02
CO756 undiluted	20.42 ± .40	0.79 ± .02	0.15 ± .01	0.04 ± .01	0.06 ± .01	3.89 ± .07	0.71 ± .01	0.20 ± .01	0.29 ± .01
CO756 1:10	20.55 ± .82	0.79 ± .02	0.15 ± .01	0.04 ± .01	0.06 ± .01	3.87 ± .10	0.71 ± .01	0.18 ± .02	0.31 ± .01
Naïve	21.40 ± 1.02	0.82 ± .03	0.15 ± .01	0.04 ± .01	0.07 ± .01	3.88 ± .09	0.68 ± .01	0.18 ± .01	0.34 ± .01
$HgCl_2$ 50 ppm	20.00 ± .60	0.83 ± .03	0.18 ± .01	0.05 ± .01	0.09 ± .01	**4.17 ± .05**	**0.88 ± .01**	**0.24 ± .01**	**0.47 ± .03**

Bolded text indicates values significantly different from respective naïve or canola oil control treatments ($P < 0.05$).

organ weights as a percentage of body weight were examined (Table 3.3). The only significant difference was observed in the $HgCl_2$ positive control group. In this group, all of the organ (liver, kidney, thymus, spleen) weights as a percentage of body weight, 4.17 ± 0.05, 0.88 ± 0.01, 0.24 ± 0.01, and 0.47 ± 0.03 respectively, were significantly increased from the naïve controls, 3.88 ± 0.09, 0.68 ± 0.01, 0.18 ± 0.01 and 0.34 ± 0.01, respectively (Table 3.3). Although these results were reassuring, in that the positive control indeed showed an effect, it was interesting to note that there were some variations between the 5 and 10 week data. One possible explanation for the changes may be the transition from an acute stage to a more chronic condition. This could explain the significant differences in all organs as a percentage of body weight at 2.5 weeks. At 5 weeks these differences are muted in that only the kidneys and spleen were significantly different. However, at 10 weeks, again, all organs studied showed a significant difference.

Treatment of A.SW mice with toxic oil did not lead to significant changes in IgG1, IgG2, or IgE antibody production.

In this study we examined the ability of A.SW mice to produce three particular antibody isotypes that have been implicated in the TOS epidemic: e.g., IgE, IgG1, and IgG2. Production of IgG1 was unchanged in A.SW mice after toxic oil treatment. When IgG1 levels were evaluated, there were no significant differences in any of the toxic oil treated groups (Table 3.4). In fact, the only

significant differences seen were in the $HgCl_2$ positive control groups. In these groups, the levels of serum IgG1 were notably elevated. At 2.5 weeks, the level of IgG1 in the $HgCl_2$ group was 8.45 ± 1.50 mg/ml compared to the naïve control group with 0.55 ± 0.05 mg/ml (Table 3.4). This pattern was repeated at 5 and 10 weeks of treatment as well. At 5 weeks, the $HgCl_2$ group had 8.60 ± 0.08 mg/ml of serum IgG1 compared to the 0.71 ± 0.05 mg/ml in the naïve control group (Table 3.4). At 10 weeks, the $HgCl_2$ group had 6.26 ± 0.05 mg/ml of IgG1 while the naïve control had 0.63 ± 0.07 mg/ml (Table 3.4). While these data shed little light on TOS, it indicated that the A.SW mouse was susceptible to mercury-induced autoimmunity.

Levels of IgG2a were also examined in the A.SW mouse following toxic oil treatment. Again, our data showed that there were no significant differences in the serum IgG2a levels between the oil treatment groups and the canola oil control groups (Table 3.4). However, the levels of serum IgG2a were elevated in the $HgCl_2$ groups (Table 3.4). At 2.5 weeks, there was 1.34 ± 0.03 mg/ml IgG2a in the $HgCl_2$ group compared to 0.31 ± 0.04 mg/ml in the naïve control group. This pattern was repeated at 5 and 10 weeks of treatment. At 5 weeks there was 2.11 ± 0.02 mg/ml IgG2a and at 10 weeks 1.55 ± 0.02 mg/ml IgG2a in the $HgCl_2$ groups (Table 3.4). This can be compared to 0.40 ± 0.06 mg/ml and 0.39 ± 0.06 mg/ml, respectively, in the naïve control groups at 5 and 10 weeks (Table 3.4). Again, these data indicated that while immunoglobulin isotypes were not altered in

Table 3.4: Immunoglobulin levels (IgG1 and IgG2a) (mg/ml; mean ± SEM) for 7 to 9 week old female A.SW mice after **2.5, 5, and 10 weeks** of treatment with canola oil, toxic oil compounds, or mercuric chloride (50 ppm). Treatments are compared statistically to their respective controls: canola oil or untreated tap water (naïve).

Group	IgG1			IgG2a		
	2.5 weeks	5 weeks	10 weeks	2.5 weeks	5 weeks	10 weeks
Canola oil	0.64 ± 0.03	0.85 ± 0.11	0.76 ± 0.12	0.38 ± 0.03	0.67 ± 0.02	0.42 ± 0.06
RSD99 undiluted	0.59 ± 0.04	0.66 ± 0.05	0.70 ± 0.03	0.36 ± 0.05	0.34 ± 0.04	0.47 ± 0.06
RSD99 1:10	0.54 ± 0.07	0.71 ± 0.09	0.70 ± 0.04	0.24 ± 0.03	0.38 ± 0.07	0.42 ± 0.04
RSA99 undiluted	0.59 ± 0.06	0.68 ± 0.09	0.72 ± 0.05	0.32 ± 0.02	0.43 ± 0.04	0.40 ± 0.04
RSA99 1:10	0.56 ± 0.05	0.54 ± 0.07	0.82 ± 0.10	0.23 ± 0.02	0.26 ± 0.04	0.46 ± 0.04
CO756 undiluted	0.62 ± 0.05	0.67 ± 0.04	0.71 ± 0.06	0.37 ± 0.07	0.34 ± 0.05	0.41 ± 0.04
CO756 1:10	0.48 ± 0.02	0.71 ± 0.05	0.98 ± 0.02	0.26 ± 0.01	0.35 ± 0.05	0.39 ± 0.04
Naïve	0.55 ± 0.05	0.71 ± 0.05	0.63 ± 0.07	0.31 ± 0.04	0.40 ± 0.06	0.39 ± 0.06
$HgCl_2$ 50 ppm	**8.45 ± 1.50**	**8.60 ± 0.08**	**6.26 ± 0.05**	**1.34 ± 0.03**	**2.11 ± 0.02**	**1.55 ± 0.02**

Bolded text indicates values significantly different from respective naive or canola oil control treatments ($p < 0.05$).

the toxic oil treated mice, mice were susceptible to mercury-induced autoimmune disease.

Levels of serum IgE, an indicator of the acute phase of TOS, were unaffected by toxic oil treatment in A.SW mice. Although we had found that there were no increases in the serum levels of IgG1 and IgG2a, we wished to examine the levels of IgE because elevations of this isotype are more characteristic of the acute phase of TOS, whereas elevated IgG isotype levels are more indicative of the chronic stage of TOS. We found that there were no significant differences in the levels of IgE in the toxic oil treated mice at 2.5, 5, and 10 weeks of treatment (Table 3.5). However, there were significant increases in the $HgCl_2$ treated groups. At 2.5 weeks, the level of serum IgE in the $HgCl_2$ group was 212.9 ± 58.9 ng/ml compared to 0.31 ± 0.12 ng/ml in the naïve control (Table 3.5). By 5 weeks of treatment, the level of IgE in the $HgCl_2$ group had dropped to 58.6 ± 13.1 ng/ml compared to 0.31 ± 0.12 ng/ml in the naïve control and by 10 weeks, this number was slightly elevated above the 5 week value. The $HgCl_2$ group at 10 weeks had 66.3 ± 10.3 ng/ml IgE while the naïve control group had 0.39 ± 0.07 ng/ml IgE. These results indicate that the acute stage of TOS was not produced in any of the toxic oil treated groups. One reason for this may be the genetic susceptibility of patients with TOS (9, 10, 15, 30-32, 36, 37, 41, 42). It is possible that these mice are not genetically vulnerable to the induction of TOS following toxic oil treatment. However, they are susceptible to the autoimmunity induced by mercury treatment.

Table 3.5: IgE antibody levels (ng/ml; mean ± SEM) for 7 to 9 week old female A.SW mice after **2.5, 5, and 10 weeks** of treatment with canola oil, toxic oil compounds, or mercuric chloride (50 ppm). Treatments are compared statistically to their respective controls: canola oil or untreated tap water (naïve).

Group	IgE		
	2.5 weeks	5 weeks	10 weeks
Canola oil	0.58 ± 0.10	0.30 ± 0.03	0.56 ± 0.10
RSD99 undiluted	0.50 ± 0.10	0.29 ± 0.13	0.36 ± 0.10
RSD99 1:10	0.34 ± 0.13	0.41 ± 0.09	0.37 ± 0.08
RSA99 undiluted	0.44 ± 0.08	0.60 ± 0.22	0.73 ± 0.27
RSA99 1:10	0.64 ± 0.22	0.23 ± 0.05	0.43 ± 0.07
CO756 undiluted	0.77 ± 0.34	0.23 ± 0.06	0.42 ± 0.23
CO756 1:10	0.84 ± 0.32	0.28 ± 0.10	0.43 ± 0.19
Naïve	0.32 ± 0.13	0.31 ± 0.12	0.39 ± 0.07
$HgCl_2$ 50 ppm	**212.9 ± 58.9**	**58.6 ± 13.1**	**66.3 ± 10.3**

Bolded text indicates values significantly different from respective naive or canola oil control treatments ($p < 0.05$).

Treatment of A.SW mice with toxic oil did not induce autoantibody production.

We examined the production of anti-nuclear and anti-native DNA antibodies by indirect immunofluorescence. Additionally, we looked for the production of anti-histone and anti-collagen type II antibodies by ELISA. We found that toxic oil treatment in the A.SW mouse did not induce autoantibody

formation. At all timepoints, 2.5, 5, and 10 weeks, there was no detection of anti-native DNA antibodies in the treated mice. Additionally, there were no anti-native DNA antibodies found in the $HgCl_2$ positive control groups (Table 3.7). Results were similar for anti-histone antibodies. Only an occasional individual mouse in the toxic oil treated groups, and in the $HgCl_2$ treated groups produced anti-histone

Table 3.6: Autoantibody production, expressed as number positive per number analyzed, for 7 to 9 week old female A.SW mice after **2.5, 5, and 10 weeks** of treatment with canola oil, toxic oil compounds, or mercuric chloride (50 ppm). Treatments are compared statistically to their respective controls: canola oil or untreated tap water (naïve).

Group	Anti-Histone			Anti-Nuclear		
	2.5 weeks	5 weeks	10 weeks	2.5 weeks	5 weeks	10 weeks
Canola oil	0 / 4	0 / 4	1 / 4	0 / 5	0 / 4	0 / 4
RSD99 undiluted	0 / 4	0 / 4	1 / 4	0 / 5	0 / 4	0 / 5
RSD99 1:10	0 / 4	0 / 4	0 / 4	0 / 5	0 / 4	0 / 4
RSA99 undiluted	0 / 4	1 / 4	0 / 4	0 / 5	0 / 4	0 / 4
RSA99 1:10	0 / 4	0 / 4	1 / 4	0 / 5	0 / 4	0 / 4
CO756 undiluted	0 / 4	0 / 4	0 / 4	0 / 5	0 / 4	0 / 4
CO756 1:10	0 / 4	0 / 4	1 / 4	0 / 5	0 / 4	0 / 3
Naïve	1 / 4	0 / 4	0 / 4	0 / 9	0 / 10	0 / 4
$HgCl_2$ 50 ppm	2 / 4	0 / 4	1 / 4	**10 / 10**	**10 / 10**	**6 / 6**

Bolded text indicates values significantly different from respective naive or canola oil control treatments.

antibodies (Table 3.6). Results for anti-nuclear antibodies were slightly different. Although no anti-nuclear antibodies were found in the mice treated with the toxic oil, the $HgCl_2$ treated mice did show anti-nuclear antibody production at 2.5 weeks (10/10), 5 weeks (10/10), and 10 weeks (6/6) (Table 3.6). Additionally, although the toxic oil treatment did not produce any anti-collagen type II antibodies, mice treated with $HgCl_2$ did (Table 3.7). At 2.5, 5 and 10 weeks, all $HgCl_2$ treated mice tested showed anti-collagen type II antibody production. These results confirmed that treatment with toxic oils did not induce autoimmune disease in A.SW mice. More specifically, the lack of autoantibody production demonstrates that the chronic stage of TOS was unable to be induced in this mouse model. However, systemic autoantibody production was detected in the $HgCl_2$ treated mice indicating that this treatment was able to induce autoimmunity.

DISCUSSION

Although the Spanish TOS epidemic occurred over twenty years ago, many people are still affected by it. The chronic stage of TOS has left many destined to a life of pain and suffering. Because the symptoms of TOS are a continued presence in those affected, research must continue so that a therapy can be developed. Currently, there are no acceptable animal models that mimic the disease progression of TOS. Researchers have been able to induce the acute phase as well as the chronic phase, but not in the same mouse model (15, 36, 37). We

Table 3.7: Autoantibody production, expressed as number positive per number analyzed, for 7 to 9 week old female A.SW mice after **2.5, 5, and 10 weeks** of treatment with canola oil, toxic oil compounds, or mercuric chloride (50 ppm). Treatments are compared statistically to their respective controls: canola oil or untreated tap water (naïve).

Group	Anti-Collagen Type II			Anti-Native DNA		
	2.5 weeks	5 weeks	10 weeks	2.5 weeks	5 weeks	10 weeks
Canola oil	0 / 4	0 / 4	0 / 4	0 / 4	0 / 4	0 / 4
RSD99 undiluted	0 / 4	0 / 4	0 / 4	0 / 5	0 / 4	0 / 4
RSD99 1:10	0 / 4	0 / 4	0 / 4	0 / 5	0 / 4	0 / 4
RSA99 undiluted	0 / 4	0 / 4	1 / 4	0 / 5	0 / 4	0 / 4
RSA99 1:10	0 / 4	0 / 4	0 / 4	0 / 5	0 / 4	0 / 4
CO756 undiluted	0 / 4	0 / 4	0 / 4	0 / 5	0 / 4	0 / 4
CO756 1:10	0 / 4	0 / 4	0 / 4	0 / 5	0 / 4	0 / 4
Naïve	0 / 4	0 / 4	0 / 4	0 / 4	0 / 4	0 / 4
$HgCl_2$ 50 ppm	**5 / 5**	**5 / 5**	**4 / 5**	0 / 6	0 / 6	0 / 6

Bolded text indicates values significantly different from respective naive or canola oil control treatments.

investigated the possibility that the A.SW mouse could develop TOS symptoms following treatment with toxic oils.

Previous researchers have shown that a genetic susceptibility is important in the induction of TOS. In mice, this has been linked to the MHC gene loci (9, 10, 15, 30-32, 36, 37, 41, 42). A/J mice (H-2a) have been shown to suffer acute

wasting disease and acute symptoms of TOS following toxic oil treatment (36, 37). C57BL/6 (H-2b) mice develop polyclonal B cell activation following toxic oil treatment, but these mice did not develop disease symptoms (37). B10.S and MRL/lpr (both H-2s) have both been shown to demonstrate some or all of the chronic symptoms of TOS following toxic oil treatment (15, 36, 38). However, in the MRL/lpr model, the increase in serum immunoglobulin levels was confounded by the variability of spontaneous induction of autoimmunity. We chose to investigate the induction of TOS in the A.SW mouse. This strain is susceptible to autoimmunity, but does not spontaneously develop it. This makes the A.SW mouse a promising strain in which to investigate chemical induced-autoimmunity because any changes seen as a result of the chemical treatment may be exacerbated given the strain's susceptibility to autoimmune disease.

T cell stimulation has seen in TOS studies (21, 40). Because one of the characteristics of the A.SW mouse is T cell hyperresponsiveness, we believed that this would be an ideal model for TOS. Following stimulation, the A.SW mouse tends to favor a Th2 response. Because this response has been implicated in the TOS epidemic, we were interested in the response these mice would have to toxic oil treatment (21). Specifically, researchers have shown that in TOS patients, there is an increase in IL-1β, IL-4, IL-5, and IL-6 (21, 22, 36, 39). This upregulation correlates well with the clinical signs of TOS. IL-1β is a potent activator of the transcription factor NF-κB (36, 43). Once NF-kB is activated, it is able to induce

transcription of a number of genes important for T cell proliferation, including IL-2. IL-4 is a cytokine that is required for IgE synthesis, whereas IL-5 is important for eosinophil production (21, 22, 44). IL-6 is a cytokine that is important in the activation of B and T cells by stimulating their migration to lymphoid tissue. In addition, IL-6 is crucial in the production of CRP from the liver. Given the importance of the Th2 response in TOS, we theorized that we would be able to see an induction of TOS symptoms in the A.SW mouse following toxic oil treatment. Unfortunately, no response occurred. Although we did not assess individual cytokine production, significant increases did not occur in IgG1, IgG2a, or IgE production. Because a Th2 response is the force behind B cell antibody production, we can infer that little to no Th2 response was generated. Additionally, autoantibodies, another characteristic of autoimmunity, were not induced. Again, this may be due to the fact that little T cell activation was generated. One of the proposed mechanisms for the generation of autoantibodies in the TOS epidemic is chronic cytotoxicity. Oxidized forms of PAP have been shown to be cytotoxic (22, 29). Induction of apoptosis following ingestion of such compounds could expose auto-reactive lymphocytes to abnormal forms of self antigens. When the level of self-recognition reaches the threshold required for T cell activation, autoimmunity would develop. The activated T cells could in turn activate the B cells to produce autoantibodies. However, since the A.SW mouse was unable to generate a potent T

cell reaction, the B cells did not receive stimulation, and no autoantibody production was seen.

Although there was no induction of autoimmune disease in the A.SW model following toxic oil treatment, there was the induction of autoimmunity following $HgCl_2$ treatment. As with TOS, there is a genetic susceptibility to mercury-induced autoimmunity (45-48). A.SW mice are highly responsive to mercury treatment, and readily develop autoimmunity. This mercury-induced autoimmunity is characterized by the increase in serum IgG1 and IgE isotypes (49, 50). Additionally, $HgCl_2$ treated mice develop anti-nucleolar antibodies (46, 51, 52). In our study with A.SW mice, following $HgCl_2$ treatment, there was a significant increase in serum IgG1, IgG2a, and IgE levels. The production of IgG1 and IgE is IL-4 dependent, indicating a role for the Th2 subset, whereas the IgG2a isotype is associated more with a Th1 response (49, 50, 53-55). It is curious that we were able to see an increase in these antibody isotypes with the mercury treatment but not with the toxic oil treatment because the same T cell subset (CD4+) is implicated. One possible explanation is that mercury generates a much more potent signal. Mercury is able to quickly activate CD4+ cells as indicated by the expression of CD69 on their surface and the production of IL-2 and the IL-2 receptor (55, 56). This is followed by a persistent increase in the number of T cells, mainly CD4+ (55). This lasting T cell population is able to readily activate B cells as indicated by an increase in CD71 on their surface (51). This immediate and

continuous activation of B cells and the presence of IL-4 lead to the increases in IgG1 and IgE isotypes. The production of IgG2a is not dependent upon IL-4 (49-51, 57). Therefore, a potent Th2 response is not required for the production of IgG2a. It is also interesting to note that anti-nucleolar antibody production is not dependent upon IL-4 (49). It is possible, therefore, that the mercury treatment was potent enough to generate a Th2 response in the A.SW mouse, while the toxic oil treatment was not.

The ability to generate a Th2 response in TOS may be linked to several factors. One such factor is dose. It is possible that a person would have to eat a certain amount of the oil in order to generate enough cytotoxicity to activate the T cells. Although unlikely, it is possible that this dose threshold was not reached in our study with the A.SW mice, and therefore, no indicators of TOS were seen. A second possibility is that there are genetic factors involved, other than the MHC genotype. In humans, researchers have begun to investigate an individual's ability to clear toxic agents. One enzyme involved in this process is N-acetyltransferase-2 (NAT2). Research has shown that there is a link between the number of defective NAT2 loci and the severity of disease (10). The more mutations that are present, the less acetylation, and the more susceptible to TOS a person could become (10). This phenomenon could explain why different mouse strains have differing reactions to toxic oil. A/J mice show acute wasting disease when they are treated with toxic oil (36). Interestingly, those mice are slow acetylators (10). Conversely,

C57/BL6 mice are fast acetylators, and show no symptoms when they are exposed to toxic oil (10, 36). This new genetic information may help to explain why mouse strains of the same MHC type do not respond similarly, if at all, to the same toxic oil treatment. It is possible that the A.SW strain used in this study is a fast acetylator and, therefore, is resistant to toxic oil induced autoimmunity. However, further research is needed to determine all of the factors involved in toxic oil induced autoimmunity.

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CHAPTER4

FLOW CYTOMETRIC ANALYSIS OF IMMUNOSTIMULATORY EFFECTS OF THREE MITOGENS ON PERIPHERAL BLOOD MONONUCLEAR CELLS FROM NORMAL DOGS

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ABSTRACT FOR CHAPTER 4

Immune function assays have been instrumental in the investigation of new treatments for disease. Through their use, cell composition, cell activation, and cell proliferation can be determined. The objective of this study was to assess mitogen-induced proliferation and dexamethasone-induced apoptosis of peripheral blood mononuclear cells (PBMCs) from healthy dogs using flow cytometry. In vitro assays were performed to determine individual mitogen function, optimal incubation time, and optimal mitogen concentration. Of the three mitogens tested [concanavalinA (conA), lipopolysaccharide (LPS), and phorbol 12-myristate 13-acetate (PMA) plus ionomycin], conA at 2.5 μg/ml induced the greatest proliferation of canine PBMCs. The highest percentage of cells proliferating was observed at 48 hours of incubation (16.1 ± 0.7 %). LPS from S. typhimurium at 50 μg/ml and PMA (1 μg/ml) / ionomycin (0.5 μg/ml) were both able to induce lymphocyte proliferation, although to a lesser degree than conA (2.0 ± 0.8 % and 20.3 ± 2.2 % respectively). Although cells stimulated with PMA/ionomycin had a slightly higher percent of cells undergoing proliferation than the conA stimulated cells, the total number of cells proliferating was greater in the conA stimulated cells. The greatest percent of cells undergoing proliferation by PMA/ionomycin and LPS stimulation was seen at 72 hours of incubation. Lymphocyte proliferation in the presence of dexamethasone (dex) was also determined. It was found that a dex concentration of 10^{-6} M was able to inhibit proliferation without inducing

overwhelming apoptosis. Similar to conA-induced proliferation, this effect was best seen at 48 hours of incubation. The optimal concentrations of conA and dex were then combined to analyze lymphocyte susceptibility to dex-induced death. It was determined that the combination of conA at 2.5 μg/ml and dex at 10^{-6} M at 48 hours of incubation yielded the best results. At this timepoint, there was a detectable inhibition of lymphocyte proliferation without overwhelming induction of apoptosis. This determination of optimal culturing and proliferation conditions for canine PBMCs using flow cytometry will allow for reliable future studies of immune function under various experimental conditions.

INTRODUCTION

The ability to measure lymphocyte proliferation and apoptosis is crucial in immune research. Numerous assays have been developed to assess immune function in humans and mice. If another species is used as an animal model to assess immune function, it is imperative that immune function assays be developed and optimized for that species.

Two categories of assays that are routinely used in immunological studies include lymphocyte proliferation assays and steroid-induced cell death assays. Lymphocyte proliferation assays use a mitogen, i.e., concanavalinA (ConA), phorbol 12-myristate 13-acetate/ionomycin (PMA/Ion), or lipopolysaccharide (LPS). Immune cells are cultured with the mitogen in vitro and then proliferation is measured by a chosen method. The prevailing method for measuring proliferation utilizes ^{3}H-thymidine incorporation into cellular DNA.

A second immune assay that is widely used to assess immune cell function is the steroid-induced cell death assay. This assay allows the investigator to determine if cells are able to resist or are susceptible to steroid-induced cell death. The steroid commonly used for these assays is dexamethasone (dex), a synthetic steroid related to hydrocortisone. Dex has a double bond between carbons 1 and 2 as well as a fluorine group added at carbon 9 and a methyl group added at carbon 18. The end result of these additions to the steroid ring of hydrocortisone is increased anti-inflammatory potency. Dex acts by inhibiting IL-2 production. IL-2

is a T-cell growth factor; thus, in the absence of IL-2, cells die rather than proliferate (1-3). By adding dex to a lymphocyte proliferation culture and then analyzing the proliferation profile, a researcher can determine the susceptibility of cells to steroid-induced cell death. This assay can be used to compare immune function of cells from animals undergoing different treatment regimens.

Although these two assays have been well documented and utilized in other species, there is less information for the canine species. A variety of mitogens have been used to assess immune cell proliferation in the dog, as well as a variety of concentrations of mitogen. ConA has been used in concentrations varying from 290 ng/ml to 500 mg/ml (4, 5). A second set of mitogens, PMA/Ion, has also been used at various concentrations in pigs and rats. PMA concentrations have ranged from 10 ng/ml to 20 ng/ml, while ionomycin concentrations have ranged from 125 ng/ml to 375 ng/ml (6, 7). Still, a third mitogen, LPS, has been used at concentrations ranging from 0.01 ng/ml to 18.3 ng/ml in the dog (4, 8). The choice of mitogen for optimum immune cell stimulation is further complicated by the differences in cell culture incubation times. Time in culture has ranged from 2 to 6 days. These variations in culture techniques make comparisons between studies difficult at best. Establishing optimal conditions for canine lymphocyte cultures will allow consistency in data interpretation in future studies.

Although conA is widely accepted to be a T cell mitogen, in some cases, its effects may be suppressive. In some species, conA has been documented to be an

activator of T-suppressor lymphocytes (4, 9-11). However, in dogs, conA is believed to be a Tcell mitogen (4, 12). To complicate things further, the concentration of conA used can have profound effects on cells in culture. High doses of conA can be toxic to cells and inhibit proliferation altogether (4, 13-15). It is therefore critical that the optimal conA concentration be determined for canine cells in culture.

Lipopolysaccharide (LPS) is the major component of gram negative bacterial cell walls. It protrudes from the surface and is composed of three components: lipid A, core polysaccharide, and the O antigen. While the lipid A and core polysaccharide are similar in all bacteria, the O antigen has great variability and determines the serotype of each bacterial species. LPS is able to stimulate an inflammatory response and, if present in high concentrations, it is able to induce shock. LPS is widely known to be a B cell activator (16, 17). This occurs following activation of a signaling cascade that culminates in activation of the nuclear transcription factor NFκB. This activation leads to the production of pro-inflammatory cytokines and immune mediators (18, 19). Although the actions of all LPS types are similar, some bacterial strains are better able to activate cells than others. In the present study, we set out to determine which type of LPS is best able to activate canine peripheral blood mononuclear cells (PBMCs).

Certain compounds activate T cells to divide without antigen exposure. In normal activation scenarios, T cells see antigen through their T cell receptors

(TCR). Following this recognition, a signaling cascade is initiated, which is characterized by activation of protein kinase C (PKC) and an increase in intracellular Ca^{2+} (7, 20-23). The culmination of these events leads to clonal expansion of T cells. Chemical activators are able to synthetically produce the same actions. We used two such compounds. PMA activates PKC, while ionomycin generates an increase in intracellular Ca^{2+} (7, 24, 25). When used together, theses compounds are able to induce T cell division. This study investigated the ability of those two compounds used together to stimulate proliferation of canine PBMCs.

The next goal was to determine the concentration of dexamathasone that induced the greatest apoptosis. Dexamethasone is a glucocortocoid that is able to induce programmed cell death, or apoptosis. When dex is administered to a cell it binds to glucocortocoid receptors located in the cytosol. This complex then translocates into the nucleus where it acts as a transcription activator. Immediately, mRNAs are produced that inhibit both glucose transport and acetate uptake into lipids. Following this immediate response, protein and RNA metabolism are inhibited, and the cell dies (2). However, it has been noted that primary and secondary immune responses have different susceptibilities to dex-induced cell death. Primary immune responses have been shown to be vulnerable to dex treatment, whereas secondary immune responses are resistant (2, 26-28). Thus, treatment of stimulated cells with dex is a way to determine if the cells of interest

are resistant or susceptible to dex-induced cell death. Before this can be ascertained however, the concentration of dex that leads to the optimal induction of apoptosis needs to be determined. At the culmination of these experiments, the data were obtained to successfully evaluate canine PBMCs in future immune studies.

MATERIALS AND METHODS

Animals

Normal adult mixed breed dogs were used as blood donors for these experiments. All were in apparent good health prior to and throughout the study. Blood was collected into heparinized vacutainer tubes via venipuncture of the jugular vein. The protocol for blood collection was reviewed and approved by the Oregon State University Animal Care and Use Committee according to the National Institutes of Health guidelines.

Peripheral Blood Mononuclear Cell Isolation

PBMCs were isolated from whole blood collected as previously described. Briefly, whole blood was diluted with equal amounts of Dulbecco's phosphate buffered saline, DPBS, (Sigma, St. Louis, MO). Approximately 10 ml of this mixture was then layered onto 3 ml of Histopaque 1077 (Sigma) in 15 ml tissue culture tubes. These tubes were centrifuged for 30 minutes at 2000 RPM without a

brake. Following the spin, the mononuclear layer was collected and placed into 15 ml centrifuge tubes. The PBMCs were washed by diluting them 3:1 with Hank's balanced salt solution, HBSS, (Sigma) and then centrifuged for 10 minutes at 1300 RPM. The supernatant was removed and the cells were resuspended in 3 ml of HBSS. Following this resuspension step, all cells were pooled into one tube. The cells were again centrifuged for 10 minutes at 1300 RPM, after which the supernatant was discarded and the cells were resuspended in phosphate buffered saline, PBS (Gibco, Grand Island, NY). At this point, total number of cells was determined from an aliquot of the cell suspension using a Coulter ZB1 Counter (Coulter Electronics, Inc., Hialeah, FL).

Cell Culture Conditions

After determining the total number of cells present, the cell suspension was centrifuged for 10 minutes at 1300 RPM. The supernatant was discarded and the cells were reconstituted to a concentration ranging from 3.5 to 6.5×10^6 cells/ml with culture media (RPMI-1640 containing L-glutamine and supplemented with 10% fetal bovine serum, 1% of 1 M HEPES buffer solution, 1% of a 10,000 μg/ml streptomycin and 10,000 units/ml penicillin) (all from Invitrogen, Carlsbad, CA). Cells (3.5 to 6.5×10^5) were plated onto 96-well tissue culture plates (Costar, Cambridge, MA) in 0.200 ml total volume.

Cells were activated with each mitogen, concanavalin A (conA), lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA) with ionomycin, or dexamethasone (dex). Four types of LPS were investigated: Escherichia coli O55:B5, Escherichia coli O111:B4, Salmonella typhimurium, and Salmonella typhosa (all from Sigma). ConA was added at the following concentrations: 2.5, 5, 10, 25, and 50 μg/ml. LPS was added at concentrations of 0.1, 1, 10, 25, and 50 μg/ml. PMA was used in conjunction with ionomycin and was plated in a checkerboard fashion. The PMA concentrations used were 0.01, 0.1, 1, 10, and 20 μg/ml, whereas the ionomycin concentrations used were 0.1, 0.25, 0.5, 1, 10, and 20 μg/ml. Dex was added at 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M. Cells cultured with conA, LPS, and dex were incubated for 24, 48, 72, or 96 hours at 37°C in 5% CO_2. Cells stimulated with PMA and ionomycin were incubated for 18, 24, 48, or 72 hours at 37°C in 5% CO_2. Additionally, cells were cultured with a combination of conA and dex. For this culture, conA was used at 1.25, 2.5, and 5 μg/ml, whereas dex was used at 10^{-5}, 10^{-6}, 10^{-7} M. These cells were cultured for 24, 48, or 72 hours at 37°C in 5% CO_2. Each cell culture was assessed in either duplicate or triplicate.

Proliferation Staining

Following the appropriate culture conditions and incubation times, plates were removed and the total number of cells per well were determined from an

aliquot using the Coulter ZB1 Counter (Coulter Electronics, Inc.). The plates were then centrifuged at 1000 RPM for 3 minutes. The supernatants were removed and the plates were briefly vortexed. The cells were washed by resuspending them in 200 µl PBS and centrifuging as above. The supernatant was again removed and the cells were washed with PBS a second time. Following supernatant removal, the cells were resuspended in 200 µl of propidium iodide staining solution (PBS with 5 mM EDTA, 5 µg/ml propidium iodide, 50 µg/ml RNAse A, and 0.3% saponin) (all from Sigma). Cells were incubated in the propidium iodide staining solution at room temperature for 30 min. Stained cells were then run on a FACSCalibur Flow Cytometer (Becton Dickinson, San Jose, CA). Data analysis was done using CellQuest™ software (Becton Dickinson) (Figure 4.1).

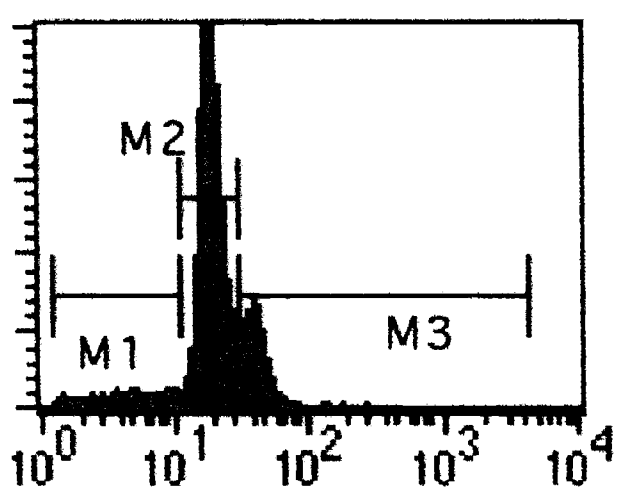

Figure 4.1: A representative sample of the propidium idodide data obtained from the flow cytometer. Cells falling within the M1 region are deemed apoptotic, cells within the M2 region are resting, and cells within the M3 region are proliferating.

RESULTS

Optimal conA concentration for in vitro stimulation is 2.5 μg/ml.

Stimulation of PBMCs with conA at a concentration of 2.5 μg/ml resulted in optimal cell proliferation. The optimal time in culture was 48 hours. At this timepoint, 16.1 ± 0.7% of cells were proliferating. This coincides with 7.4 ± $0.3x10^4$ total cells proliferating (Figure 4.2). Additionally, this time point (48 hours) yielded the lowest percentage of apoptotic cells, which was similar to media alone (Figure 4.3). Conversely, the highest conA concentration, 50 μg/ml, showed little proliferation (Figure 4.2). Both the percentage of cells proliferating and the total number of cells proliferating at this concentration were similar to that of media alone. The 50 μg/ml concentration also yielded the highest percent and highest total number of apoptotic cells, 25.1 ± 0.4% and $6.0 \pm 0.1x10^4$, respectively (Figure 4.3).

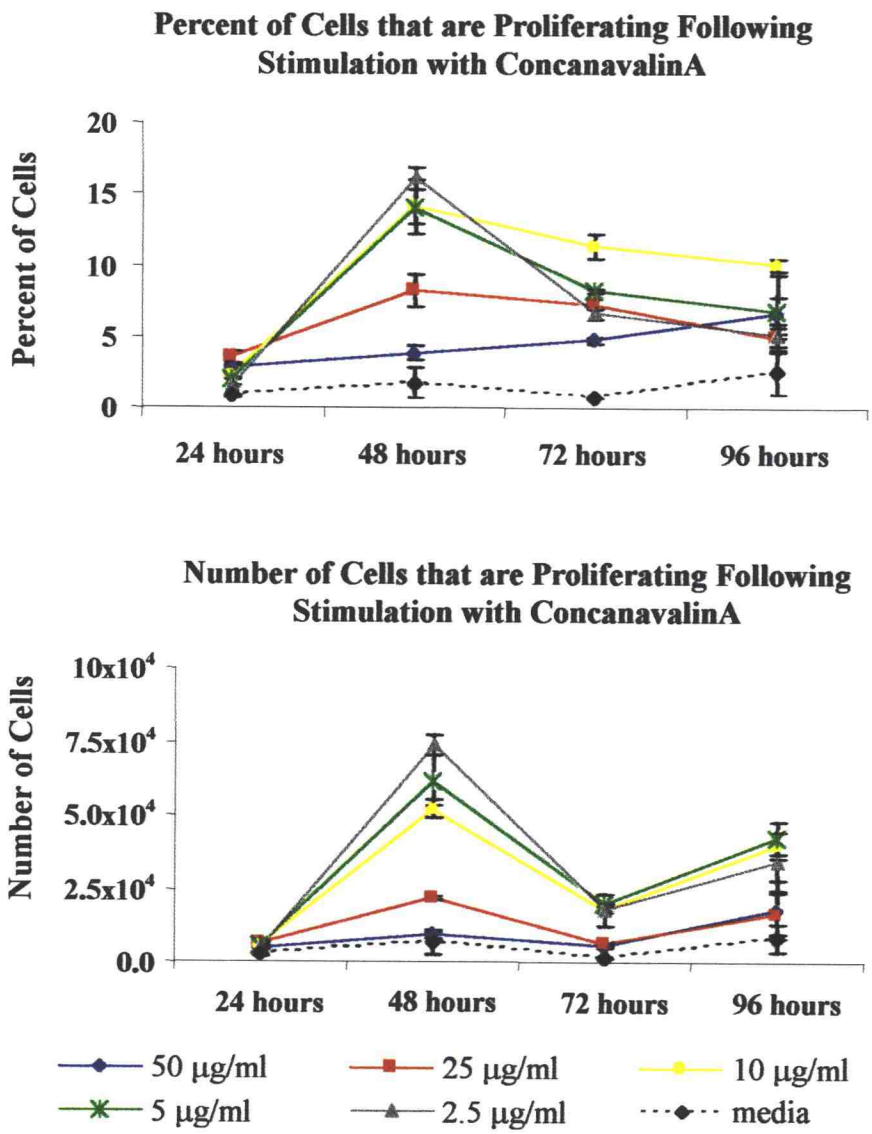

Figure 4.2: Percent and number of proliferating cells following stimulation with concanavalinA (conA). In vitro cell cultures were stimulated with various concentrations of conA. The cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide and then analyzed by flow cytometry. Means ± SEM are shown.

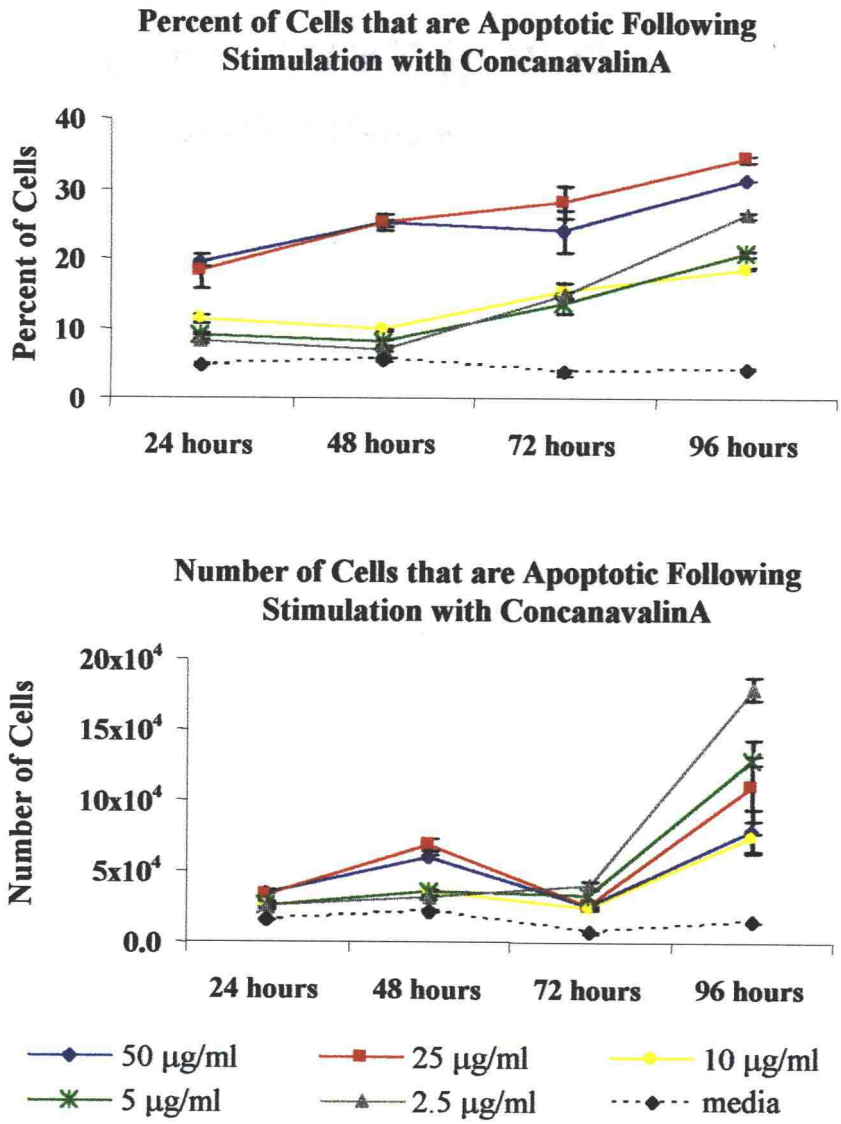

Figure 4.3: Percent and number of apoptotic cells following stimulation with concanavalinA (conA). In vitro cell cultures were stimulated with various concentrations of conA. These cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and then analyzed by flow cytometry. Means ± SEM are shown.

LPS from Salmonella typhimurium at a concentration of 50 μg/ml provides the best LPS stimulation.

We tested four different types of LPS: E. coli O55:B5, E. coli O111:B4, S. typhimurium, and S. typhosa. Each type of LPS was tested at various concentrations of 50, 25, 10, 1.0, and 0.1 μg/ml. Although little proliferation was seen with any type of LPS, certain types induced cell division better than others. We found that stimulation with LPS from S. typhimurium showed the greatest percent proliferation. At 72 hours, S. typhimurium at 50 μg/ml induced 2.0 ± 0.8% of cells to proliferate, or $5.6 \pm 2.8 \times 10^3$ total cells proliferating (Figures 4.4 and 4.5). The next best strain was E. coli O111:B4, followed by E. coli O55:B5, and lastly S. typhosa. When the percent and number of apoptotic cells was examined, all species of LPS were virtually equivalent (Figures 4.4 and 4.5). Thus, LPS from S. typhimurium at a concentration of 50 μg/ml and an incubation time of 72 hours gave the best proliferation response.

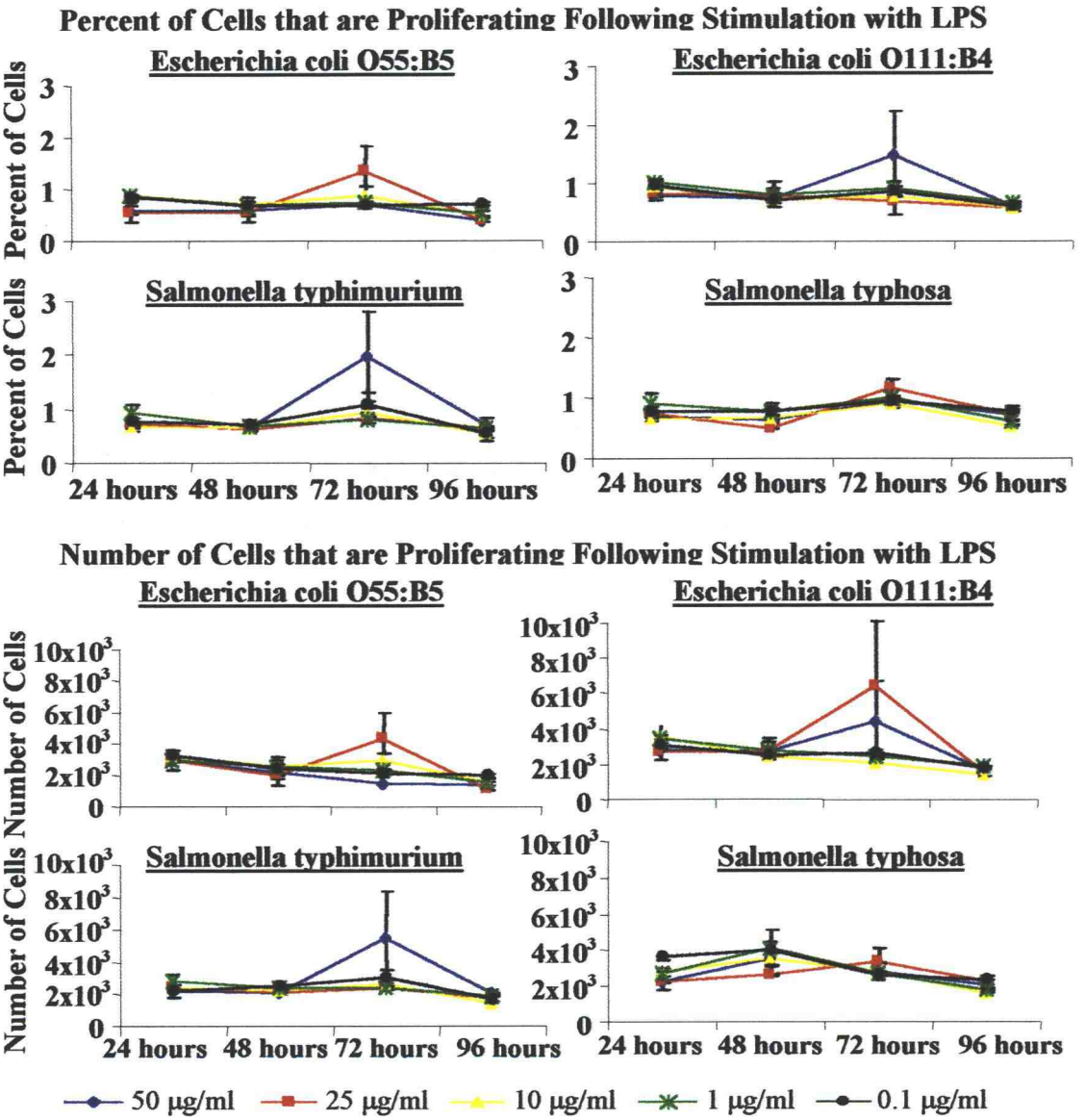

Figure 4.4: Percent and number of proliferating cells following stimulation with LPS. In vitro cell cultures were stimulated with LPS from E. coli O55:B5, E. coli O111:B4, S. typhimurium, or S. typhosa at varying concentrations. The cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and then analyzed by flow cytometry. Means ± SEM are shown.

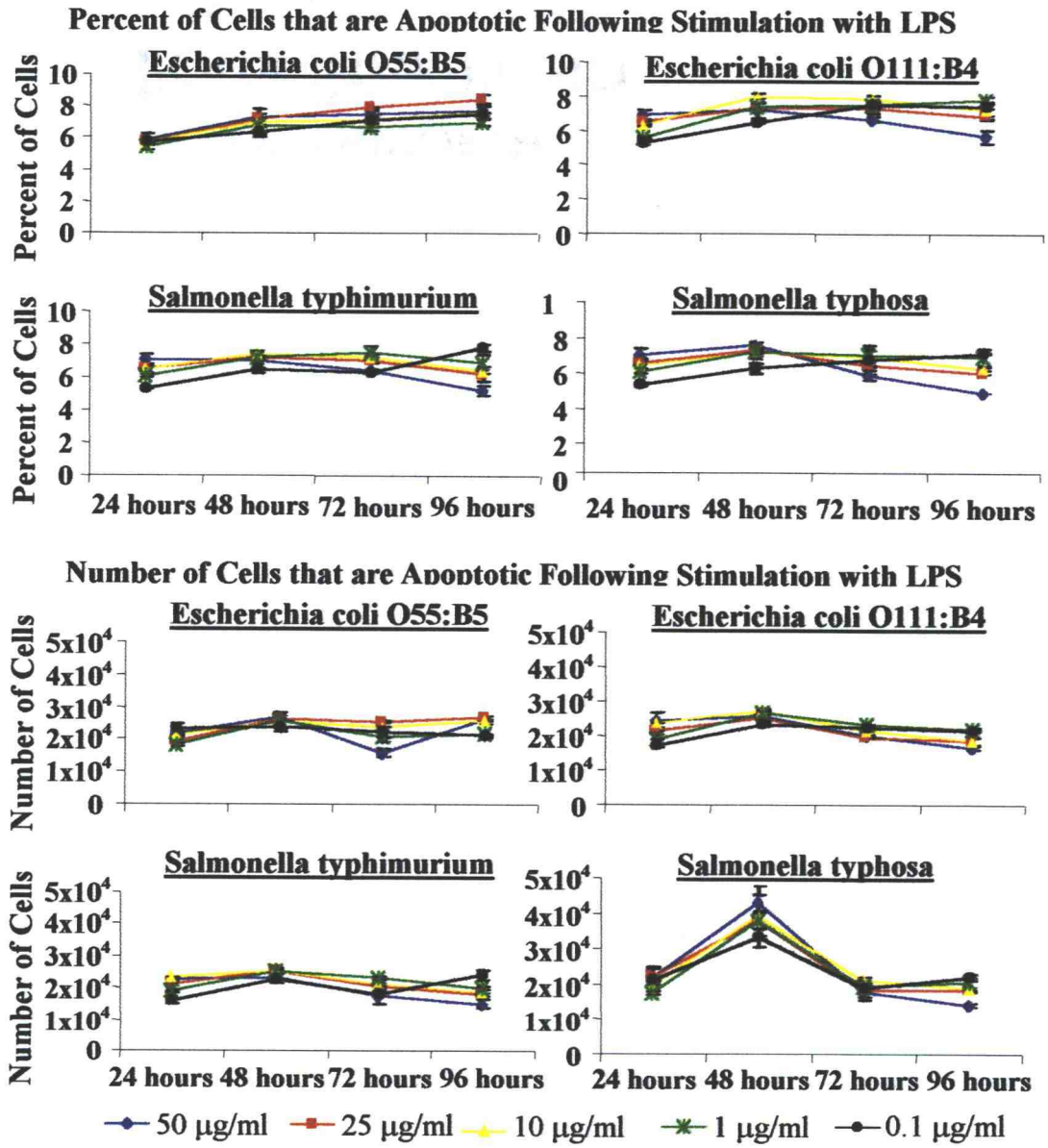

Figure 4.5: Percent and number of apoptotic cells following stimulation with LPS. In vitro cell cultures were stimulated with LPS from E. coli O55:B5, E. coli O111:B4, S. typhimurium, or S. typhosa at varying concentrations. The cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

Stimulation with PMA at 1 μg/ml and Ionomycin at 0.5 μg/ml yields the best proliferation.

We examined the proliferative capacity of these mitogens at 5 (PMA) or 6 (ionomycin) different concentrations. PMA was examined at concentrations of 0.01, 0.1, 1, 10, and 20 μg/ml. Ionomycin was added at concentrations of 0.1, 0.25, 0.5, 1, 10, and 20 μg/ml. These experiments were done in a checkerboard pattern so that every combination was tested. We found that optimal proliferation following PMA and ionomycin stimulation occurred at 72 hours. There was little effect at 18 or 24 hours (Appendix A). For these mitogens, we found that optimal proliferation occurred when PMA was used at a concentration of 1 μg/ml and ionomycin was used at a concentration 0.5 μg/ml. When these compounds were used together at these concentrations, at 72 hours 20.3 ± 2.2% of the cells were proliferating. If absolute numbers are calculated, $5.3 \pm 1.0 \times 10^4$ cells were proliferating at this time point (Table 4.1). Additionally, only 8.9 ± 0.3% of the cells treated with 1 μg/ml PMA and 0.5 μg/ml ionomycin were apoptotic at 72 hours (Table 4.1). These experiments also demonstrated that at concentrations of 10 μg/ml or higher, PMA exhibits a toxic effect (Appendix B). Of the cells that were in the latter treatment groups, between 20% and 45% of them were apoptotic (Appendix B). Thus, these results show that it is imperative to use PMA and ionomycin at optimal concentrations of 1 μg/ml for PMA and 0.5 μg/ml for ionomycin. Higher concentrations are detrimental to cell viability.

Table 4.1: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with PMA and ionomycin. PMA was used at concentrations of 1.0, 0.1, and 0.01 μg/ml, whereas ionomycin was used at concentrations of 20, 10, 1.0, 0.5, 0.25, and 0.1 μg/ml. Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM at 48 and 72 hours are shown.

48 Hours	PMA Concentration											
	1.0 μg/ml				0.1 μg/ml				0.01 μg/ml			
Ionomycin Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
20 μg/ml	13.8 ± 1.0	3.5 ± 0.4	2.7 ± 0.4	0.7 ± 0.1	12.7 ± 0.8	2.8 ± 0.1	2.1 ± 0.1	0.5 ± 0.0	12.8 ± 2.0	3.4 ± 0.8	2.0 ± 0.2	0.5 ± 0.1
10 μg/ml	10.5 ± 0.3	2.2 ± 0.2	3.0 ± 0.3	0.6 ± 0.0	10.4 ± 0.7	2.4 ± 0.1	3.0 ± 0.2	0.7 ± 0.1	10.9 ± 0.3	2.5 ± 0.2	3.2 ± 0.2	0.7 ± 0.1
1 μg/ml	8.9 ± 0.4	2.3 ± 0.2	12.4 ± 1.0	3.2 ± 0.3	9.7 ± 1.5	2.4 ± 0.5	10.1 ± 1.0	2.5 ± 0.1	11.0 ± 1.5	2.7 ± 0.4	8.5 ± 0.5	2.0 ± 0.1
0.5 μg/ml	12.0 ± 0.7	2.9 ± 0.4	11.5 ± 0.7	2.8 ± 0.1	12.5 ± 0.7	2.6 ± 0.0	10.5 ± 0.2	2.2 ± 0.1	13.4 ± 1.5	2.5 ± 0.1	9.9 ± 0.1	1.9 ± 0.1
0.25 μg/ml	11.5 ± 0.8	2.5 ± 0.2	9.7 ± 1.1	2.1 ± 0.3	11.1 ± 0.7	2.5 ± 0.2	9.2 ± 0.8	2.0 ± 0.2	11.4 ± 0.6	2.7 ± 0.1	7.1 ± 0.2	1.7 ± 0.0
0.10 μg/ml	11.6 ± 0.9	2.7 ± 0.3	5.7 ± 0.5	1.3 ± 0.1	11.4 ± 0.3	2.5 ± 0.1	5.5 ± 0.5	1.2 ± 0.1	12.9 ± 0.3	2.8 ± 0.1	6.1 ± 0.02	1.3 ± 0.0
Media only	18.5 ± 0.5	4.1 ± 0.1	3.1 ± 0.1	0.7 ± 0.0								

Table 4.1 continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with PMA and ionomycin. PMA was used at concentrations of 1.0, 0.1, and 0.01 μg/ml, whereas ionomycin was used at concentrations of 20, 10, 1.0, 0.5, 0.25, and 0.1 μg/ml. Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM at 48 and 72 hours are shown.

72 Hours	PMA Concentration											
	1.0 μg/ml				0.1 μg/ml				0.01 μg/ml			
Ionomycin Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
20 μg/ml	18.1 ± 0.3	5.1 ± 0.3	3.9 ± 0.2	1.1 ± 0.1	18.1 ± 0.6	5.1 ± 0.5	4.1 ± 0.2	1.1 ± 0.1	18.2 ± 0.8	4.9 ± 0.4	4.0 ± 0.4	1.1 ± 0.1
10 μg/ml	13.0 ± 1.0	3.7 ± 0.3	5.7 ± 2.1	1.6 ± 0.6	17.4 ± 2.0	4.2 ± 0.5	4.4 ± 0.5	1.1 ± 0.1	16.1 ± 1.3	3.9 ± 0.4	5.1 ± 1.3	1.3 ± 0.4
1 μg/ml	10.46 ± 1.3	3.2 ± 0.5	14.8 ± 0.2	4.6 ± 0.1	14.2 ± 2.7	4.2 ± 0.9	11.9 ± 3.3	3.4 ± 0.8	14.8 ± 0.4	3.9 ± 0.1	9.6 ± 0.8	2.5 ± 0.2
0.5 μg/ml	8.9 ± 0.3	2.3± 0.1	20.3 ± 2.2	5.3 ± 1.0	9.6 ± 0.4	2.2 ± 0.4	17.9 ± 0.7	4.1 ± 0.4	10.6 ± 0.5	1.8 ± 0.2	16.2 ± 1.2	2.7 ± 0.0
0.25 μg/ml	8.7 ± 0.4	1.9 ± 0.1	13.1 ± 2.1	2.9 ± 0.5	8.6 ± 0.7	1.7 ± 0.1	10.5 ± 1.4	2.1 ± 0.3	9.7 ± 0.6	1.7 ± 0.0	8.3 ± 0.6	1.4 ± 0.2
0.10 μg/ml	8.8 ± 0.4	2.0 ± 0.2	8.0 ± 0.02	1.9 ± 0.1	9.3 ± 0.4	2.0 ± 0.2	7.4 ± 0.3	1.6 ± 0.0	10.1 ± 0.5	2.1 ± 0.0	6.6 ± 0.05	1.4 ± 0.1
Media only	12.6 ± 0.3	1.9 ± 0.1	6.5 ± 0.7	1.0 ± 0.0								

Optimal steroid-induced death occurs when dex is used at 10^{-6} M.

Cultured cells were treated with concentrations of dex that ranged from 10^{-5} to 10^{-10} M. Apoptosis was determined at 24, 48, 72, and 96 hours by propidium iodide staining. We found that that optimal time point for assaying dex-induced cell death was 48 hours, as this was the time with the greatest percent and number of apoptotic cells (Figure 4.6). We did see some groups with greater values for percent and number of apoptotic cells at 96 hours, however, we concluded that this was because of prolonged incubation time rather than dex treatment. We therefore chose 48 hours as the time point to analyze for subsequent dex concentration studies. At this time point, dex at 10^{-6} M produced the greatest percentage of apoptotic cells, 26.0 ± 0.6%, as well as the greatest number of apoptotic cells, 5.2 ± $0.2x10^{4}$. Our results also showed that dex at 10^{-8}, 10^{-9}, and 10^{-10} had no effect on the cells. In fact, these treatment concentrations had less apopotosis than the media alone control (Figure 4.6).

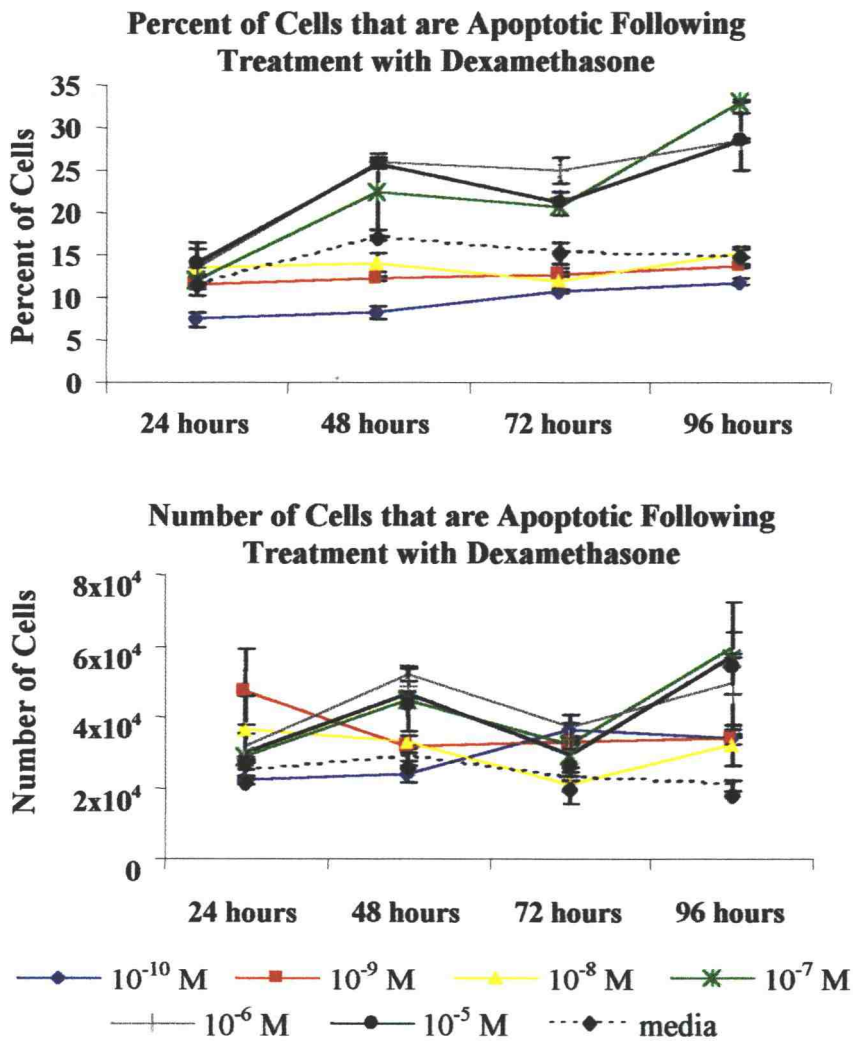

Figure 4.6: Percent and number of apoptotic cells following treatment with dexamethasone (dex). In vitro cell cultres were treated with varying concentrations of dex for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

Susceptibility to dex-induced death is best determined with conA at 2.5 μg/ml and dex and 10^{-6} M.

Once the optimal concentration and incubation time for conA had been determined, as well as the optimal concentration of dex, we wanted to find the best combination of conA plus dex with which to culture and incubate cells in order to determine susceptibility of conA activated cells to dex-induced cell death. We cultured cells with various concentrations of both conA and dex. ConA was used at concentrations of 1.25, 2.5, and 5.0 μg/ml as determined from the previously discussed conA experiment. Dex was used at concentrations of 10^{-5}, 10^{-6}, and 10^{-7} M as determined in the previous dex experiment. Cells were cultured for 24, 48, and 72 hours.

Apoptosis was not affected by the combination of conA and dex at 24 hours (Appendix C). Consequently, we found that 48 hours was the best timepoint to assess susceptibility to dex-induced cell death. At this timepoint, the greatest percentage of cells were undergoing conA stimulated proliferation (Table 4.2). This was in agreement with previous experiments, which had also shown 48 hours to be the best timepoint. Again, optimal proliferation was seen with the conA concentration of 2.5 μg/ml. When dex was added into this equation, the greatest inhibition of proliferation occurred with a dex concentration of 10^{-6} M. With this combination, conA at 2.5 μg/ml and dex at 10^{-6} M, 17.1 ± 1.7% of cells were

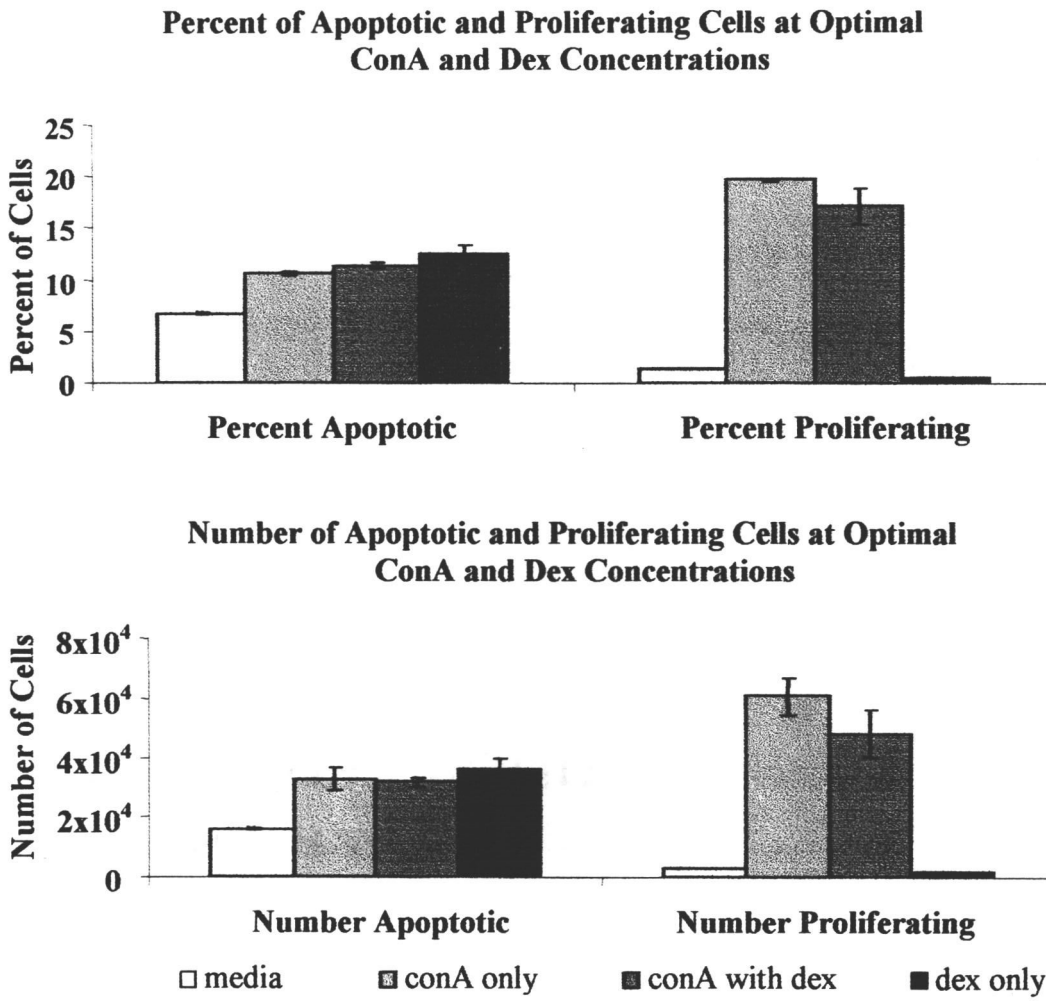

Figure 4.7: Percent and number of apoptotic and proliferating cells following stimulation with the optimal concentration of conA and treatment with the optimal dex concentration. Cells were cultured with media alone, conA only at 2.5 mg/ml, dex only at 10^{-6} M, or conA at 2.5 mg/ml with dex at 10^{-6} M. Following a 48 hour incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

proliferating (Figure 4.7 and Table 4.2). This was a 2.5% decrease from the percent of cells proliferating with conA alone (Figure 4.7 and Table 4.2). Additionally, there was a 20.6% decrease in the number of cells proliferating with this combination of conA and dex. Cultures stimulated with conA only had $6.1 \pm 0.6 \times 10^4$ proliferating cells, whereas cultures treated with conA and dex had only $4.8 \pm 0.8 \times 10^4$ proliferating cells (Figure 4.7 and Table 4.2). Thus, conA at 2.5 μg/ml and dex at 10^{-6}M were optimal for assessing susceptibility of conA activated cells to dex-induced cell death.

DISCUSSION

To our knowledge, no one has investigated the kinetics of proliferation and apoptosis of PBMCs with various mitogens in the dog using flow cytometry. To date, the majority of proliferation assays performed utilized tridiated-thymidine (^{3}H-T) (4-6, 8, 29-32). However, because many different culture conditions were used, cross-comparisons between studies were difficult, if not impossible. We decided to determine the optimum culture conditions for canine PBMCs to assist in the interpretation of future research studies that use dogs as the experimental model.

We found that the optimal conA concentration to stimulate proliferation in PBMCs in vitro was 2.5 μg/ml. The optimal culture time was 48 hours. At this timepoint and concentration of conA, proliferation was increased 9 fold over media

Table 4.2: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with conA and treatment with dex. Cells were cultured with various concentrations of conA and dex for 24, 48, or 72 hours (only 48 and 72 hours are shown). Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

48 Hours	ConA Concentration											
	1.25 μg/ml				2.5 μg/ml				5.0 μg/ml			
Dex Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
10^{-5} M	8.2 ± 0.0	2.0 ± 0.0	14.8 ± 0.2	3.6 ± 0.0	11.3 ± 0.1	3.1 ± 0.1	17.6 ± 0.2	4.8 ± 0.2	11.5 ± 0.1	3.2± 0.2	15.8 ± 0.7	4.4 ± 0.1
10^{-6} M	8.5 ± 0.1	2.2 ± 0.0	15.6 ± 0.1	4.1 ± 0.0	11.4 ± 0.3	3.2 ± 0.2	17.1 ± 1.7	4.8 ± 0.8	12.1 ± 0.0	3.5 ± 0.4	15.8 ± 0.8	4.5 ± 0.2
10^{-7} M	8.4 ± 0.0	2.2 ± 0.0	17.0 ± 0.2	4.4 ± 0.0	10.9 ± 0.2	3.2 ± 0.2	19.6 ± 0.3	5.7 ± 0.1	12.1 ± 0.7	3.4 ± 0.0	16.7 ± 0.3	4.7 ± 0.4
ConA only	8.4 ± 0.1	2.5 ± 0.0	18.4 ± 0.0	5.5 ± 0.0	10.6 ± 0.2	3.3 ± 0.4	19.6 ± 0.2	6.1 ± 0.6	11.6 ± 0.1	3.6 ± 0.1	17.5 ± 0.2	5.4 ± 0.2
10^{-6} M Dex only	12.5 ± 0.8	3.6 ± 0.4	0.5 ± 0.0	0.2 ± 0.0								
Media only	6.7 ± 0.1	1.6 ± 0.0	1.3 ± 0.01	0.3 ± 0.0								

Table 4.2 continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with conA and treatment with dex. Cells were cultured with various concentrations of conA and dex for 24, 48, or 72 hours (only 48 and 72 hours are shown). Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

72 Hours	ConA Concentration											
	1.25 μg/ml				2.5 μg/ml				5.0 μg/ml			
Dex Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
10^{-5} M	9.8 ± 0.1	4.5 ± 0.0	14.8 ± 2.1	6.8 ± 1.0	10.2 ± 0.5	4.3 ± 0.4	13.6 ± 0.3	5.7 ± 0.2	13.2 ± 0.5	4.7 ± 0.2	13.8 ± 0.5	5.0 ± 0.2
10^{-6} M	10.2 ± 1.6	4.5 ± 0.5	14.7 ± 0.5	6.4 ± 0.1	9.8 ± 0.3	3.7 ± 0.1	13.9 ± 0.8	5.3 ± 0.0	13.1 ± 1.4	4.8 ± 0.8	13.4 ± 0.5	4.8 ± 0.4
10^{-7} M	9.3 ± 0.8	4.4 ± 0.3	14.3 ± 0.7	6.7 ± 0.2	10.4 ± 0.3	4.0 ± 0.1	16.8 ± 0.9	6.4 ± 0.7	12.5 ± 0.0	4.5 ± 0.0	16.1 ± 0.2	5.8 ± 0.1
ConA only	9.1 ± 0.5	4.5 ± 0.3	16.5 ± 2.5	8.1 ± 1.3	10.7 ± 0.1	4.3 ± 0.1	18.1 ± 1.0	7.3 ± 0.4	13.3 ± 0.2	5.9 ± 0.1	16.5 ± 0.1	7.3 ± 0.1
10^{-6} M Dex only	11.8 ± 0.6	2.2 ± 0.0	0.6 ± 0.2	0.1 ± 0.0								
Media only	6.3 ± 0.0	1.4 ± 0.0	0.7 ± 0.0	0.1 ± 0.0								

alone. At concentrations of conA above and below 2.5 μg/ml, proliferation was less. These experiments give credence to the theory that there is a concentration dependent ability of conA to either stimulate or inhibit proliferation of PBMCs. Our finding that conA generates the best proliferative response of all the mitogens investigated here agrees with previous studies (Figure 4.8) (4, 30). Although the percent of cells proliferating with PMA and ionomycin at 72 hours was greater than the percent of cells proliferating with conA stimulation, the actual numbers of proliferating cells was less (Figure 4.8). Previous studies investigating mitogens that induced proliferation utilizing ^{3}H-T uptake and different conA concentrations found conA to be the best stimulator of proliferation of PBMCs (5, 32). However, the concentration of conA used was 200 times greater than the concentration used in our study (5). Additionally, one mitogen that was not investigated in our study, phytohemaglutinin, was investigated by others and was shown to induce even greater proliferation than conA (5). It is possible that their conA concentration of 500 μg/ml elicited a toxic effect on the cells and led to suboptimal proliferation. Because all those studies measured proliferation by ^{3}H-T uptake, absolute numbers of proliferating cells were not reported (5, 32).

We found that not all species of bacteria producing LPS are able to activate canine PBMCs equally. We found that LPS from S. typhimurium at a concentration of 50 μg/ml generated the greatest proliferation of canine PBMCs.

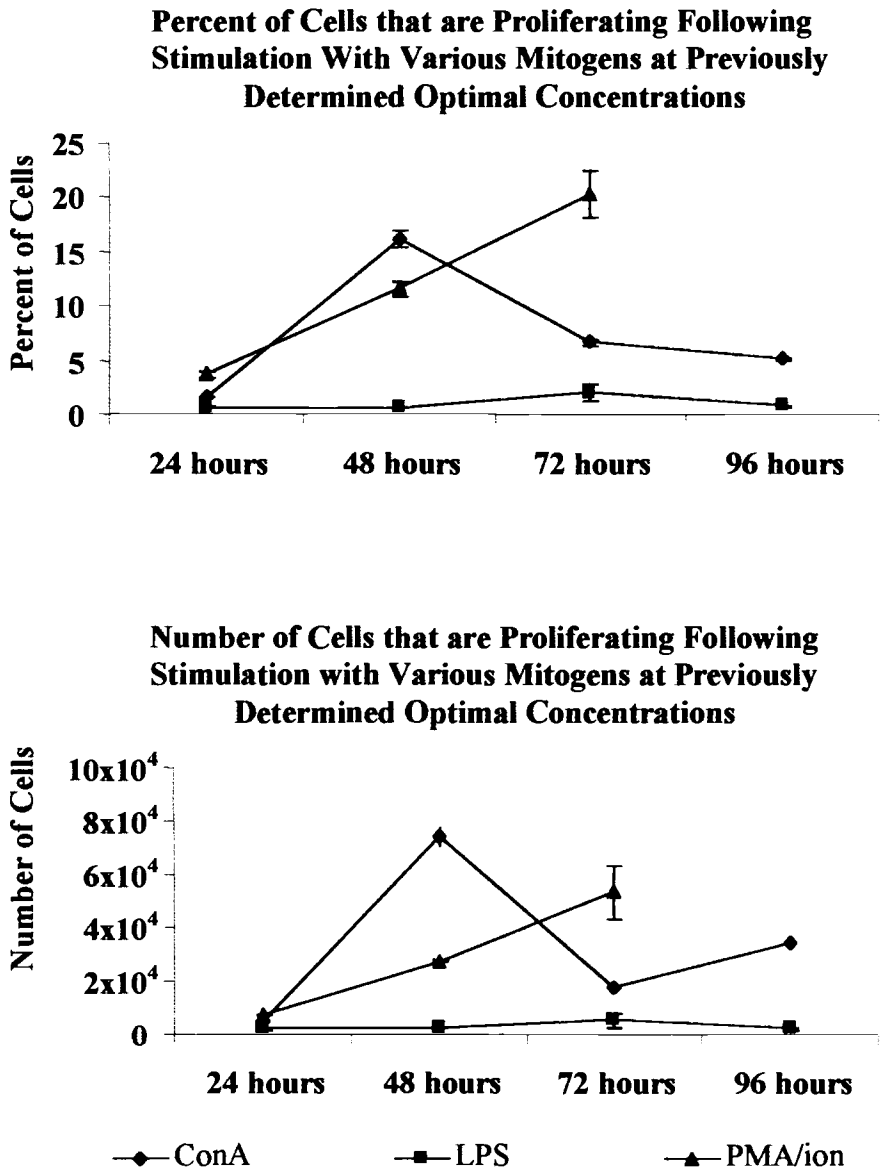

Figure 4.8: Comparison of percent and number of cells proliferating following stimulation with various mitogens. In vitro cell cultures were stimulated with either conA at 2.5 μg/ml, LPS from S. typhimurium at 50 μg/ml, or PMA at 1 μg/ml with ionomycin at 0.5 μg/ml. PMA with ionomycin stimulation was not analyzed at 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and then analyzed by flow cytometry. Means ± SEM are shown.

Although LPS did not stimulate cells as well as conA, its stimulation was rather constant over time. Previous studies using the LPS from S. typhosa showed similar results: sub-optimal, but consistent stimulation (8).

We found that PBMCs stimulated with 1 μg/ml PMA and 0.5 μg/ml ionomycin showed optimal proliferation. This occurred at the 72 hour timepoint. Because of the scarcity of research in this area, it is difficult to compare these results to other studies. However, these studies will now allow for future comparisons using these T cell activators in the dog.

We determined that at a concentration of 10^{-6} M, dex inhibited the proliferation of conA stimulated cells. This effect was greatest at 48 hours of incubation. Interestingly, previous studies performed with canine cancer cells showed various responses to dex treatment (33). In the latter studies, it was found that cutaneous mast cell tumors were susceptible to dex inhibition, while intestinal mast cell tumors were not. However, is it noted that one characteristic of cancer cells is mutated glucocorticoid receptors (33). In this study, we verified that canine PBMCs were susceptible to dex-induced inhibition.

These mitogen studies, coupled with the dex study, allowed us the means to assess steroid-induced cell death in canine PBMCs. For this assay, we found that conA at 2.5 μg/ml and dex at 10^{-6} M generated the greatest effect. We found that this inhibitory effect peaked at the 48 hour timepoint. These results can now be used in future studies to assess the ability of other cells to resist dex-induced cell

death. This assay could be of particular interest when investigating the reactivity of treated versus control cells.

Although in vitro proliferation experiments using canine peripheral blood mononuclear cells have been performed by other researchers, consistency in methodology is lacking. We have developed a new method for canine proliferation assays that utilizes propidium iodide staining and a flow cytometer. This method allows for the calculation of the exact number of cells undergoing proliferation or apoptosis, a measurement lacking in ^{3}H-T studies. These experiments also establish standards for canine in vitro culturing conditions that will allow for future comparative immune investigations. Through the use of flow cytometry, many determinations can be made about immune function. For example, cell composition in a culture can be determined, as well as expression of cell surface molecules. These analyses can be accomplished through the use of fluorochrome-conjugated antibodies to individual cell markers (i.e., CD4, CD8 and B cell). Surface molecule expression can also give an estimate of cell activation. One example of this is CD69 expression (34). This molecule is an indicator of lymphocyte activation that can be labeled by fluorochrome staining and identified by flow cytometry. The culture conditions outlined in these studies have set the necessary ground work for this type of future research.

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CHAPTER 5
CONCLUDING THOUGHTS

It is clear that there is a delicate balance between helpful immunity and autoimmunity. The immune system must be able to eradicate pathogens, but must not attack "self." Fundamental to this challenge are understanding the signals involved in these processes. What signals are given that lead to autoimmunity? Are these signals fundamentally different from signals involved in constructive immunity? In order to answer these questions, assays need to be established in a different species so that these questions can be studied in the future.

Studies with OX40 have led to interesting advances in understanding immune function. Investigations reported in this thesis demonstrate that even with multiple costimulatory signals, antigen-activated T cells are susceptible to antigen-induced cell death. These results lend credence to the "danger theory" i.e., without an inflammatory response, increased cell longevity is not seen. Additionally, OX40 ligation promotes expression of several important survival proteins , Bcl-xL and Bcl-2 (1). These proteins are able to inhibit apoptosis, and thus, allow for increased cell survival. However, even production of these anti-apoptotic proteins does not prevent activation-induced cell death. These studies show that there is an overwhelming shut-off signal, and that even additional costimulation is unable to counter it.

If such a potent signal for shutting down a normal immune response exists, then why are some substances able to induce autoimmunity? The "danger signal" hypothesizes that substances capable of inducing large inflammatory responses are able to lead to prolonged lymphocyte survival (2). This survival may then be the forerunner of autoimmunity. LPS has been implicated in this process. Additional substances that may be able to induce a similar survival property are rapeseed oil and aromatic anilides. These substances are capable of inducing an inflammatory response, which in the case of "Toxic Oil Syndrome" led to autoimmunity. The question that arises around this phenomenon is what signal is present in this scenario that is not present with costimulatory molecules? Why are immune responses that are mounted in the presence of these inflammatory agents able to persist?

Research has led to some elucidation of the signaling cascade of LPS. It is now understood that following administration of LPS, it interacts with the LPS binding protein (LBP) which shuttles LPS molecules to the receptor CD14 (3-6). CD14 is present both in the cytosol and as a membrane-bound protein on monocytes and macrophages (5-7). The soluble CD14 is thought to mediate the effects of LPS on $CD14^-$ cells such as lymphocytes, endothelial cells, and granulocytes (8-10). However, this protein has no intracellular signaling domain, and therefore a third component is required for LPS signaling. Toll-like receptors (TLRs) have recently been identified as the proteins responsible for transferring the

activation signal to the nucleus. Of particular importance in LPS signaling are TLR2 and TLR4. Both of these receptors have been shown to mediate activation of the transcription factors AP-1 and NF-κB by activating a kinase cascade that leads to the phosphorylation of a repressor protein IκB that sequesters the NF-κB transcription factor in the cytoplasm. The phosphorylation of IκB targets it for ubiquitination and subsequent degradation. This degradation releases the transcription factor to translocate to the nucleus where it leads to the transcription of numerous genes for proinflammatory cytokines including TNF-α, IL-1, IL-6, IL-8, ICAM-1 and E-selectin (6, 10-17) (Figure 5.1). The production of these inflammatory mediators may be the driving force behind the damage that is implicated in the "danger theory".

Further research in the area of lipid signaling, using the inflammatory agent rapeseed oil or the "toxic oil" utilized in this study, may lead to new discoveries about the initiation and progression of TOS. It would be interesting to investigate whether TOS is inducible in CD14 knockout mice. Because these mice are resistant to the effects of LPS, including endotoxic shock, it is possible that they would not be susceptible to TOS mediated autoimmunity (18, 19). Additional investigations could also be done using mice with site-directed mutagenesis of the TLRs. Because other genetic factors have been implicated in TOS susceptibility, it is possible that differences in expression or mutations in these genes may play a role.

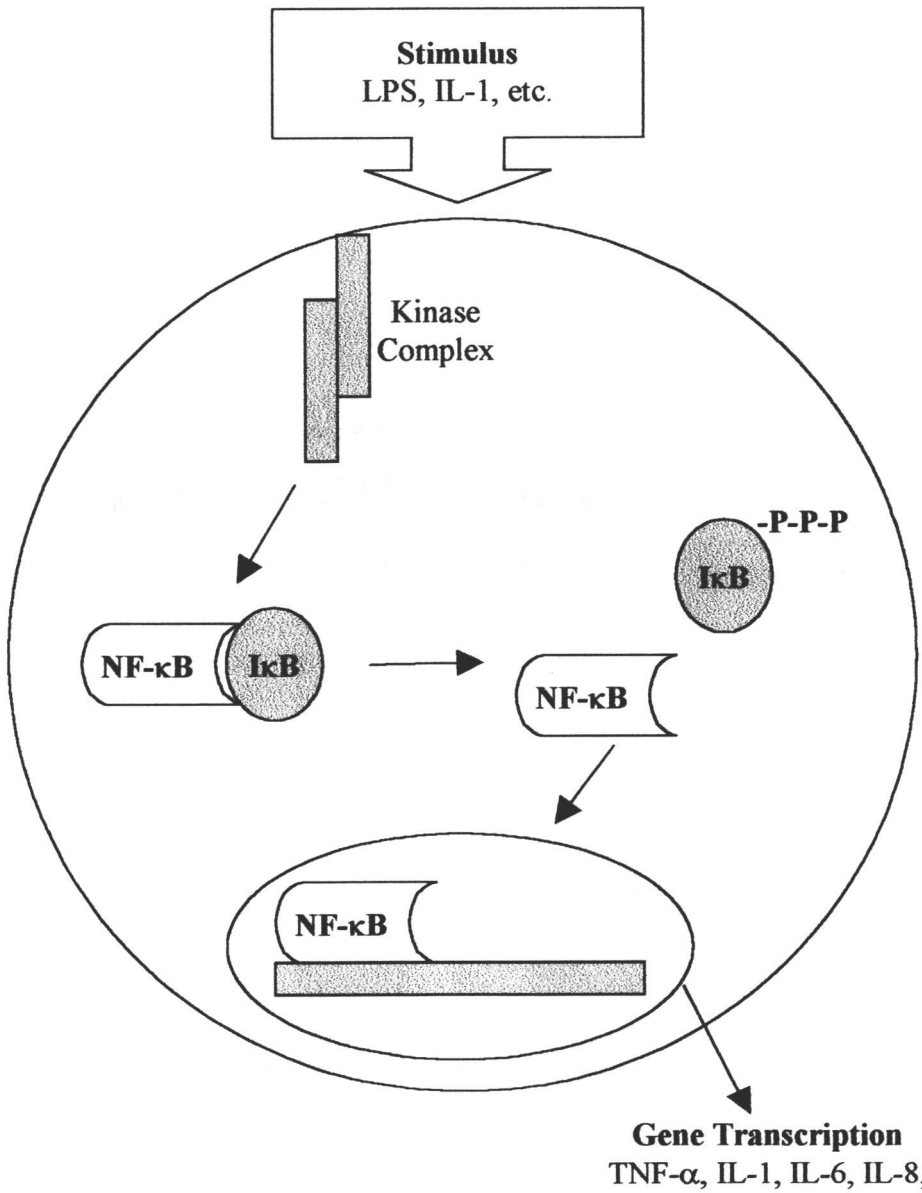

Figure 5.1: Signaling cascade leading to NF-κB activation. The first step in NF-κB activation is the binding of some stimulus to a receptor on the surface of the cell. This binding leads to the activation of a kinase cascade that is able to phosphorylate the suppressor protein IκB. This phosphorylation targets this protein for ubiquitination and degradation. This releases the NF-κB transcription factor. It is then able to translocate into the nucleus where it activates transcription of a variety of genes including TNF-α, IL-1, IL-6, and IL-8.

Not only are lipids able to induce autoimmunity, but also heavy metals are able to induce autoimmunity. The mechanism by which mercury induces autoimmunity is somewhat different from the mechanism discussed above, although the outcome is similar. Mercury is thought to activate self-reactive T cells by binding to protein side chains and creating metal-protein complexes (20, 21). These complexes can be so stable that they affect normal protein processing and presentation. As a result, cryptic self proteins are presented to T cells. However, given the acceptance of the two signal requirement for full T cell activation, a second signal would still be needed for generation of T cell effector function. One possibility is that mercury is able to induce expression of the costimulatory molecule B7 on APCs, although this possibility has not been investigated. A second possibility is that mercury treatment may lead to localized cell death, and therefore, induce a local inflammatory response (21, 22). This inflammatory response would then stimulate the APCs, thereby giving them the ability to deliver signal two to the responding T cells. These self-reacting T cells would then be able to stimulate antibody production by self-reacting B cells and lead to autoantibody production, a hallmark of mercury-induced autoimmune disease. Interestingly, a third mechanism may exist for costimulation following mercury treatment. It has been noted that mercury is able to directly induce IL-1 production (21, 23). Interestingly, IL-1 is also able to directly activate the transcription factor NF-κB (10, 24, 25). By binding to the IL-1 receptor on the cell surface, IL-1 initiates the

signaling cascade that culminates in the activation of NF-κB (13, 26-28). This activation again leads to the production of proinflammatory cytokines and an inflammatory response. This inflammatory response may then supply the "danger signal" leading to increased longevity of the self-reacting lymphocytes and autoimmunity.

These varied responses and the complexity of immune function and autoimmunity necessitates the development of new models. Although significant advances have already been made using primarily mouse models, further studies are needed. One species that may be useful in further advancing knowledge of the immune system is the dog. The studies outlined in this thesis lay the foundation for this research. These studies provide the standard conditions for immune function assays in the canine species. Further investigations utilizing these assays may lead to additional information and understanding of functional immune responses and autoimmunity.

Why is continued research into immunity necessary? Many diseases have an immune component. A complete understanding of the signals involved may lead to advances in therapy. Some examples are cancer therapy and treatment for systemic autoimmune diseases such as systemic lupus erythematosis. In the case of neoplastic cells, the immune system is no longer able to fight the defective cells. It would be of great benefit if those tolerant immune cells could be re-activated to fight the tumor cells. In the case of systemic lupus erythematosis, self-reacting T

cells are generating autoantibody production. The ability to turn off self-reacting T cells would allow for the amelioration of autoimmune disease. Through continued research, these treatments may become a reality.

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APPENDICES

Appendix A: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with PMA and ionomycin. PMA was used at concentrations of 1.0, 0.1, and 0.01 μg.ml, whereas ionomycin was used as concentrations of 20, 10, 1.0, 0.5, 0.25, and 0.1 μg/ml. Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown at 18 hours of incubation.

18 Hours	PMA Concentration											
	1.0 μg/ml				0.1 μg/ml				0.01 μg/ml			
Ionomycin Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
20 μg/ml	10.4 ± 0.8	2.5 ± 0.2	3.0 ± 0.0	0.7 ± 0.0	10.5 ± 0.2	2.3 ± 0.0	2.4 ± 0.1	0.5 ± 0.0	10.8 ± 0.1	2.4 ± 0.0	3.3 ± 0.4	0.7 ± 0.1
10 μg/ml	9.5 ± 0.8	2.2 ± 0.3	2.4 ± 0.3	0.6 ± 0.1	9.7 ± 0.2	2.2 ± 0.1	2.5 ± 0.4	0.6 ± 0.1	9.8 ± 1.2	2.2 ± 0.3	2.0 ± 0.4	0.4 ± 0.1
1 μg/ml	8.8 ± 0.2	2.0 ± 0.1	2.3 ± 0.0	0.5 ± 0.0	9.57 ± 0.6	2.1 ± 0.2	2.8 ± 0.0	0.6 ± 0.0	9.4 ± 0.6	2.2 ± 0.0	2.6 ± 0.2	0.6 ± 0.1
0.5 μg/ml	9.8 ± 1.0	2.2 ± 0.2	4.0 ± 0.5	0.9 ± 0.1	10.1 ± 1.0	2.2 ± 0.2	3.3 ± 0.2	0.7 ± 0.1	10.8 ± 1.4	2.0 ± 0.2	3.7 ± 0.4	0.7 ± 0.1
0.25 μg/ml	10.5 ± 0.6	2.8 ± 0.0	3.6 ± 0.1	1.0 ± 0.1	10.8 ± 0.4	3.6 ± 0.0	3.5 ± 0.2	1.2 ± 0.0	12.0 ± 0.2	2.9 ± 0.5	3.4 ± 0.5	0.8 ± 0.0
0.10 μg/ml	9.2 ± 1.0	2.4 ± 0.3	3.0 ± 0.4	0.8 ± 0.1	10.3 ± 0.3	2.5 ± 0.0	2.9 ± 0.3	0.7 ± 0.1	11.9 ± 0.2	2.9 ± 0.4	4.5 ± 0.2	0.1 ± 0.0
Media only	12.7 ± 0.5	3.0 ± 0.1	3.4 ± 0.5	0.8 ± 0.1								

Appendix A continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with PMA and ionomycin. PMA was used at concentrations of 1.0, 0.1, and 0.01 μg.ml, whereas ionomycin was used as concentrations of 20, 10, 1.0, 0.5, 0.25, and 0.1 μg/ml. Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown at 24 hours of incubation.

24 Hours	PMA Concentration											
	1.0 μg/ml				0.1 μg/ml				0.01 μg/ml			
Ionomycin Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
20 μg/ml	11.9 ± 0.2	2.6 ± 0.3	2.6 ± 0.4	0.6 ± 0.1	11.5 ± 0.1	2.4 ± 0.1	2.7 ± 0.2	0.6 ± 0.0	10.7 ± 0.5	2.2 ± 0.0	2.5 ± 0.6	0.5 ± 0.2
10 μg/ml	9.4 ± 0.0	2.3 ± 0.0	2.6 ± 0.2	0.6 ± 0.0	9.6 ± 0.5	2.2 ± 0.2	2.7 ± 0.2	0.6 ± 0.0	8.7 ± 0.0	1.8 ± 0.3	2.5 ± 0.1	0.5 ± 0.0
1 μg/ml	8.0 ± 0.3	1.8 ± 0.0	2.3 ± 0.0	0.5 ± 0.0	8.9 ± 0.5	1.9 ± 0.0	2.6 ± 0.2	0.6 ± 0.0	7.7 ± 0.5	1.3 ± 0.3	2.8 ± 0.1	0.5 ± 0.1
0.5 μg/ml	9.1 ± 0.5	1.7 ± 0.2	3.7 ± 0.2	0.7 ± 0.0	9.7 ± 0.3	1.9 ± 0.2	3.7 ± 0.6	0.7 ± 0.1	10.2 ± 1.4	1.6 ± 0.3	3.8 ± 0.2	0.6 ± 0.0
0.25 μg/ml	9.9 ± 0.2	1.7 ± 0.0	4.7 ± 0.4	0.8 ± 0.1	9.8 ± 0.9	1.9 ± 0.3	4.0 ± 0.1	0.8 ± 0.1	10.4 ± 2.2	1.7 ± 0.4	3.6 ± 0.2	5.8 ± 0.4
0.10 μg/ml	10.0 ± 0.8	1.8 ± 0.3	3.7 ± 0.1	0.7 ± 0.1	10.3 ± 1.1	1.9 ± 0.0	3.4 ± 0.3	0.7 ± 0.0	12.3 ± 0.6	2.0 ± 0.2	3.1 ± 0.0	5.0 ± 0.3
Media only	9.2 ± 0.1	1.5 ± 0.0	3.0 ± 0.1	0.5 ± 0.0								

Appendix A continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with PMA and ionomycin. PMA was used at concentrations of 1.0, 0.1, and 0.01 μg/ml, whereas ionomycin was used at concentrations of 20, 10, 1.0, 0.5, 0.25, and 0.1 μg/ml. Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM at 48 and 72 hours are shown.

48 Hours	PMA Concentration											
	1.0 μg/ml				0.1 μg/ml				0.01 μg/ml			
Ionomycin Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
20 μg/ml	13.8 ± 1.0	3.5 ± 0.4	2.7 ± 0.4	0.7 ± 0.1	12.7 ± 0.8	2.8 ± 0.1	2.1 ± 0.1	0.5 ± 0.0	12.8 ± 2.0	3.4 ± 0.8	2.0 ± 0.2	0.5 ± 0.1
10 μg/ml	10.5 ± 0.3	2.2 ± 0.2	3.0 ± 0.3	0.6 ± 0.0	10.4 ± 0.7	2.4 ± 0.1	3.0 ± 0.2	0.7 ± 0.1	10.9 ± 0.3	2.5 ± 0.2	3.2 ± 0.2	0.7 ± 0.1
1 μg/ml	8.9 ± 0.4	2.3 ± 0.2	12.4 ± 1.0	3.2 ± 0.3	9.7 ± 1.5	2.4 ± 0.5	10.1 ± 1.0	2.5 ± 0.1	11.0 ± 1.5	2.7 ± 0.4	8.5 ± 0.5	2.0 ± 0.1
0.5 μg/ml	12.0 ± 0.7	2.9 ± 0.4	11.5 ± 0.7	2.8 ± 0.1	12.5 ± 0.7	2.6 ± 0.0	10.5 ± 0.2	2.2 ± 0.1	13.4 ± 1.5	2.5 ± 0.1	9.9 ± 0.1	1.9 ± 0.1
0.25 μg/ml	11.5 ± 0.8	2.5 ± 0.2	9.7 ± 1.1	2.1 ± 0.3	11.1 ± 0.7	2.5 ± 0.2	9.2 ± 0.8	2.0 ± 0.2	11.4 ± 0.6	2.7 ± 0.1	7.1 ± 0.2	1.7 ± 0.0
0.10 μg/ml	11.6 ± 0.9	2.7 ± 0.3	5.7 ± 0.5	1.3 ± 0.1	11.4 ± 0.3	2.5 ± 0.1	5.5 ± 0.5	1.2 ± 0.1	12.9 ± 0.3	2.8 ± 0.1	6.1 ± 0.02	1.3 ± 0.0
Media only	18.5 ± 0.5	4.1 ± 0.1	3.1 ± 0.1	0.7 ± 0.0								

Appendix A continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with PMA and ionomycin. PMA was used at concentrations of 1.0, 0.1, and 0.01 μg/ml, whereas ionomycin was used at concentrations of 20, 10, 1.0, 0.5, 0.25, and 0.1 μg/ml. Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM at 48 and 72 hours are shown.

72 Hours	PMA Concentration											
	1.0 μg/ml				0.1 μg/ml				0.01 μg/ml			
Ionomycin Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
20 μg/ml	18.1 ± 0.3	5.1 ± 0.3	3.9 ± 0.2	1.1 ± 0.1	18.1 ± 0.6	5.1 ± 0.5	4.1 ± 0.2	1.1 ± 0.1	18.2 ± 0.8	4.9 ± 0.4	4.0 ± 0.4	1.1 ± 0.1
10 μg/ml	13.0 ± 1.0	3.7 ± 0.3	5.7 ± 2.1	1.6 ± 0.6	17.4 ± 2.0	4.2 ± 0.5	4.4 ± 0.5	1.1 ± 0.1	16.1 ± 1.3	3.9 ± 0.4	5.1 ± 1.3	1.3 ± 0.4
1 μg/ml	10.46 ± 1.3	3.2 ± 0.5	14.8 ± 0.2	4.6 ± 0.1	14.2 ± 2.7	4.2 ± 0.9	11.9 ± 3.3	3.4 ± 0.8	14.8 ± 0.4	3.9 ± 0.1	9.6 ± 0.8	2.5 ± 0.2
0.5 μg/ml	8.9 ± 0.3	2.3± 0.1	20.3 ± 2.2	5.3 ± 1.0	9.6 ± 0.4	2.2 ± 0.4	17.9 ± 0.7	4.1 ± 0.4	10.6 ± 0.5	1.8 ± 0.2	16.2 ± 1.2	2.7 ± 0.0
0.25 μg/ml	8.7 ± 0.4	1.9 ± 0.1	13.1 ± 2.1	2.9 ± 0.5	8.6 ± 0.7	1.7 ± 0.1	10.5 ± 1.4	2.1 ± 0.3	9.7 ± 0.6	1.7 ± 0.0	8.3 ± 0.6	1.4 ± 0.2
0.10 μg/ml	8.8 ± 0.4	2.0 ± 0.2	8.0 ± 0.02	1.9 ± 0.1	9.3 ± 0.4	2.0 ± 0.2	7.4 ± 0.3	1.6 ± 0.0	10.1 ± 0.5	2.1 ± 0.0	6.6 ± 0.05	1.4 ± 0.1
Media only	12.6 ± 0.3	1.9 ± 0.1	6.5 ± 0.7	9.7 ± 0.0								

Appendix B: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with PMA and ionomycin. PMA was used at concentrations of 20 and 10 μg.ml, whereas ionomycin was used as concentrations of 20, 10, 1.0, 0.5, 0.25, and 0.1 μg/ml. Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown at 18 and 24 hours of incubation.

18 Hours	PMA Concentration							
	20 μg/ml				10 μg/ml			
Ionomycin Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
20 μg/ml	23.8 ± 0.0	6.7 ± 0.0	3.6 ± 0.6	1.0 ± 0.2	37.2 ± 2.8	7.4 ± 0.6	3.7 ± 0.3	0.8 ± 0.1
10 μg/ml	28.4 ± 1.1	7.1 ± 0.6	3.7 ± 0.3	0.9 ± 0.1	47.3 ± 1.2	9.0 ± 0.2	3.0 ± 0.1	0.6 ± 0.1
1 μg/ml	24.0 ± 1.7	6.0 ± 0.2	3.9 ± 0.5	1.0 ± 0.2	40.0 ± 1.3	8.7 ± 0.5	2.4 ± 0.2	0.5 ± 0.0
0.5 μg/ml	20.9 ± 1.0	6.1 ± 1.3	4.1 ± 0.6	1.2 ± 0.4	36.1± 9.2	8.2 ± 1.8	3.1 ± 0.4	0.7 ± 0.1
0.25 μg/ml	22.1 ± 1.0	5.1 ± 0.0	3.7 ± 0.2	0.9 ± 0.0	24.2 ± 1.1	6.3 ± 0.3	3.2 ± 0.0	0.8 ± 0.0
0.10 μg/ml	20.1 ± 0.8	4.6 ± 0.4	4.3 ± 0.9	1.0 ± 0.2	23.8 ± 2.7	5.9 ± 0.4	2.8 ± 0.5	0.7 ± 0.1
Media only	12.7 ± 0.5	3.0 ± 0.1	3.4 ± 0.5	0.8 ± 0.1				

24 Hours	PMA Concentration							
	20 μg/ml				10 μg/ml			
Ionomycin Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
20 μg/ml	22.3 ± 0.3	6.7 ± 0.1	3.2 ± 0.1	1.0 ± 0.0	36.7 ± 0.4	7.0 ± 0.3	2.9 ± 0.2	0.6 ± 0.0
10 μg/ml	25.6 ± 0.8	6.7 ± 0.7	3.5 ± 0.2	0.9 ± 0.1	45.0 ± 2.8	7.6 ± 0.0	3.2 ± 0.5	0.5 ± 0.1
1 μg/ml	19.0 ± 1.1	5.1 ± 0.1	4.6 ± 0.4	1.2± 0.1	40.3 ± 0.6	7.7 ± 0.5	2.7 ± 0.4	0.5 ± 0.1
0.5 μg/ml	19.2 ± 0.8	6.0 ± 0.4	4.6 ± 0.2	1.4 ± 0.1	35.8± 0.5	7.5 ± 0.2	3.8 ± 0.2	0.8 ± 0.1
0.25 μg/ml	19.7 ± 0.1	4.1 ± 0.2	4.3 ± 0.1	0.9 ± 0.0	29.4 ± 0.5	5.9 ± 0.1	5.1 ± 0.3	1.0 ± 0.1
0.10 μg/ml	18.8 ± 1.1	3.9 ± 0.0	5.2 ± 0.9	1.1 ± 0.1	28.1 ± 0.1	5.9 ± 0.3	3.4 ± 0.1	0.7 ± 0.0
Media only	9.2 ± 0.1	1.5 ± 0.0	3.0 ± 0.1	0.5 ± 0.0				

Appendix B continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with PMA and ionomycin. PMA was used at concentrations of 20 and 10 μg.ml, whereas ionomycin was used as concentrations of 20, 10, 1.0, 0.5, 0.25, and 0.1 μg/ml. Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown at 48 hours of incubation.

48 Hours	PMA Concentration							
	20 μg/ml				10 μg/ml			
Ionomycin Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
20 μg/ml	22.2 ± 0.2	8.6 ± 0.2	1.4 ± 0.1	0.5 ± 0.0	34.3 ± 1.9	7.9 ± 0.1	1.9 ± 0.2	0.4 ± 0.1
10 μg/ml	24.4 ± 0.4	8.5 ± 0.4	1.9 ± 0.0	0.7 ± 0.0	41.4 ± 0.3	8.3 ± 0.9	2.4 ± 0.0	0.5 ± 0.1
1 μg/ml	19.8 ± 0.3	5.7 ± 0.1	2.2 ± 0.1	0.6 ± 0.1	38.7 ± 0.3	7.7 ± 0.1	1.6 ± 0.1	0.3 ± 0.0
0.5 μg/ml	18.8 ± 0.9	5.2 ± 0.2	2.3 ± 0.5	0.7 ± 0.2	30.3± 0.1	6.1 ± 0.0	1.3 ± 0.2	0.3 ± 0.0
0.25 μg/ml	15.8 ± 0.4	3.9 ± 0.1	4.3 ± 0.3	1.1 ± 0.0	30.1 ± 0.0	6.6 ± 0.0	3.5 ± 0.4	0.8 ± 0.1
0.10 μg/ml	16.3 ± 0.5	3.9 ± 0.1	3.1 ± 0.0	0.7 ± 0.0	30.0 ± 0.7	6.0 ± 0.1	3.3 ± 0.2	0.7 ± 0.0
Media only	18.5 ± 0.5	4.1 ± 0.1	3.1 ± 0.1	0.7 ± 0.0				

72 Hours	PMA Concentration							
	20 μg/ml				10 μg/ml			
Ionomycin Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
20 μg/ml	22.8 ± 0.2	9.1 ± 0.1	2.1 ± 0.2	0.8 ± 0.1	34.1 ± 0.9	8.5 ± 0.1	1.6 ± 0.1	0.4 ± 0.0
10 μg/ml	19.9 ± 1.6	6.8 ± 0.5	2.0 ± 0.2	0.7 ± 0.1	35.0 ± 0.2	7.7 ± 0.0	2.1 ± 0.1	0.5 ± 0.0
1 μg/ml	20.4 ± 0.9	6.7 ± 0.1	2.4 ± 0.0	0.8 ± 0.0	35.2 ± 0.6	7.8 ± 0.8	2.7 ± 0.1	0.6 ± 0.0
0.5 μg/ml	20.6 ± 0.3	7.8 ± 0.1	3.1 ± 0.5	1.2 ± 0.2	31.8± 0.4	7.3 ± 0.4	2.6 ± 0.3	0.6 ± 0.1
0.25 μg/ml	18.8 ± 0.9	6.4 ± 0.1	3.8 ± 0.7	1.3 ± 0.3	25.8 ± 2.4	5.7 ± 0.5	3.3 ± 0.4	0.7 ± 0.1
0.10 μg/ml	16.7 ± 0.5	4.8 ± 0.0	3.1 ± 0.4	0.9 ± 0.2	25.6 ± 0.3	5.6 ± 0.5	3.4 ± 0.1	0.8± 0.1
Media only	12.6 ± 0.3	1.9 ± 0.1	6.5 ± 0.7	1.0 ± 0.0				

Appendix C: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with conA and treatment with dex. Cells were cultured with varying concentrations of conA and dex for 24 hours. Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

24 Hours	ConA Concentration											
	1.25 μg/ml				2.5 μg/ml				5.0 μg/ml			
Dex Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
10^{-5} M	7.4 ± 0.0	2.1 ± 0.1	2.6 ± 0.1	0.7 ± 0.1	8.8 ± 0.4	2.4 ± 0.2	2.7 ± 0.2	0.7 ± 0.1	9.7 ± 0.9	2.3 ± 0.2	2.8 ± 0.1	0.7 ± 0.0
10^{-6} M	9.1 ± 1.2	2.8 ± 0.5	2.8 ± 0.1	0.9 ± 0.0	8.9 ± 1.3	2.6 ± 0.5	2.9 ± 0.1	0.8 ± 0.0	8.6 ± 0.2	2.0 ± 0.0	2.7 ± 0.2	0.6 ± 0.1
10^{-7} M	6.5 ± 0.1	1.8 ± 0.0	2.5 ± 0.0	0.7 ± 0.0	7.8 ± 0.4	2.0 ± 0.0	2.6 ± 0.2	0.7 ± 0.0	7.7 ± 0.0	2.0 ± 0.0	2.7 ± 0.0	0.7 ± 0.0
ConA only	6.5 ± 0.2	1.9 ± 0.0	3.1 ± 0.0	0.9 ± 0.0	7.1 ± 0.1	1.8 ± 0.2	2.4 ± 0.0	0.6 ± 0.1	9.6 ± 1.2	2.2 ± 0.4	2.4 ± 0.4	0.6± 0.1
10^{-6} M Dex only	14.2 ± 1.1	3.8 ± 0.2	1.0 ± 0.2	0.3 ± 0.0								
Media only	8.0 ± 0.0	2.4 ± 0.0	1.3 ± 0.0	0.2 ± 0.0								

Appendix C continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with conA and treatment with dex. Cells were cultured with varying concentrations of conA and dex for 24, 48, or 72 hours (only 48 and 72 hours are shown). Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

48 Hours	ConA Concentration											
	1.25 μg/ml				2.5 μg/ml				5.0 μg/ml			
Dex Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
10^{-5} M	8.2 ± 0.0	2.0 ± 0.0	14.8 ± 0.2	3.6 ± 0.0	11.3 ± 0.1	3.1 ± 0.1	17.6 ± 0.2	4.8 ± 0.2	11.5 ± 0.1	3.2± 0.2	15.8 ± 0.7	4.4 ± 0.1
10^{-6} M	8.5 ± 0.1	2.2 ± 0.0	15.6 ± 0.1	4.1 ± 0.0	11.4 ± 0.3	3.2 ± 0.2	17.1 ± 1.7	4.8 ± 0.8	12.1 ± 0.0	3.5 ± 0.4	15.8 ± 0.8	4.5 ± 0.2
10^{-7} M	8.4 ± 0.0	2.2 ± 0.0	17.0 ± 0.2	4.4 ± 0.0	10.9 ± 0.2	3.2 ± 0.2	19.6 ± 0.3	5.7 ± 0.1	12.1 ± 0.7	3.4 ± 0.0	16.7 ± 0.3	4.7 ± 0.4
ConA only	8.4 ± 0.1	2.5 ± 0.0	18.4 ± 0.0	5.5 ± 0.0	10.6 ± 0.2	3.3 ± 0.4	19.6 ± 0.2	6.1 ± 0.6	11.6 ± 0.1	3.6 ± 0.1	17.5 ± 0.2	5.4 ± 0.2
10^{-6} M Dex only	12.5 ± 0.8	3.6 ± 0.4	0.5 ± 0.0	0.2 ± 0.0								
Media only	6.7 ± 0.1	1.6 ± 0.0	1.3 ± 0.01	0.3 ± 0.0								

Appendix C continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with conA and treatment with dex. Cells were cultured with varying concentrations of conA and dex for 24, 48, or 72 hours (only 48 and 72 hours are shown). Following incubation, cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

72 Hours	ConA Concentration											
	1.25 μg/ml				2.5 μg/ml				5.0 μg/ml			
Dex Concentration	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif	% Apop	# Apop	% Prolif	# Prolif
10^{-5} M	9.8 ± 0.1	4.5 ± 0.0	14.8 ± 2.1	6.8 ± 1.0	10.2 ± 0.5	4.3 ± 0.4	13.6 ± 0.3	5.7 ± 0.2	13.2 ± 0.5	4.7 ± 0.2	13.8 ± 0.5	5.0 ± 0.2
10^{-6} M	10.2 ± 1.6	4.5 ± 0.5	14.7 ± 0.5	6.4 ± 0.1	9.8 ± 0.3	3.7 ± 0.1	13.9 ± 0.8	5.3 ± 0.0	13.1 ± 1.4	4.8 ± 0.8	13.4 ± 0.5	4.8 ± 0.4
10^{-7} M	9.3 ± 0.8	4.4 ± 0.3	14.3 ± 0.7	6.7 ± 0.2	10.4 ± 0.3	4.0 ± 0.1	16.8 ± 0.9	6.4 ± 0.7	12.5 ± 0.0	4.5 ± 0.0	16.1 ± 0.2	5.8 ± 0.1
ConA only	9.1 ± 0.5	4.5 ± 0.3	16.5 ± 2.5	8.1 ± 1.3	10.7 ± 0.1	4.3 ± 0.1	18.1 ± 1.0	7.3 ± 0.4	13.3 ± 0.2	5.9 ± 0.1	16.5 ± 0.1	7.3 ± 0.1
10^{-6} M Dex only	11.8 ± 0.6	2.2 ± 0.0	0.6 ± 0.2	0.1 ± 0.0								
Media only	6.3 ± 0.0	1.4 ± 0.0	0.7 ± 0.0	0.1 ± 0.0								

Appendix D: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with concanavalinA (conA). In vitro cell cultures were stimulated with varying concentrations of conA. These cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and then analyzed by flow cytometry. Means ± SEM are shown.

24 hours				
ConA concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	19.4 ± 0.4	3.5 ± 0.1	2.8 ± 0.1	0.5 ± 0.0
25 μg/ml	18.2 ± 2.4	3.3 ± 0.4	3.5 ± 0.4	0.6 ± 0.1
10 μg/ml	11.2 ± 0.5	2.6 ± 0.2	2.3 ± 0.7	0.5 ± 0.1
5 μg/ml	9.1 ± 0.1	2.6 ± 0.2	2.1 ± 0.0	0.6 ± 0.0
2.5 μg/ml	8.2 ± 0.3	2.6 ± 0.0	1.6 ± 0.0	0.5 ± 0.0
media	4.7 ± 0.1	1.6 ± 0.0	0.8 ± 0.1	0.3 ± 0.0

48 hours				
ConA concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	25.1 ± 0.4	6.0 ± 0.1	3.9 ± 0.5	0.9 ± 0.1
25 μg/ml	25.3 ± 1.2	6.8 ± 0.4	8.2 ± 1.1	2.2 ± 0.1
10 μg/ml	9.7 ± 0.2	3.6 ± 0.0	14.1 ± 1.2	5.2 ± 0.3
5 μg/ml	8.1 ± 0.5	3.5 ± 0.2	14.1 ± 1.9	6.2 ± 0.8
2.5 μg/ml	6.9 ± 0.2	3.2 ± 0.1	16.1 ± 0.7	7.4 ± 0.3
media	5.5 ± 0.2	2.2 ± 0.1	1.8 ± 1.4	0.7 ± 0.4

Appendix D continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with concanavalinA (conA). In vitro cell cultures were stimulated with varying concentrations of conA. These cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and then analyzed by flow cytometry. Means ± SEM are shown.

72 hours				
ConA concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	24.1 ± 3.0	2.6 ± 0.1	4.8 ± 0.3	0.5 ± 0.0
25 μg/ml	28.0 ± 2.3	2.5 ± 0.1	7.3 ± 0.4	0.6 ± 0.0
10 μg/ml	15.4 ± 1.1	2.4 ± 0.1	11.4 ± 0.8	1.8 ± 0.1
5 μg/ml	13.7 ± 1.6	3.3 ± 0.4	8.3 ± 0.1	2.0 ± 0.0
2.5 μg/ml	14.9 ± 0.3	4.0 ± 0.2	6.6 ± 0.3	1.8 ± 0.1
media	3.7 ± 0.4	0.7 ± 0.1	0.7 ± 0.1	0.1 ± 0.0

96 hours				
ConA concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	31.3 ± 0.1	7.8 ± 1.5	6.7 ± 2.7	1.8 ± 1.0
25 μg/ml	34.4 ± 0.5	11.0 ± 3.3	5.0 ± 0.7	1.7 ± 0.7
10 μg/ml	18.6 ± 0.1	7.5 ± 1.1	10.1 ± 0.4	4.0 ± 0.5
5 μg/ml	20.8 ± 0.4	12.9 ± 0.2	6.7 ± 0.9	4.3 ± 0.6
2.5 μg/ml	26.4 ± 0.4	17.9 ± 0.8	5.1 ± 0.1	3.5 ± 0.2
media	4.2 ± 0.1	1.4 ± 0.0	2.6 ± 1.6	0.8 ± 0.5

Appendix E: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with LPS. In vitro cell cultures were stimulated with LPS from E. coli O111:B4, E. coli O55:B5, S. typhimurium, or S. typhosa at varying concentrations. The cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

24 hours	Escherichia coli O111:B4			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	6.9 ± 0.3	2.4 ± 0.0	0.8 ± 0.0	0.3 ± 0.0
25 μg/ml	6.5 ± 0.2	2.2 ± 0.1	0.8 ± 0.0	0.3 ± 0.0
10 μg/ml	6.3 ± 0.0	2.4 ± 0.3	0.9 ± 0.0	0.4 ± 0.1
1 μg/ml	5.6 ± 0.1	1.9 ± 0.0	1.0 ± 0.0	0.3 ± 0.0
0.1 μg/ml	5.3 ± 0.1	1.7 ± 0.0	1.0 ± 0.0	0.3 ± 0.0
media	5.3 ± 0.0	2.1 ± 0.0	0.9 ± 0.0	0.4 ± 0.0

48 hours	Escherichia coli O111:B4			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	7.2 ± 0.6	2.6 ± 0.2	0.8 ± 0.1	0.3 ± 0.0
25 μg/ml	7.3 ± 0.6	2.6 ± 0.1	0.8 ± 0.1	0.3 ± 0.0
10 μg/ml	8.0 ± 0.2	2.7 ± 0.1	0.7 ± 0.0	0.2 ± 0.0
1 μg/ml	7.4 ± 0.2	2.7 ± 0.1	0.8 ± 0.2	0.3 ± 0.1
0.1 μg/ml	6.5 ± 0.1	2.3 ± 0.0	0.7 ± 0.0	0.3 ± 0.0
media	6.0 ± 0.0	2.3 ± 0.0	0.8 ± 0.0	0.3 ± 0.0

Appendix E continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with LPS. In vitro cell cultures were stimulated with LPS from E. coli O111:B4, E. coli O55:B5, S. typhimurium, or S. typhosa at varying concentrations. The cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

72 hours	Escherichia coli O111:B4			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	6.7 ± 0.1	2.0 ± 0.0	1.5 ± 0.7	0.4 ± 0.2
25 μg/ml	7.3 ± 0.2	2.0 ± 0.0	0.7 ± 0.3	0.6 ± 0.3
10 μg/ml	7.9 ± 0.2	2.1 ± 0.1	0.8 ± 0.0	0.2 ± 0.0
1 μg/ml	7.5 ± 0.2	2.3 ± 0.0	0.9 ± 0.1	0.2 ± 0.0
0.1 μg/ml	7.5 ± 0.1	2.3 ± 0.0	0.9 ± 0.1	0.3 ± 0.0
media	6.4 ± 0.5	3.2 ± 0.3	1.0 ± 0.2	0.5 ± 0.1

96 hours	Escherichia coli O111:B4			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	5.7 ± 0.4	1.7 ± 0.1	0.6 ± 0.0	0.2 ± 0.0
25 μg/ml	6.8 ± 0.2	1.8 ± 0.1	0.6 ± 0.1	0.2 ± 0.0
10 μg/ml	7.2 ± 0.4	1.9 ± 0.1	0.6 ± 0.0	0.1 ± 0.0
1 μg/ml	7.8 ± 0.2	2.2 ± 0.0	0.7 ± 0.0	0.2 ± 0.0
0.1 μg/ml	7.4 ± 0.1	2.2 ± 0.0	0.6 ± 0.0	0.2 ± 0.0
media	6.6 ± 0.4	2.3 ± 0.0	0.9 ± 0.1	0.3 ± 0.0

Appendix E continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with LPS. In vitro cell cultures were stimulated with LPS from E. coli O111:B4, E. coli O55:B5, S. typhimurium, or S. typhosa at varying concentrations. The cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

24 hours	Escherichia coli O55:B5			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	5.8 ± 0.0	2.2 ± 0.0	0.6 ± 0.1	0.3 ± 0.0
25 μg/ml	5.8 ± 0.2	1.9 ± 0.1	0.6 ± 0.2	0.3 ± 0.1
10 μg/ml	5.8 ± 0.1	2.1 ± 0.0	0.9 ± 0.1	0.3 ± 0.0
1 μg/ml	5.4 ± 0.2	1.8 ± 0.1	0.9 ± 0.0	0.3 ± 0.0
0.1 μg/ml	5.8 ± 0.5	2.3 ± 0.2	0.8 ± 0.1	0.3 ± 0.0
media	5.3 ± 0.0	2.1 ± 0.0	0.9 ± 0.0	0.4 ± 0.0

48 hours	Escherichia coli O55:B5			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	7.3 ± 0.1	2.7 ± 0.1	0.6 ± 0.1	0.2 ± 0.0
25 μg/ml	7.2 ± 0.4	2.7 ± 0.1	0.6 ± 0.2	0.2 ± 0.1
10 μg/ml	7.0 ± 0.8	2.5 ± 0.3	0.7 ± 0.1	0.3 ± 0.0
1 μg/ml	6.8 ± 0.5	2.6 ± 0.2	0.7 ± 0.2	0.3 ± 0.1
0.1 μg/ml	6.4 ± 0.3	2.4 ± 0.0	0.7 ± 0.1	0.2 ± 0.0
media	6.0 ± 0.0	2.3 ± 0.0	0.8 ± 0.0	0.3 ± 0.0

Appendix E continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with LPS. In vitro cell cultures were stimulated with LPS from E. coli O111:B4, E. coli O55:B5, S. typhimurium, or S. typhosa at varying concentrations. The cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

72 hours	Escherichia coli O55:B5			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	7.5 ± 0.2	1.6 ± 0.1	0.7 ± 0.0	0.2 ± 0.0
25 μg/ml	7.9 ± 0.1	2.5 ± 0.0	1.4 ± 0.5	0.4 ± 0.2
10 μg/ml	7.0 ± 0.1	2.4 ± 0.2	0.9 ± 0.2	0.3 ± 0.0
1 μg/ml	6.7 ± 0.2	2.1 ± 0.1	0.8 ± 0.0	0.2 ± 0.0
0.1 μg/ml	7.1 ± 0.3	2.2 ± 0.2	0.7 ± 0.0	0.2 ± 0.0
media	6.4 ± 0.5	3.2 ± 0.3	1.0 ± 0.2	0.5 ± 0.1

96 hours	Escherichia coli O55:B5			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	7.7 ± 0.5	2.6 ± 0.0	0.4 ± 0.0	0.1 ± 0.0
25 μg/ml	8.4 ± 0.3	2.7 ± 0.1	0.4 ± 0.0	0.1 ± 0.0
10 μg/ml	7.6 ± 0.4	2.6 ± 0.1	0.5 ± 0.0	0.2 ± 0.0
1 μg/ml	7.0 ± 0.2	2.2 ± 0.0	0.5 ± 0.1	0.2 ± 0.0
0.1 μg/ml	7.5 ± 0.1	2.1 ± 0.0	0.7 ± 0.0	0.2 ± 0.0
media	6.6 ± 0.4	2.3 ± 0.0	0.9 ± 0.1	0.3 ± 0.0

Appendix E continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with LPS. In vitro cell cultures were stimulated with LPS from E. coli O111:B4, E. coli O55:B5, S. typhimurium, or S. typhosa at varying concentrations. The cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

24 hours	Salmonella typhimurium			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 µg/ml	7.1 ± 0.4	2.3 ± 0.0	0.7 ± 0.1	0.2 ± 0.0
25 µg/ml	6.6 ± 0.3	2.1 ± 0.0	0.8 ± 0.1	0.2 ± 0.0
10 µg/ml	6.5 ± 0.3	2.3 ± 0.0	0.7 ± 0.0	0.2 ± 0.0
1 µg/ml	6.1 ± 0.1	1.9 ± 0.0	0.9 ± 0.2	0.3 ± 0.0
0.1 µg/ml	5.3 ± 0.1	1.6 ± 0.0	0.8 ± 0.1	0.2 ± 0.0
media	5.3 ± 0.0	2.1 ± 0.0	0.9 ± 0.0	0.4 ± 0.0

48 hours	Salmonella typhimurium			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 µg/ml	7.0 ± 0.2	2.3 ± 0.1	0.7 ± 0.0	0.2 ± 0.0
25 µg/ml	7.2 ± 0.4	2.5 ± 0.1	0.6 ± 0.0	0.2 ± 0.0
10 µg/ml	7.4 ± 0.2	2.5 ± 0.1	0.7 ± 0.1	0.2 ± 0.0
1 µg/ml	7.2 ± 0.1	2.5 ± 0.1	0.7 ± 0.1	0.2 ± 0.0
0.1 µg/ml	6.5 ± 0.1	2.3 ± 0.0	0.7 ± 0.0	0.3 ± 0.0
media	6.0 ± 0.0	2.3 ± 0.0	0.8 ± 0.0	0.3 ± 0.0

Appendix E continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with LPS. In vitro cell cultures were stimulated with LPS from E. coli O111:B4, E. coli O55:B5, S. typhimurium, or S. typhosa at varying concentrations. The cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

72 hours	Salmonella typhimurium			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 µg/ml	6.4 ± 0.1	1.7 ± 0.2	2.0 ± 0.8	0.6 ± 0.3
25 µg/ml	7.0 ± 0.1	2.0 ± 0.1	0.8 ± 0.0	0.2 ± 0.0
10 µg/ml	7.2 ± 0.2	2.1 ± 0.0	0.9 ± 0.1	0.3 ± 0.0
1 µg/ml	7.5 ± 0.4	2.3 ± 0.1	0.8 ± 0.1	0.2 ± 0.0
0.1 µg/ml	6.3 ± 0.0	1.8 ± 0.1	1.1 ± 0.2	0.3 ± 0.1
media	6.4 ± 0.5	3.2 ± 0.3	1.0 ± 0.2	0.5 ± 0.1

96 hours	Salmonella typhimurium			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 µg/ml	5.2 ± 0.3	1.5 ± 0.1	0.7 ± 0.1	0.2 ± 0.0
25 µg/ml	6.2 ± 0.5	1.8 ± 0.1	0.6 ± 0.1	0.2 ± 0.0
10 µg/ml	6.4 ± 0.1	1.9 ± 0.0	0.6 ± 0.1	0.2 ± 0.0
1 µg/ml	7.0 ± 0.4	2.0 ± 0.1	0.7 ± 0.0	0.2 ± 0.0
0.1 µg/ml	7.8 ± 0.2	2.4 ± 0.1	0.6 ± 0.1	0.2 ± 0.0
media	6.6 ± 0.4	2.3 ± 0.0	0.9 ± 0.1	0.3 ± 0.0

Appendix E continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with LPS. In vitro cell cultures were stimulated with LPS from E. coli O111:B4, E. coli O55:B5, S. typhimurium, or S. typhosa at varying concentrations. The cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

24 hours	Salmonella typhosa			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	7.1 ± 0.4	2.2 ± 0.3	0.7 ± 0.1	0.2 ± 0.0
25 μg/ml	6.6 ± 0.3	2.2 ± 0.2	0.8 ± 0.1	0.2 ± 0.1
10 μg/ml	6.5 ± 0.3	2.0 ± 0.1	0.7 ± 0.0	0.3 ± 0.0
1 μg/ml	6.1 ± 0.1	1.8 ± 0.0	0.9 ± 9.2	0.3 ± 0.0
0.1 μg/ml	5.3 ± 0.1	2.1 ± 0.0	0.8 ± 0.1	0.4 ± 0.0
media	5.3 ± 0.0	2.1 ± 0.0	0.9 ± 0.0	0.4 ± 0.0

48 hours	Salmonella typhosa			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	7.6 ± 0.0	4.3 ± 0.2	0.6 ± 0.1	0.4 ± 0.0
25 μg/ml	7.3 ± 0.5	3.8 ± 0.2	0.5 ± 0.0	0.3 ± 0.0
10 μg/ml	7.2 ± 0.2	3.9 ± 0.8	0.7 ± 0.0	0.4 ± 0.1
1 μg/ml	7.2 ± 0.1	3.8 ± 0.3	0.8 ± 0.1	0.4 ± 0.1
0.1 μg/ml	6.3 ± 0.2	3.3 ± 0.1	0.8 ± 0.1	0.4 ± 0.0
media	6.0 ± 0.0	2.3 ± 0.0	0.8 ± 0.0	0.3 ± 0.0

Appendix E continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following stimulation with LPS. In vitro cell cultures were stimulated with LPS from E. coli O111:B4, E. coli O55:B5, S. typhimurium, or S. typhosa at varying concentrations. The cells were incubated for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

72 hours	Salmonella typhosa			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	6.0 ± 0.3	1.8 ± 0.2	0.9 ± 0.1	0.3 ± 0.0
25 μg/ml	6.5 ± 0.5	1.9 ± 0.1	1.2 ± 0.2	0.3 ± 0.0
10 μg/ml	6.9 ± 0.4	2.1 ± 0.1	0.9 ± 0.0	0.3 ± 0.0
1 μg/ml	7.0 ± 0.6	2.0 ± 0.2	1.0 ± 0.0	0.3 ± 0.0
0.1 μg/ml	6.7 ± 0.7	1.9 ± 0.2	0.9 ± 0.1	0.3 ± 0.0
media	6.4 ± 0.5	3.2 ± 0.3	1.0 ± 0.2	0.5 ± 0.1

96 hours	Salmonella typhosa			
LPS concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
50 μg/ml	4.9 ± 0.0	1.4 ± 0.0	0.7 ± 0.1	0.2 ± 0.0
25 μg/ml	6.0 ± 0.0	1.9 ± 0.1	0.7 ± 0.1	0.2 ± 0.0
10 μg/ml	6.3 ± 0.1	1.9 ± 0.0	0.5 ± 0.0	0.2 ± 0.0
1 μg/ml	6.9 ± 0.5	2.1 ± 0.2	0.6 ± 0.0	0.2 ± 0.0
0.1 μg/ml	7.2 ± 0.0	2.2 ± 0.1	0.8 ± 0.0	0.2 ± 0.0
media	6.6 ± 0.4	2.3 ± 0.0	0.9 ± 0.1	0.3 ± 0.0

Appendix F: Percent and number ($x10^4$) of proliferating and apoptotic cells following treatment with dexamethasone (dex). In vitro cell cultres were treated with varying concentrations of dex for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

24 hours				
Dex concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
10^{-10} M	7.4 ± 0.9	2.2 ± 0.1	3.9 ±0.0	1.2 ± 0.1
10^{-9} M	11.5 ± 0.2	4.7 ± 1.1	3.0 ± 0.2	1.2 ± 0.4
10^{-8} M	13.4 ± 3.0	3.7 ± 1.0	3.4 ± 0.6	0.9 ± 0.1
10^{-7} M	11.9 ± 0.9	2.8 ± 0.0	3.1 ± 0.6	0.7 ± 0.1
10^{-6} M	13.5 ± 2.2	3.1 ± 0.6	4.2 ± 0.1	1.0 ± 0.0
10^{-5} M	14.1 ± 1.6	3.0 ± 0.8	3.8 ± 0.3	0.8 ± 0.1
media	11.6 ± 0.1	2.4 ± 0.3	4.3 ± 0.0	0.9 ± 0.1

48 hours				
Dex concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
10^{-10} M	8.3 ± 0.7	2.4 ± 0.2	2.7 ± 0.4	0.8 ± 0.1
10^{-9} M	12.2 ± 0.3	3.2 ± 0.1	2.7 ± 0.4	0.7 ± 0.1
10^{-8} M	14.1 ± 1.1	3.3 ± 0.2	2.3 ± 0.2	0.5 ± 0.1
10^{-7} M	22.5 ± 4.5	4.5 ± 0.9	4.4 ± 1.1	0.6 ± 0.2
10^{-6} M	26.0 ± 0.6	5.2 ± 0.2	3.6 ± 0.9	0.6 ± 0.2
10^{-5} M	25.8 ± 0.5	4.6 ± 0.1	4.2 ± 0.1	0.8 ± 0.0
media	17.1 ± 0.3	2.9 ± 0.1	3.6 ± 0.4	0.7 ± 0.1

Appendix F continued: Percent and number ($x10^4$) of proliferating and apoptotic cells following treatment with dexamethasone (dex). In vitro cell cultres were treated with varying concentrations of dex for 24, 48, 72, or 96 hours. Following incubation, the cells were washed, stained with propidium iodide, and analyzed by flow cytometry. Means ± SEM are shown.

72 hours				
Dex concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
10^{-10} M	10.8 ± 0.3	3.7 ± 0.2	2.3 ± 0.4	0.9 ± 0.1
10^{-9} M	12.8 ± 0.1	3.3 ± 0.0	1.5 ± 0.2	0.4 ± 0.1
10^{-8} M	12.0 ± 1.4	2.1 ± 0.5	4.8 ± 2.0	0.6 ± 0.3
10^{-7} M	20.7 ± 0.8	3.2 ± 0.1	4.3 ± 0.4	0.7 ± 0.1
10^{-6} M	25.1 ± 1.5	3.7 ± 0.4	4.4 ± 0.4	0.6 ± 0.1
10^{-5} M	21.2 ± 1.3	2.9 ± 0.5	4.7 ± 0.6	0.7 ± 0.0
media	15.2 ± 1.3	2.3 ± 0.0	5.3 ± 0.2	0.8 ± 0.0

96 hours				
Dex concentration	Percent Apoptotic	Number Apoptotic	Percent Proliferating	Number Proliferating
10^{-10} M	11.9 ± 0.3	3.4 ± 0.0	1.0 ± 0.0	0.3 ± 0.0
10^{-9} M	13.7 ± 0.4	3.4 ± 0.2	0.8 ± 0.1	0.2 ± 0.0
10^{-8} M	15.3 ± 0.5	3.2 ± 0.6	1.5 ± 0.9	0.3 ± 0.1
10^{-7} M	33.1 ± 0.2	6.0 ± 1.3	3.0 ± 0.5	0.6 ± 0.2
10^{-6} M	28.4 ± 3.4	4.9 ± 1.4	3.0 ± 0.4	0.5 ± 0.2
10^{-5} M	28.6 ± 0.3	5.7 ± 0.1	3.9 ± 0.6	0.8 ± 0.1
media	14.9 ± 1.1	2.1 ± 0.2	5.7 ± 0.6	0.8 ± 0.1