

AN ABSTRACT OF THE THESIS OF

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Abstract approved: _____


Anthony T. Vella

T cells are one of the key cells in the immune system. Although they are not the first line of defense against a pathogen, their functions can greatly enhance the phagocytosis and destruction of pathogens as well as the development of antibody responses. Furthermore, even when responding T cells have facilitated the clearance of the pathogen, they can avoid death to become long-lived cells that “remember” encountering the pathogen for years afterward. This long-term memory allows subsequent immune responses to improve with each exposure, ultimately preventing disease upon reinfection. The activation of these T cells depends on specific recognition of antigen along with a costimulatory signal. This activation process is well studied, but not completely understood. Additionally, the mechanism behind memory T cell development is still very much unknown. In the work presented in this thesis, delivery of costimulatory signals via CD40 and OX40 were studied using an in vivo superantigen (SAg) model of T cell stimulation. In the context of this two-signal (SAg + costimulation) model, both CD40 and OX40 could deliver signals that enhanced SAg-reactive T cell clonal expansion, but they

could only partially prevent T cell death. Coadministration of the inflammatory agent lipopolysaccharide (LPS), however, could keep increased responder T cell populations alive for at least two months. Interestingly, this three-signal (S_{Ag} + costimulation + LPS) induced survival was not dependent on proinflammatory cytokines or activation of the transcription factor NF- κ B, but was sensitive to the immunosuppressant cyclosporin A (CsA). The mode of action of CsA may point to the mechanism driving long-term T cell survival. Additionally, examination of early time points after three-signal stimulation suggested more clues to the mechanism of survival induction. The cytokines IL-2 and TNF- α seem to be involved early on, but for now, little is known about their complete role. Thus, the goal of this work was to investigate the costimulatory and adjuvant-mediated signals required for memory T cell development. Ultimately, an understanding of how memory T cells can be generated could be used to enhance vaccine efficacy or shut off autoimmune conditions.

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The Role of Costimulation and Adjuvants in the Development of T Cell Effector
and Memory Responses

by
Joseph R. Maxwell

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CONTRIBUTION OF AUTHORS

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THE ROLE OF COSTIMULATION AND ADJUVANTS IN THE DEVELOPMENT OF T CELL EFFECTOR AND MEMORY RESPONSES

INTRODUCTION AND BACKGROUND

The concept of immunity has been understood by various civilizations for thousands of years. As far back as ancient Greece, the protection that illness can have on reinfection was known (1). The modern science of immunology, however, really began to develop after the initial realization by Jenner in the late 1700s that exposure to cowpox could prevent the development of smallpox, a very serious and often life-threatening condition up until the late 20th century. This led ultimately to the development of modern vaccine technology and the complete eradication of smallpox from the planet. Many other diseases have been controlled by the administration of vaccines, yet what is perhaps most ironic is that although the idea behind vaccination has been around for quite some time, and has led to some truly astonishing accomplishments, humans still do not fully understand how vaccines work.

The immune system is composed of a generalized non-specific innate response and a highly specific adaptive response, the hallmark of which is memory (2). Memory refers to the ability of an organism's immune system to "remember" encountering a pathogen (3, 4). The initial immune response to an invader may be relatively slow, yet effective; however, upon reexposure to the pathogen, the

immune system will respond much better and much faster, ultimately clearing the infection with no or minor symptoms of disease.

By exposing the immune system to debilitated pathogens and the molecules they express, vaccines take advantage of this very important aspect of adaptive immunity called memory. Currently, there is much interest in learning how to generate memory responses. Ideally, vaccines that induce a better memory response would lead to better protection against infection. In order to manipulate the immune system in this way, however, it is important to understand which cells constitute the immune system and how those cells interact with each other. The primary cells of interest: the antigen presenting cell (APC) and the T cell.

THE ANTIGEN PRESENTING CELL

APCs do exactly what their name implies. They present antigens on their surface, and the cells that recognize these presented antigens are T cells. An antigen, loosely defined, is any molecule that can elicit an immune response. For a B cell, an antigen would be anything that the antibody surface receptor recognizes. For a T cell, an antigen is usually a short peptide that can be bound to molecules of the major histocompatibility complex (MHC) and then presented to the T cell.

The MHC is a collection of many genes each of which possess many alleles, thus APCs contain great diversity in the MHC molecules they express (5, 6). The primary function of MHC is to present small portions of proteins and other molecules to T cells. There are two kinds of MHC molecules, termed class I and class II (Fig. 1.1) (7, 8). MHC class I is a heterodimer of an α -chain non-

covalently attached to β_2 -microglobulin. MHC class II is composed of an α - and a β - chain.

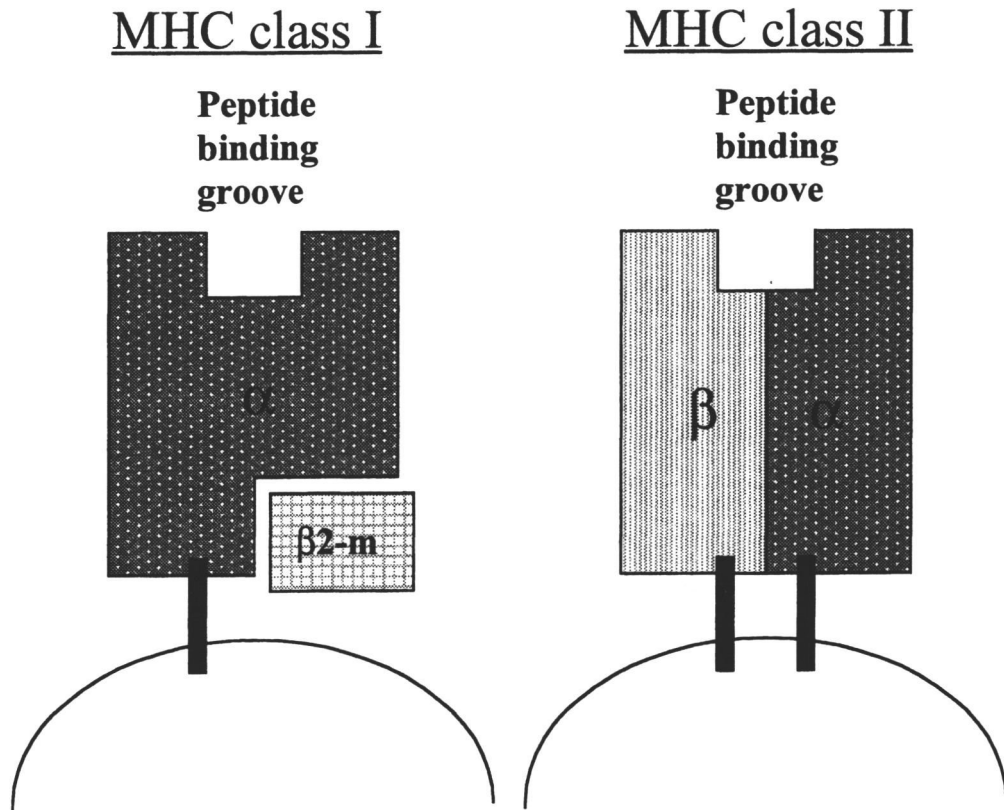


Figure 1.1: General structure of MHC molecules. Class I MHC consists of an α -chain non-covalently bound to β_2 -microglobulin (β_2 -m) while class II MHC consists of an α -chain and a β -chain.

Both MHC molecules form an antigen-binding cleft (9, 10). This cleft is composed of two parallel α -helices positioned above an antiparallel β -pleated sheet. The α -helices serve as the walls of the cleft, while the β -sheet serves as its floor. Proteins are digested within the APC into short peptides that are bound within the peptide-binding cleft of either class I or class II MHC (11). The peptides

that bind to a particular MHC molecule may have different sequences, but will possess identical amino acid anchor residues that serve to lock them in the groove. The general rule of thumb, although not absolute, is that peptides generated from intracellular sources such as a viral infection are presented by MHC class I, while extracellular proteins are presented by class II (12). These differences are important for influencing the proper T cell response for fighting off the pathogen.

There are three main cells that function as APCs for T cells. Each of these cells has different importance and function within the immune system. By enhancing a T cell response, each cell can serve to enhance its own activity.

Dendritic cells are considered by many immunologists to be the premier APC, often being referred to as “professional” APCs (13, 14). Their ability to capture many antigens and present them for T cell stimulation is unmatched (15, 16). These cells are often identified as having long branched processes that extend from the cell body. These processes can house many MHC/peptide complexes and can allow for effective scanning of these complexes by T cells (17). Many of these dendritic cells are found in the lymphoid tissues where T cells and B cells are stimulated. Generally, these dendritic cells are initially found in peripheral tissues where they are referred to as Langerhans cells. Langerhans cells phagocytose antigens and infectious agents and then migrate to the lymphoid tissues for T cell presentation (18). Overall, the main function of these cells is to present antigens.

Macrophages are phagocytic cells that express many receptors on their surface that recognize various bacterial structures or dying cells (19-22). These

receptors can assist the macrophage in binding to the cell and internalizing it in a phagosome (23). Once a macrophage has engulfed a pathogen or infected/dying cell, it will destroy it by dumping lysosomal contents into the phagosome. Besides destroying the pathogen, another outcome of receptor binding and phagocytosis is cytokine secretion. Activated macrophages secrete proinflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-12, IL-6, IL-15 and IL-1 β (24, 25). These cytokines serve to recruit and activate other phagocytic and innate cells into the infected area to help fight the infection. Additionally, internalization and presentation of protein antigens allows the macrophage to function as an APC.

B cells, like T cells, are part of the adaptive immune system. B cells express a Y-shaped antigen receptor called an antibody on their surface which can be secreted (26, 27). Each arm of the antibody recognizes the same folded pattern or linear sequence on an antigen called an epitope, thus allowing an antibody to bind two epitopes that it is specific for. This region of the antibody is termed the variable region because different highly variable combinations of protein chains can come together to form it (Fig. 1.2). Additionally, each of these variable combinations can further mutate during an immune response to allow for increased specificity toward the antigen (28, 29).

The base of the antibody molecule is termed the constant region and possesses the effector function of the antibody (Fig. 1.2). This portion has little variability and can be made up of five different protein chains that yield an antibody with an IgM, IgD, IgG, IgA, or IgE isotype.

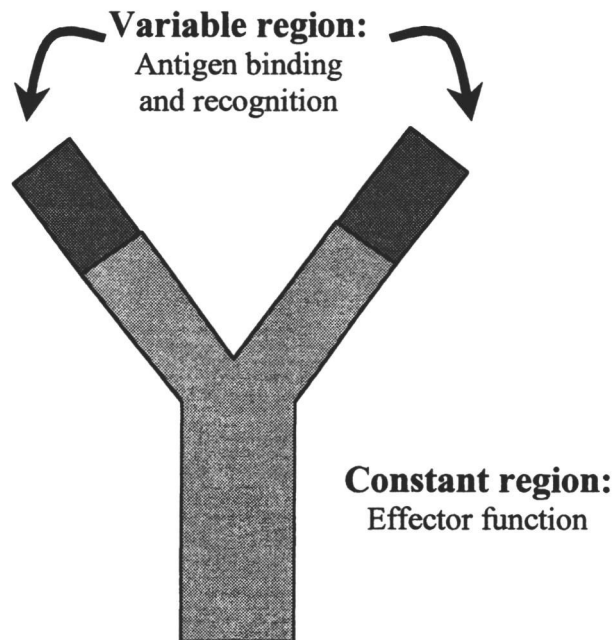


Figure 1.2: Antibody structure. The variable region at the end of each arm of the antibody is the antigen binding site. Effector functions induced by the antibody molecule are triggered by cell surface receptors binding to the constant region.

Each isotype performs some or all of three main functions. *Neutralization* is the ability of the antibody to bind to and neutralize receptors or ligands on the surface of pathogens that are important for their function such as entry into cells or binding of effector molecules (30). *Opsonization* occurs when an antibody binds to a pathogen and facilitates its engulfment and destruction by a phagocytic cell such as a macrophage (23). Finally, antibody binding can promote *complement activation*, an innate defense mechanism that ultimately leads to opsonization or direct lysis of the cell (31). Additionally, antigen binding to antibody on the surface of a B cell will be internalized and presented on the B cell surface. Thus, like dendritic cells and macrophages, B cells serve as APCs.

THE T CELL

T cells are the other component of the adaptive immune system that also display the enhanced function upon reexposure to a particular antigen that is typical of a memory response (3, 4). The T cell antigen receptor (TCR) is composed of an α -chain and a β -chain (Fig. 1.3) (32, 33). In a fashion that is similar to an antibody, different combinations of α - and β -chains can be paired together to produce a broad range of variable region specificities. Unlike an antibody, however, the TCR does not possess dual binding sites for antigens and it is not secreted. Neither does the TCR mutate to increase affinity for the antigen. The main factor that appears to contribute to a memory T cell's enhanced responses is its activation requirements, which may be less restricted than a naïve T cell, allowing for faster activation and effector function.

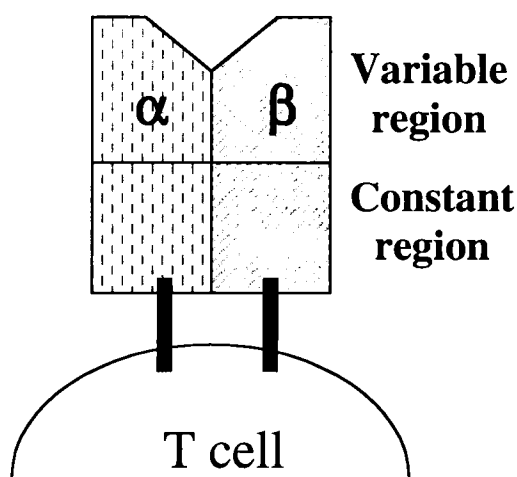


Figure 1.3: Structure of the T cell receptor. Both α - and β -chains form a variable and constant region. The variable portion of the β -chain is the site of superantigen binding.

THE T CELL

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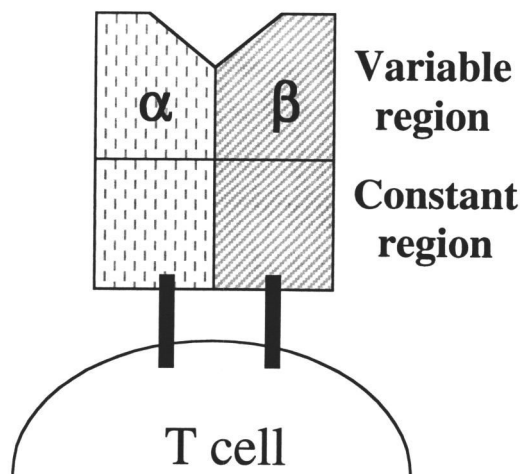


Figure 1.3: Structure of the T cell receptor. Both α - and β -chains form a variable and constant region. The variable portion of the β -chain is the site of superantigen binding.

Every mature T cell in the adaptive immune system expresses one of two coreceptors called CD4 or CD8. These coreceptors interact with the TCR to promote activation of the T cell under appropriate conditions (34, 35). T cells expressing each coreceptor have differing functions (36, 37).

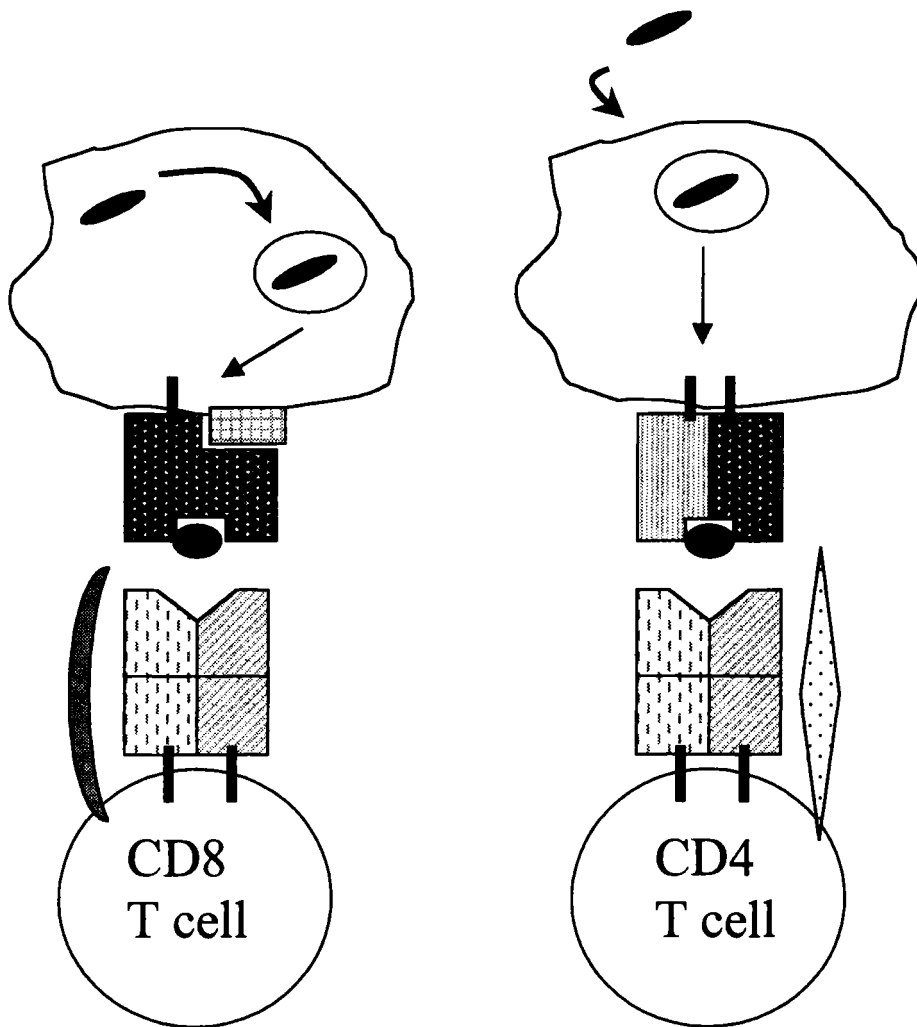
CD8 T cells are cytotoxic T cells that play a role in killing pathogen-infected cells. They do this either by inducing a programmed cell death pathway known as apoptosis via ligation of Fas on the surface of the target cell, or by releasing granular contents into the cell such as perforin and granzyme (38).

CD4 T cells are known as the helper T cells. They assist other immune cells in the activation process. CD4 T cells are essential for further activating macrophages, or in the primary induction of CD8 and B cell function (39, 40). Without CD4 T cells many immune responses are impaired. Thus, these T cells are important not only for induction of an adaptive immune response, but also for fueling the innate response.

The activation of T cells is an essential part of any adaptive immune response (Fig. 1.4). This activation process is intimately dependent on cells expressing the appropriate MHC molecule. Antigens presented by the class I pathway are recognized by CD8 T cells while antigens presented by the class II pathway are recognized by CD4 T cells (41, 42). This makes sense in that class I antigens are typically derived from intracellular pathogens such as viruses. To prevent the spread of a viral infection, the best mechanism the immune system has is to kill the infected cells. CD8 T cells mediate this process. Additionally, the

Intracellular pathogen

Extracellular pathogen



Cytotoxic cell

Kill infected cells

Helper cell

Assist antibody production
Assist macrophage activation

Figure 1.4: Antigen processing and presentation to T cells. Intracellular pathogens are presented by class I MHC to CD8 T cells that can kill infected cells. Extracellular pathogens are presented by class II MHC to CD4 T cells that assist in the function of other cells.

rather ubiquitous expression of MHC class I molecules means that nearly every cell in the body can be destroyed by CD8 T cells if infected. Alternatively, class II MHC generally presents antigens from extracellular pathogens. This stimulates CD4 T cells, which activate B cells to produce antibodies that can bind up pathogens in the serum or peripheral tissues. Additionally, CD4 T cells augment the activity of macrophages to further enhance the ability of these innate cells to phagocytose and destroy extracellular pathogens. The expression of MHC class II is quite restricted, being limited primarily to APCs. This prevents unwanted antibody and inflammatory responses directed against normal host cells. Thus, the central role of T cells in the immune system is obvious. The primary question is how are these T cells activated?

T CELL ACTIVATION

Within a host organism's body, MHC can just as easily present self-antigens to T cells as foreign molecules. It has been established for quite some time that antigens expressed early in development are tolerated by the immune system, while those arising later can lead to an immune response (43).

Contributions by Bevan and Zinkernagel showed that T cells are restricted in their responses by the MHC molecules they recognize (44-46). Only T cells that recognize antigens presented by self-MHC molecules with a moderate affinity will live, a process in the thymus now known as positive selection. Additionally, further work showed that if a T cell recognizes self-antigens presented by MHC in the thymus with a very high affinity, that T cell will be deleted by negative

selection (47, 48). Only if a T cell can recognize self-MHC, but not self-peptides complexed to it, will it reach full maturity and be released into the periphery.

Many self-antigens are presented in the thymus, but not all of them. Various antigens are expressed only at specific times in development or in a tissue-specific manner. Thus, one of the great questions in immunology is how do peripheral T cells know which antigens to attack, and which to ignore (49)?

Peripheral T cell activation is generally agreed upon to require at least two signals. This current *two-signal hypothesis* is founded on an initial model of self-nonself discrimination first developed by Bretscher and Cohn in the late 1960s (50, 51). This model centers on the notion that the immune system is organized to attack anything that is not self. In its initial form, the model described how “antibody-producing cells” were activated. It hypothesized that an antibody-producing cell that bound to antigen via its surface antibody would become paralyzed or inactivated. If, however, another antibody found in the serum or bound to another cell also recognized the same antigen, the dual interaction would induce a structural change in the surface antibody on the producing cell and overcome paralysis in favor of activation.

The model in its current form has been modified into a two-step, two-signal hypothesis that accounts for T cell activation (Fig. 1.5) (52, 53). In the first step, a naive T cell that receives only one signal, the MHC/peptide signal, will be inactivated. If this T cell receives a costimulatory signal constitutively expressed on particular APCs, the T cell will undergo proliferation and develop into a

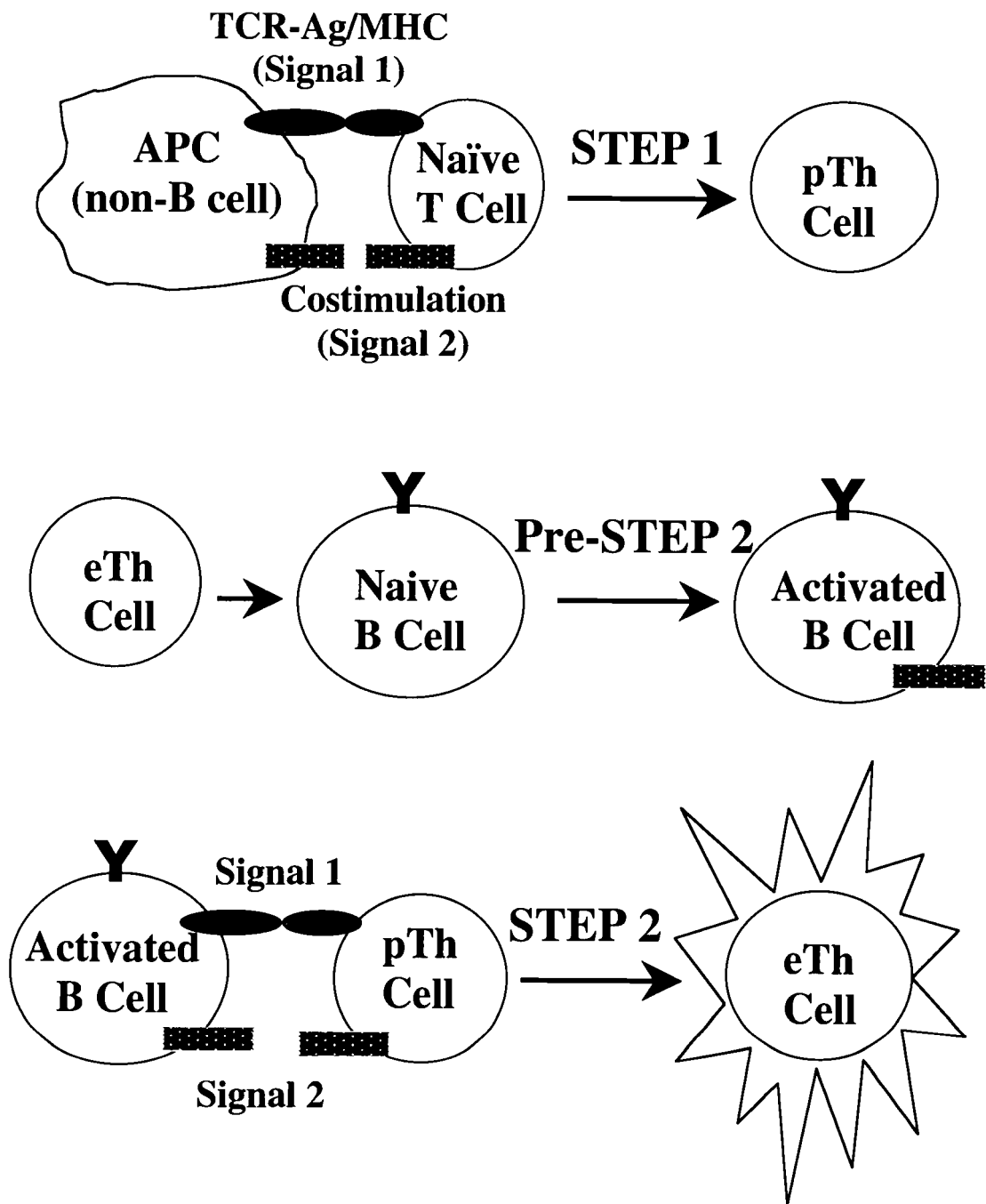


Figure 1.5: Bretscher's two-step, two-signal hypothesis. The model describes how a naïve T helper cell becomes an effector T helper cell (eTh) through a precursor T cell (pTh) intermediate step. The eTh is what drives further T cell, B cell and APC activation. Details are in the text.

precursor T helper cell. In the next step, this precursor T helper cell must further recognize MHC/peptide complexes presented by a B cell that has been activated by a previously formed effector T cell to express costimulatory molecules. Upon such dual stimulation, the precursor T cell can become an effector T cell. Thus a T cell must receive two signals in two different steps in order to become activated. Self-antigens would not be able to generate the effector T cells that are necessary to activate a B cell and thus would not be attacked.

This two step, two signal model, being centered on the immune system distinguishing between self and nonself describes an effective control mechanism for ensuring that the immune system only attacks foreign antigens that both T and B cells recognize, a concept known as linked-recognition. However, its main difficulty is describing how the initial effector T cells arise that can stimulate the B cell to drive further effector T cell development. Bretscher claims that this is due to a spontaneous formation of effector T cells, a process that could very easily lead to autoimmune disease, and that seems to override any exquisite self/nonself controls the body has developed to prevent such an occurrence.

A more current model for T cell activation was laid out by Matzinger in 1994. This theory, called the Danger Theory, was influenced very heavily by Janeway (54) and shifts attention away from self/nonself discrimination, and instead focuses on what is dangerous (14, 55). According to the Danger Theory, any self- or nonself-antigen can activate the immune system as long as it is seen as dangerous. The model itself does not make clear what these “danger signals”

specifically are, but in terms of T cell activation, it describes the process well.

Here, two signals are again required for a T cell response. The first signal is the MHC/peptide binding to the TCR. The second signal is a costimulatory signal that is induced on APCs when they sense some level of danger.

According to the Danger Theory, only APCs can activate a T cell.

Peripheral tissues cannot stimulate a T cell because although they express MHC, they do not express many costimulatory molecules. Thus self-antigens, primarily those expressed in a tissue-specific fashion, will usually tolerize T cells to the antigen because they will be expressed by MHC in the absence of costimulation. During an infection, some autoreactive T cells may become activated by encountering self-antigen/MHC on activated APCs expressing costimulatory molecules. Matzinger believes that activated T cells have a short lifespan, where after a short time, they will either die or revert back to a resting state. This gives cells directed against a foreign molecule time to perform their functions, but keeps both their activity and the activity of T cells directed against self-molecules under control so they do not get out of hand. After the response has waned, foreign antigen-specific T cells will die or remain in a resting state, while residual self antigen-specific T cells will be tolerized to their antigen expressed on self tissues in the absence of costimulation.

It is important to note that not all antigens need to be presented by MHC to stimulate a T cell. Superantigens are a special class of antigen that stimulate a polyclonal population of T cells based solely on the variable β -chain they express

on their TCR (Fig. 1.3) (56). Superantigens also bind MHC class II molecules, yet they can stimulate CD8 T cells as well as CD4 T cells because of their β -chain specificity. Such binding stimulates all T cells expressing the appropriate TCR to proliferate (57). After the T cell population clonally expands for a couple of days, most of the responding cells die off. Despite a “non-classical” antigenic mechanism of T cell stimulation, superantigens also rely on costimulatory molecules to induce T cell activation.

Thus, costimulation is believed to be at the heart of an adaptive immune response. Without this important signal, T cells cannot be efficiently activated. Without T cells, B cell function and many innate functions will not be enhanced. The nature of costimulation bears some discussion.

COSTIMULATORY SIGNALS

The requirement for some accessory signal for T cell activation was first described in the mid-1980s. In a seminal work, Jenkins and Schwartz showed that APCs that were chemically treated, yet which could present antigen, caused inactivation of T cells in vitro, and to some degree in vivo (58). They suggested that some additional signal must be necessary to activate a T cell. Thus, the two-signal hypothesis began to be applied to T cell responses and the search went out for what these costimulatory molecules were. There are many surface molecules that are known to be costimulatory (59). CD28, CD40 and OX40 are three of the most prominent costimulatory molecules.

The CD28/B7 interaction

CD28 is the prototypical T cell costimulatory molecule (60). Its role in T cell costimulation was first hinted at by studies which found that administration of an antagonistic anti-CD28 monoclonal antibody (mAb) inhibited T cell responses in vitro (61, 62). Subsequent work found that signaling via CD28 on the T cell stabilized mRNAs of many T cell cytokines (63). Further work ultimately showed that costimulation via CD28 could enhance IL-2 production in T cells, preventing tolerance induction in favor of proliferation (64-66).

Increasing knowledge about the importance of CD28 in T cell responses fueled a search for its natural ligand, B7. B7 was initially identified as a B cell marker, but it was not until about ten years later that it was fully recognized as the ligand for CD28 (67, 68). B7 was found to be expressed on dendritic cells, monocytes and B cells (69-71). Blockade of this molecule mimicked CD28 blockade, causing reduced T cell responses and B cell help (39).

Eventually, it was discovered that B7 was actually two related molecules, B7-1 (CD80) and B7-2 (CD86) (72). Both of these molecules can costimulate T cells but have somewhat different expression patterns (73, 74). B7-2 is more constitutively expressed, while B7-1 tends to be upregulated after activating stimuli. Some studies suggest that signals delivered through B7-1 or B7-2 can yield different outcomes (75, 76), but there has been no consensus on any contrasting functions of these molecules (77). What is certain is that both B7-1 and B7-2 costimulate T cells.

Finally, a molecule related to CD28, named cytotoxic T lymphocyte antigen (CTLA)-4, was also shown to bind B7, but with much higher affinity (78). Its functions were initially thought to kill cells or shut off T cell responses (79). Current research suggests that CTLA-4 may actually activate regulatory cells (80). The high affinity of CTLA-4 for B7 molecules means that it can outcompete CD28 for binding and thus inhibit costimulation. This fact has proven useful for the treatment of diseases and for transplant rejection (81).

The CD40/CD40L interaction

CD40 is a costimulatory molecule that belongs to the tumor necrosis factor receptor (TNFR) family (82-84). This family consists of a growing number of proteins that are important for signaling cellular death such as Fas and TNFR, or activation such as OX40 and 4-1BB (85). Expression of CD40 is primarily on APCs.

The natural ligand for CD40 is CD40L (CD154, gp139) (86). CD40L is found mainly on activated CD4 T cells whose ability to function as helpers for B cell antibody production is heavily dependent the CD40/CD40L interaction (87). This dependence is manifested most visibly in people with X-linked hyper IgM syndrome (88). Patients afflicted with this condition lack functional CD40L, and as a result have defective B cell responses, culminating in elevated serum levels of IgM, but very little antibodies of other isotypes. Additionally, the dependence of CD8 T cell activation on CD4 T cell help has been suggested to result from the

CD4 T cells stimulating CD40 on the APC. Such stimulation can make the APC more effective at costimulating the CD8 T cell (40, 89, 90).

CD40L is generally expressed as a multimeric complex on the surface of a cell, but can be released in a shorter soluble form that may act as a cytokine (91). CD40L expression on T cells is stabilized by CD28 stimulation, and under chronic inflammatory conditions can be detected in large amounts in affected tissues (92). Thus, this interaction is a critical component of T cell-APC interactions.

Activation of APCs via CD40 triggers many responses (84). Surface expression of B7-1 and B7-2 are elevated which facilitates better T cell costimulation (93). Many cytokines are secreted that are important for the activation, migration and differentiation of T cells, and other APCs in the vicinity. Nitric oxide is produced which can activate macrophages and facilitate bacterial destruction. Additionally, apoptosis, or programmed cell death, of the APCs is inhibited after ligation of CD40 (94, 95).

Although T cells can benefit from the enhanced costimulatory capacity and cytokine secretion of the APCs, they also may benefit from direct signals through CD40L itself (96). The intracellular signaling pathway of CD40L on T cells is poorly understood, but it is well documented that in the absence of this signaling, T cell activation and memory development are impaired (97, 98).

The OX40/OX40L interaction

OX40 is another costimulatory molecule that, like CD40, is a member of the TNFR family (99). OX40 is expressed transiently after activation of CD4 T

cells, and to a lesser extent, CD8 T cells (100). Enhanced OX40 expression is facilitated by CD28 ligation, again showing the importance of CD28 in T cell responses. Furthermore, the costimulatory ability of OX40 on its own is rather weak in the absence of B7 expression (101). Thus, the combination of OX40 and CD28 signals appears to greatly enhance T cell activation over either one alone.

OX40 ligand (OX40L) is found on activated APCs in low levels (102). Expression of OX40L can be enhanced by CD40 stimulation of the APC (103). This, coupled with the ability of CD40 to enhance B7 expression on APCs further enhances T cell activation. Although OX40 can be expressed in quite high levels on T cells, OX40L expression, even after APC stimulation is often still somewhat low. Thus, availability of OX40L is often considered the limiting factor in T cell responses via this receptor-ligand interaction (104).

OX40 is a molecule that has long been established as a marker for antigen-specific T cells in a number of autoimmune diseases such as inflammatory bowel disease and experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (105, 106). In inflamed tissues, the autoreactive T cells are “marked” by the fact that they express OX40 (107). In EAE, the myelin-specific T cells express large amounts of OX40, while naïve or non-reactive T cells do not. With this knowledge, Weinberg and colleagues linked the toxic compound ricin to a mAb that recognized OX40 (108). Thus, the autoreactive T cells that were expressing OX40 would bind the antibody and be killed by the ricin. This strategy

alleviated EAE in mice and has shown how OX40 may be useful in combating inflammatory and autoimmune conditions.

OX40 has also been suggested to be important in T cell memory responses (109). Studies done with OX40-deficient mice found that the deficient T cells survived poorly during their initial expansion phase and could not generate a substantial population of memory cells. This was partly attributed to the ability of OX40 to enhance T cell proliferation. If more cells accumulate, there are more cells that may survive. OX40 was reasoned to enhance the expansion phase and assist in the long-term survival of those T cells.

Being that OX40 is so prevalent on T cells in inflamed tissues, it is not surprising that its expression is tightly controlled. Naïve T cells do not express OX40, and when they become activated, the expression is transient, and the response limited by the amount of OX40L on the APCs. Thus, this molecule is also viewed as very important to the immune system. Its binding can enhance T cell responses that contribute to assisting the responses of other cells, while its deregulation could potentially be deadly.

TRANSCRIPTION FACTORS IN T CELL ACTIVATION

In the midst of all of these costimulatory signals, there is another important aspect to T cell activation. All of the costimulatory molecules that affect T cell responses must activate the transcription of specific genes. Two of the most important transcription factors in the immune system are NF- κ B and NFAT.

The NF- κ B pathway

Nuclear factor-kappa binding (NF- κ B) was initially discovered as a protein complex that bound to the enhancer region of the kappa light chain gene in B cells (110, 111). Since then, its integral role in the functions of all hematopoietic cells has been well established.

NF- κ B is a dimeric molecule formed by the interaction of Rel family proteins (112-114). There are five proteins in the Rel family: NF- κ B1 (p105/p50), NF- κ B2 (p100/p52), c-Rel, RelA (p65) and RelB. Different combinations of these proteins yield different NF- κ B dimers whose functions may not be entirely redundant. Many transgenic and gene knockout studies suggest that each subunit may have specific functions (115).

The activation of NF- κ B is somewhat complex, but demonstrates a remarkable control system over one of the immune system's most versatile transcription factors (Fig. 1.6). NF- κ B is normally sequestered in the cytoplasm complexed to a repressor called I κ B (116). During some stimulus, a kinase phosphorylates I κ B, facilitating the ubiquitination of this repressor and its subsequent degradation (117, 118). Once NF- κ B is released from I κ B, it translocates into the nucleus where it activates many genes. Cytokine genes such as TNF- α and IL-6 are transcribed by NF- κ B, as are many survival genes such as Bcl-x_L, A1 and A20 (119-121). Additionally, more I κ B is transcribed which will serve to resequester NF- κ B and shut off transcription (122).

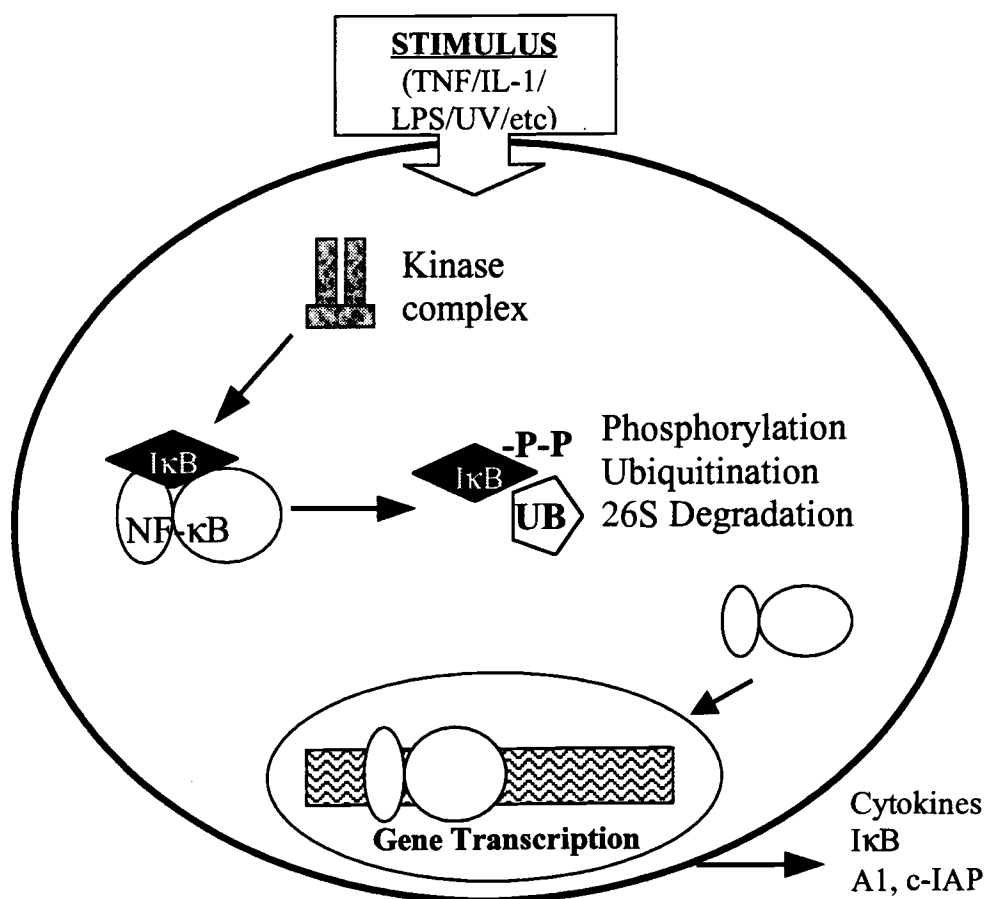


Figure 1.6: The NF-κB activation pathway. Stimuli that degrade the repressor IκB facilitate NF-κB nuclear translocation and DNA transcription. Details are in the text.

The NFAT pathway

Nuclear factor of activated T cells (NFAT) is another important transcription factor, that although first discovered in T cells, is not exclusively expressed there (123). The NFAT family of proteins consists of four main groups of proteins termed NFAT1 through NFAT4, each of which has multiple isoforms. These proteins are primarily activated by a calcium-dependent pathway that is susceptible to inhibition by the immunosuppressive drug cyclosporin A (124, 125).

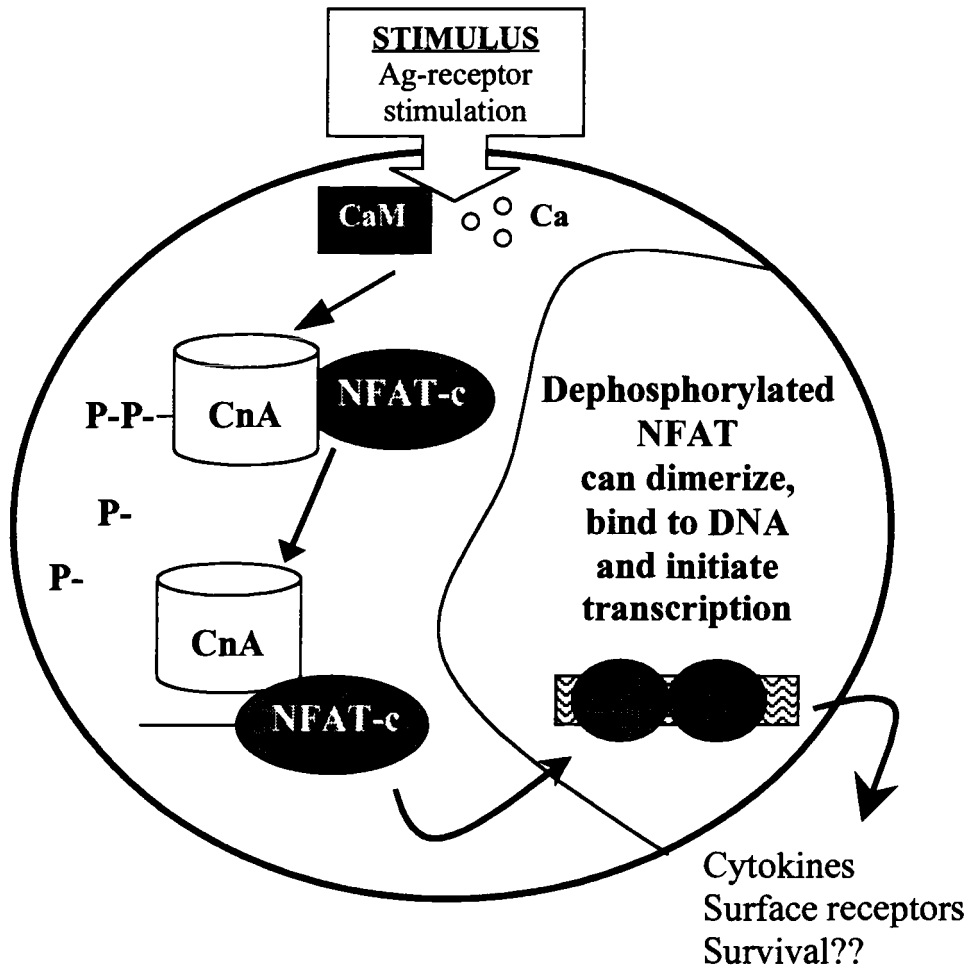


Figure 1.7: The NFAT activation pathway. Calcium (Ca) stimulates calmodulin (CaM) to activate calcineurin (CnA). Active CnA dephosphorylates NFAT, which can translocate to the nucleus and activate transcription. More details in the text.

The activation of NFAT (Fig. 1.7) is similar to that of NF- κ B in that both transcription factors are sequestered in the cytoplasm. The cytoplasmic form of NFAT, NFAT-c, is sequestered by complexing to calcineurin and other proteins (126). Specific phosphorylated residues on NFAT-c help to mask the nuclear localization sequence. During TCR or B cell antigen receptor stimulation, intracellular calcium stores are released. This calcium binds to calmodulin, which

activates calcineurin. Activated calcineurin dephosphorylates NFAT-c, which exposes the nuclear localization sequence and residues necessary for DNA binding, and allows for translocation of NFAT-c to the nucleus where it can dimerize with a nuclear component (NFAT-n) or AP-1 family members to activate transcription (127, 128). Transcription by NFAT is important for cytokine and cell surface receptor production (123). Specific cellular survival signals induced by NFAT are not well characterized, but may involve Bcl-2 family members (129).

DANGER, ADJUVANTS AND THE THIRD SIGNAL

One of the major influences of Matzinger's Danger Theory was Janeway's notion that bacterial molecules are required to activate a T cell response (130). This stimulus was considered something dangerous because microbial products could induce an immune response against an antigen that alone would stimulate only a weak response. These microbial products are important constituents of adjuvants.

Adjuvants are compounds that Janeway termed "an immunologist's dirty little secret (54)." These are compounds that can greatly enhance T cell activation and generate increased levels of long-term survival. Most adjuvants contain a bacterial component. Complete Freund's Adjuvant (CFA), for example, is killed mycobacterium immersed in oil. Just how, exactly, adjuvants enhance immunity is unknown, but may be the key to T cell memory.

Lipopolysaccharide (LPS) is another very potent adjuvant (131, 132). LPS is a normal component of Gram-negative bacterial outer membranes and is released

upon bacterial lysis, hence its alternative name, endotoxin. LPS consists of an O-specific chain, a core region and lipid A. The core region serves primarily as the linker between the highly variable O-specific chain and the more conserved lipid A structure. Most of the toxic effects of LPS result from the activity of lipid A, as synthetic lipid A can mediate identical responses as LPS (133).

LPS is bound by CD14 on the surface of macrophages (134). Other cells that do not express high levels of CD14 such as B cells may still respond to LPS via the interaction of soluble CD14 and LPS-binding protein in the serum. The confusing aspect of LPS/CD14 signaling is that CD14 does not have intracellular signaling capabilities, thus it was reasoned that it must interact with some other receptor to deliver an LPS-induced signal into the cell. This other receptor was recently discovered and named the Toll-like receptor (TLR) (135, 136). TLRs have the ability to bind LPS directly or to interact with CD14 to signal when LPS has been bound.

The effects of LPS are primarily on APCs. LPS is a very potent inflammatory agent and inducer of APC activation. LPS can induce proliferation, cytokine production and adhesion molecule expression on APCs (131, 137). Knowledge of its effects on T cells has been rather limited. Studies by Sprent and colleagues showed that LPS could induce non-specific T cell proliferation but that TLR expression on APCs was necessary for the response (138). Other studies, however, showed that antigen-specific T cell survival could be enhanced by LPS treatment in vivo (139). The primary result of this work suggested that LPS could

keep activated T cells alive for a long time in vivo. With this study, the means to study T cell memory development improved.

The work presented in this dissertation serves to better understand the requirements that T cells need to undergo an optimal response. This optimal response not only entails activation, but long-term survival as well. The idea that three signals (antigen, costimulation and danger) are important for T cell immunity is central to this work. Additionally, the studies presented herein raise many questions about how LPS and adjuvants signal within the immune system.

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Chapter 2

CD40 Activation Boosts T Cell Immunity In Vivo by Enhancing T Cell Clonal Expansion and Delaying Peripheral T Cell Deletion

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ABSTRACT FOR CHAPTER 2

In this report we show that activation of APCs with an agonist anti-CD40 mAb profoundly alters the behavior of CD4 T cells in vivo. Stimulation of mice with anti-CD40 2 days before, but not 1 day after, administration of superantigen (SAg) enhanced CD4 and CD8 T cell clonal expansion by approximately threefold. Further, CD40 activation also delayed peripheral T cell deletion after activation. Dying, activated T cells were quantitated by detecting extracellular phosphatidylserine with concomitant staining for SAg-reactive T cells using a TCR V β -specific mAb. Upon close examination, it was shown that CD40 activation delayed the death of the activated T cells. Additionally, it was found that enhanced survival of CD4 T cells was equally dependent on APC expression of B7-1 and B7-2. This is in contrast to CD8 T cells, which did not depend as much on B7-1 as B7-2. Thus, CD40 activation indirectly promotes T cell growth and delays the death of SAg-stimulated CD4 T cells in vivo. These data suggest that one way CD40 activation promotes a more robust immune response is by indirectly increasing the production of effector T cells and by keeping them alive for longer periods of time.

INTRODUCTION

The immune system functions through a complex network of signals. This complexity is most apparent when generating a productive immune response. For an optimal T cell response, many checkpoints must be passed. At minimum, delivery of two signals is required to activate T lymphocytes against an Ag (1, 2). The first signal involves engagement of the TCR by peptides presented by the MHC molecules. The second signal provides costimulation and involves ligation of another receptor on the T cell surface (3, 4). This second signal is generally described as CD28 on T cells and its ligand B7-1 (CD80) or B7-2 (CD86) on APCs (5-10).

To mount an optimal T cell response against Ag, it is thought that APC deliver both signals to T cells. These two signals activate T cells and drive T cell clonal expansion. As a result, many T cells are generated, and, of those, many become effector T cells (11). Normally, non-APCs do not bear the B7 molecules and thus cannot deliver signal two (12). Under such conditions, based on in vitro data with T cell clones, delivery of signal one without signal two does not activate the T cell fully, but instead directs it to a non-responsive state known as anergy (3, 13). Perhaps surprisingly, little is known about T cell activation in vivo. In part this is due to the complexity of APC/T cell interactions and the vast number of signals each cell can deliver to each other. These include not only “costimulatory” signals but also the variety of cytokines that are secreted.

Although in an optimal T cell response clonal expansion is important for fighting pathogens, just as important is the ability to down-regulate the T cell response after the pathogen is cleared. T cell populations must decline in number or else autoimmunity and immunopathology can occur (14, 15). Some cells must survive, however, if T cell memory is to develop. Thus, a complex balance must be maintained between sustained immunity and tolerance in the immune system.

Efficient monitoring of the T cell response, both expansion and deletion, has been performed with the use of staphylococcal enterotoxin A (SEA). SEA is a SA_g that binds MHC class II and selectively engages all TCRs that contain the V β 3 chain (16-20). Injection of B10.Br mice with SEA promotes the clonal expansion of CD4 and CD8 T cell populations that bear V β 3; however, these cells very soon afterward decrease in number to below normal uninjected levels (21, 22).

In this study, we set out to use the SEA model to investigate a very important molecule whose role in the immune system is only beginning to be made clear: CD40 (23). CD40 is a member of the TNF receptor family (24). It is expressed on all APCs such as B cells (24), macrophages (25) and dendritic cells (26) and has also been reported on T cells (27, 28). CD40 ligand (CD40L, CD154, gp39) is a member of the TNF family as well (29, 30). It is expressed mainly on CD4 T cells (31) but is also found on CD8 T cells (31-33) as well as other cells such as eosinophils and NK cells (34, 35).

Ligation of CD40 has been shown to yield many effects on APCs. Several groups have shown that CD40 ligation can enhance the costimulatory abilities of

APCs (36-38). In fact, other studies have shown that lack of CD40 ligation can actually promote T cell tolerance as a result of poor B7 expression (39, 40). Many groups have investigated the role this tolerance mechanism plays in autoimmunity and transplant rejection. For example, autoimmune conditions such as experimental allergic encephalomyelitis (41, 42), collagen-induced arthritis and insulin-dependent diabetes mellitus (43, 44) are augmented by CD40 activation. Other studies have found that stimulation of CD40 plays a role in graft transplant rejection (45-47).

Effects of CD40 activation on T cell activation have also been reported. For example, IL-12-dependent Th1 differentiation has been shown to be modulated by CD40 activation (38, 48). Most recently, CD40 ligation has been shown to circumvent the requirement for T helper cells in the priming of CTL (49-51). CD8 T cell responses are thus also affected by CD40 ligation. Observed effects include improved antitumor-killing abilities (52, 53) and memory CTL responses (53, 54). Little has been reported on death susceptibility of T cells after CD40 ligation; however, recent reports have shown that CD40 activation of B cells, monocytes and dendritic cells can block apoptosis within these populations (55, 56).

To examine the effects of CD40 stimulation on T cell clonal expansion and deletion upon response to an Ag, an anti-CD40 agonistic mAb was used in conjunction with SEA injection. We found that activation of CD40 not only enhanced CD4 and CD8 T cell clonal expansion but also delayed, but did not prevent, their subsequent deletion. Thus, CD40 enhances an immune response in

vivo by increasing the number of effector T cells and delaying their subsequent death.

MATERIALS AND METHODS

Mice

Female B10.Br/SgSnJ and B10.A/Cr mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and the National Cancer Institute (Frederick, MD), respectively, and maintained in our animal facility under specific pathogen-free conditions. In all experiments, mice between the ages of 6 and 12 wk were used.

Reagents, experimental protocols and Abs

SEA was purchased from Toxin Technology (Madison, WI) and administered to mice as i.p. injections of 0.15 or 0.30 μ g. The anti-CD40-producing hybridoma FGK45.5 was a kind gift from Dr. A. Rolink (Basel Institute, Switzerland) (57). The Ab was purified from hybridoma supernatants over protein G columns (Pharmacia, Piscataway, NJ). As a control, rat IgG (Sigma, St. Louis, MO) was injected in doses equal to the anti-CD40.

In experiments designed to block interactions between CD28 and B7-1/B7-2, CTLA4-Ig (58) or an isotype-matched control chimeric Ab, L6, was injected i.p. at 0.50 mg/injection. These were both kind gifts from Dr. Peter Linsley (Bristol Myers-Squibb, Seattle, WA). Alternatively, Abs directed against B7-1 (16-10A1; ref 59) or B7-2 (GL1; ref 6) were used at doses of 1 mg/injection. These

hybridomas were obtained from ATCC (Manassas, VA), and the resulting Abs were purified separately from hybridoma supernatants over protein G columns (Pharmacia).

Anti-TCR V β 3 (KJ25-607.7; ref 59) and anti-IE^k (14.4.4; ref 60) were purified separately from hybridoma supernatants over protein G columns (Pharmacia). These Abs were FITC conjugated by us. FITC-conjugated annexin V, FITC-conjugated anti-V β 14, PE-conjugated anti-CD4, PE-conjugated streptavidin and biotinylated anti-TCR V β 3 were all purchased from PharMingen (San Diego, CA). PE-conjugated anti-B7-1, PE-conjugated anti-B7-2, PE-conjugated anti-macrophage Ab (F4/80) and PE-conjugated anti-CD45R (B220) were all purchased from Caltag (Burlingame, CA). Red 613-conjugated anti-CD4 and Red 613-conjugated anti-CD8 were purchased from Life Technologies (Grand Island, NY).

Injection Schedule

Injection of SEA was at time 0 h, and all other injections were done in relation to SEA. Injections before SEA injection were designated as negative days, while injections after SEA were designated as positive days. The anti-CD40 Ab was injected on day -2 before SEA unless otherwise stated. Rat IgG was always given to a control group at the same time as anti-CD40 was given to an experimental group. The molecules L6, CTLA4-Ig, anti-B7-1 and anti-B7-2 were all given 2 h before SEA. All injections were i.p.

Cell processing and flow cytometry

Spleens were removed and teased through nylon mesh (Falcon, Becton Dickinson, Franklin Lakes, NJ) and subjected to ammonium chloride to lyse red blood cells. Peripheral LNs (inguinal, axillary and bronchial) were teased into single cell suspensions and washed with balanced salt solution (BSS). T cells from spleen or LN populations were purified on nylon wool columns as described previously (61). Briefly, 3-cc syringes were filled with 0.12 to 0.15 g of washed and brushed nylon wool. The columns were prepared with warm BSS 5% FBS, after which the cells were loaded in a 0.5 ml volume and incubated for 30 min at 37 °C. After draining 0.5 ml away, the columns were incubated an additional 30 min, followed by elution with BSS 5% FBS.

For two- and three-color staining, cells were incubated on ice with the primary Abs in the presence of 5% normal mouse serum, culture supernatant from hybridoma cells producing an anti-mouse Fc receptor mAb (24.G2; ref 60) and 10 µg/ml human γ -globulin (Sigma) to block non-specific binding. After a 30 min incubation on ice in staining buffer (BSS, 3% FBS, 0.1% sodium azide) with primary Abs, the cells were washed twice and analyzed by flow cytometry, or, if a second staining step was necessary, the incubation and wash procedures were repeated. Flow cytometry was conducted on an EPICS XL flow cytometer (Coulter Electronics, Miami, FL). Greater than 5000 viable cells were analyzed with WinList software (Verity Software House, Topsham, ME).

Histochemistry

The mesenteric LN and spleen from each mouse were fixed in PBS 4% paraformaldehyde. They were transferred into OmniSette tissue cassettes (Fisher Scientific, Pittsburgh, PA) and washed with distilled water for 2-3 h and then soaked in 70% ethanol overnight. The tissues were embedded in paraffin after a 1 h wash in 85% ethanol, three 1 h washes in 95% ethanol, three 1 h washes in 100% ethanol and three 1 h washes in xylene. Paraffin-embedded tissues were sectioned and baked onto Superfrost/Plus microscope slides (Fisher Scientific) overnight. Tissues were stained using the Apoptosis Detection System, Fluorescein from Promega (Madison, WI), which uses the TdT-mediated dUTP Nick-End Labeling (TUNEL) assay (62). Briefly, tissues were deparaffinized with organic solvents and permeabilized with proteinase K. The tissues were then incubated with TdT enzyme and a nucleotide mixture containing FITC-labeled dUTP. This was done in a humidified chamber for 1 h at 37 °C. Tissues were then washed, counterstained with propidium iodide, and then sealed under a cover slip. Confocal images were captured at x20 magnification using a Leica TCS 4D confocal microscope (Heidelberg, Germany) and combined using Adobe Photoshop software (Mountainview, CA).

RESULTS

Activation of CD40 inhibits Ag-induced T cell deletion in lymph nodes and spleen

Initial studies sought to examine the effects CD40 activation would have on T cell populations in the presence of stimulating Ag. To examine this issue, mice were treated with SEA. SEA is a SAg that stimulates T cells bearing TCR V β 3 chains (16); therefore, SAg-stimulated T cells can be directly analyzed after SEA treatment. Mice were injected with SEA alone or with SEA and anti-CD40. As a control for the SEA/anti-CD40 group, a third group was injected with SEA and rat IgG. A final group was left uninjected (normal) as a negative control. Fourteen days after SEA injection, the peripheral (inguinal, axillary and bronchial) LN and the mesenteric (mucosal) LN, as well as the spleen, were removed, and the T cells were isolated by nylon wool fractionation separately from each tissue. The T cells were stained for CD4 V β 3 and CD8 V β 3 expression and analyzed by flow cytometry.

The results show that the presence of Ag alone causes significant deletion of both CD4 and CD8 T cells bearing V β 3 in each tissue examined 14 days after SEA (Fig. 2.1). Percentages of each T cell population were two- to fourfold lower in SEA-injected mice than in uninjected controls. In contrast, injection of anti-CD40 in SEA-treated mice potently inhibited Ag-induced T cell deletion in every tissue examined. CD4 V β 3 and CD8 V β 3 percentages in these mice were generally

as high as, if not higher than those in the uninjected control, and in all cases higher than those in the mice injected with SEA alone.

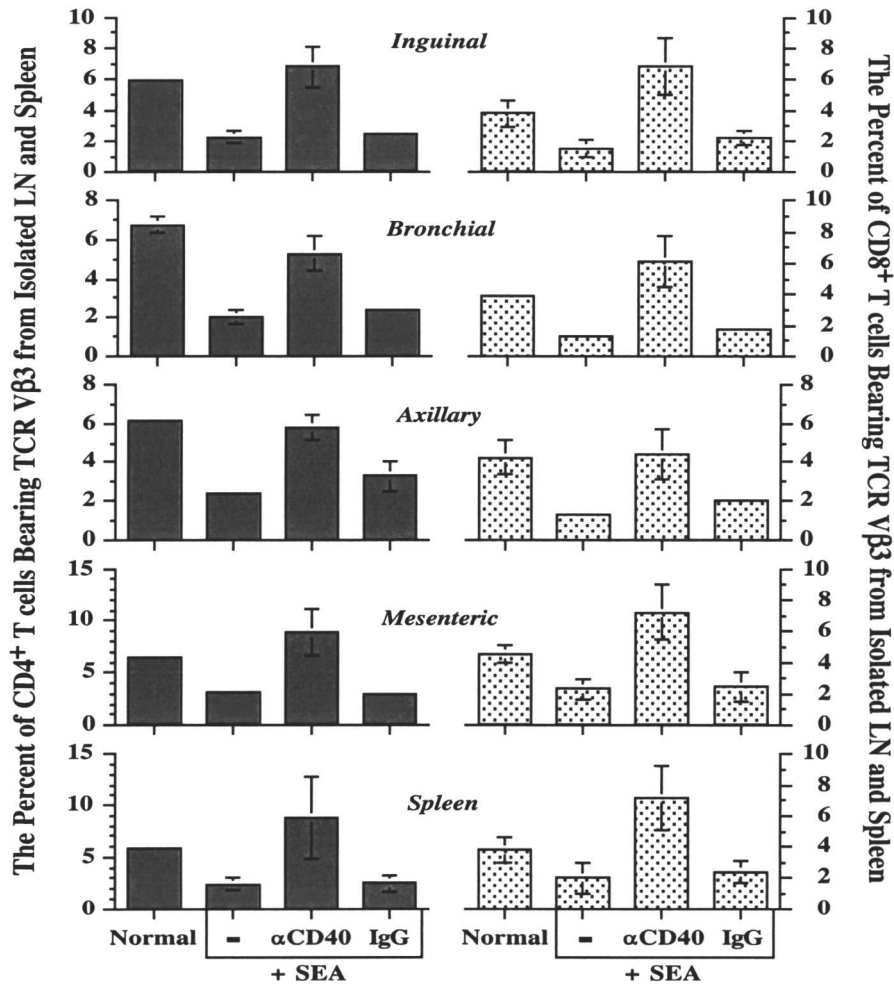


Figure 2.1: CD40 activation blocks Ag-specific T cell deletion in peripheral and mucosal LN and spleen populations. Two groups of B10.Br mice were injected with either 0.5 mg of anti-CD40 or rat IgG 24 h before, and concurrent with, injection of 0.15 μ g of SEA. Two other groups received no injection or SEA only. Fourteen days after SEA injection, T cells were isolated from the inguinal, bronchial, axillary, and mesenteric LN, as well as the spleen. These cells were stained for CD4 V β 3 (left panel) and CD8 V β 3 (right panel) and analyzed by flow cytometry. These data represent the mean percentages \pm SEM from three or four mice over two separate experiments combined.

To test whether T cell rescue from deletion was due to CD40 stimulation and not to a nonspecific effect from the injected Ab, rat IgG was injected with SEA (Fig. 2.1). The results showed the same amount of T cell deletion that was observed with SEA alone, strongly suggesting that CD40 activation blocks SAg-induced T cell deletion. Percentages can be misleading due to migratory effects and bystander T cell death, so it was important to examine the absolute number of T cells in the various lymphoid organs. Data shown in Table 2.1 confirm that deletion was inhibited, since CD4 V β 3 numbers are elevated in the anti-CD40- and SEA-treated mice compared with SEA alone. An additional control experiment was performed where anti-CD40 alone was injected. The results showed no differences in T cell percentages when compared with an uninjected control (data not shown). Furthermore, staining of the T cells from each tissue for TCR V β 14 chains, a subset of T cells that does not respond to SEA, showed no significant difference in their percentages when compared with the uninjected control (data not shown).

Table 2.1: Absolute numbers of CD4 V β 3 T cells in various lymphoid tissues*

Treatment	Inguinal LN	Bronchial LN	Axillary LN	Mesenteric LN	Spleen
Normal	1.7 \pm 0.5	2.2 \pm 0.2	2.2 \pm 0.2	5.8 \pm 1.6	8.3 \pm 1.5
SEA	0.8 \pm 0.4	0.6 \pm 0.2	0.8 \pm 0.2	2.7 \pm 0.6	3.1 \pm 1.4
SEA/αCD40	3.0 \pm 1.2	2.4 \pm 0.2	2.6 \pm 1.2	8.1 \pm 0.6	11.6 \pm 5.0
SEA/IgG	0.7 \pm 0.1	0.9 \pm 0.4	1.1 \pm 0.3	1.9 \pm 0.5	2.8 \pm 1.0

* Data represent the numbers of CD4 V β 3 T cells ($\times 10^{-5}$) \pm SEM from the experiment described in Fig. 2.1.

Optimization of anti-CD40 injection

To standardize future experiments with anti-CD40, the optimal conditions for anti-CD40 injection were determined. Experiments were set up in which B10.Br mice were injected with 1 mg of anti-CD40 at different times in relation to SEA. Anti-CD40 injections were performed at 5 days before SEA injection (day -5) and up to 1 day after (day +1). Eight days after SEA injection, T cells from the LN (Fig 2.2) and spleen (not shown) were counted, stained for CD4 V β 3 and CD8 V β 3, and analyzed by flow cytometry.

T cell percentages declined after SEA injection in comparison with uninjected control mice (Fig. 2.2, A and C). Most injections of anti-CD40 were effective at preventing the deletion, regardless of the day injected relative to SEA. Deletion was most efficiently prevented when anti-CD40 was injected before SEA. For example, when anti-CD40 was injected on day +1, the percentage of CD4 V β 3 T cells was $3.8 \pm 0.9\%$; when anti-CD40 was injected on days -2, -3 and -4, however, this rose to $11.6 \pm 2.4\%$, $9.0 \pm 2.5\%$ and $9.1 \pm 3.1\%$, respectively (Fig. 2.2A). The percentages of CD8 V β 3 T cells were above normal in every case, except when anti-CD40 was injected 5 days before SEA (Fig. 2.2C). The CD4 V β 3 percentages were near or above normal, except when anti-CD40 was given on day -5 and day +1. All SEA/anti-CD40 injections consistently led to greater V β 3 T cell percentages than those observed with SEA alone. Based on percentages, day -2 was the best day for anti-CD40 injection. On this day, CD4 V β 3 percentages rose to $11.6 \pm 2.4\%$, almost six times the level observed in SEA alone-treated mice

($2.1 \pm 0.5\%$) (Fig. 2.2A). CD8 V β 3 percentages rose from a $1.5 \pm 0.6\%$ population found in SEA injected mice to $9.4 \pm 1.6\%$ (Fig. 2.2C).

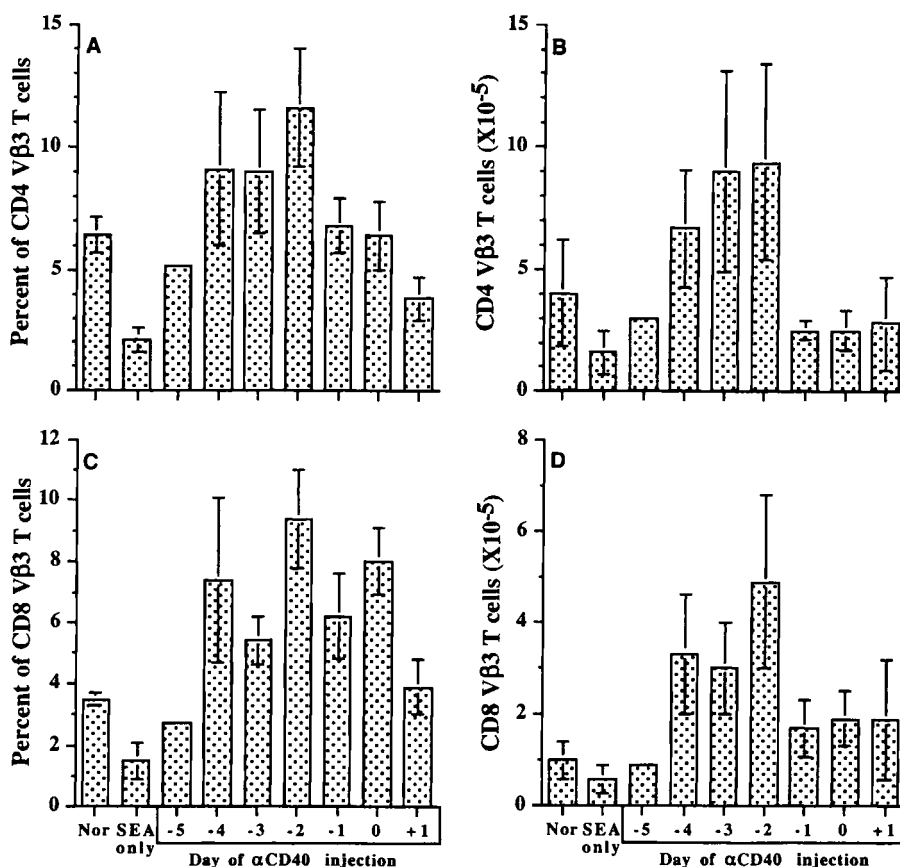


Figure 2.2: The optimal day for anti-CD40 injection is two days before SEA administration. Female B10.Br mice were injected with $0.15 \mu\text{g}$ of SEA at time 0. At various times relative to SEA injection, 1 mg of anti-CD40 was injected. Control groups were also set up that received no injections or SEA only. LN T cells were isolated eight days after SEA injection and were stained and counted for CD4 V β 3 (A, B) and CD8 V β 3 (C, D). Each bar represents data from four or five mice, collected over five separate experiments except the day -5 bar, which was collected from one mouse. Except for day -5 , these data represent mean percentages, as determined by flow cytometry, and numbers \pm SEM.

The total numbers of CD4 V β 3 (Fig. 2.2B) and CD8 V β 3 (Fig. 2.2D) T cells were calculated. Days -2 , -3 and -4 had the highest total numbers of V β 3 T

cells after SEA/anti-CD40 treatment. Day -2 was still the best, providing counts of $9.4 \times 10^5 \pm 4.0$ and $4.9 \times 10^5 \pm 1.9$ CD4 and CD8 T cells bearing V β 3, respectively (Fig. 2.2, B and D). Based on these results, day -2 was chosen as the standard day of injection of anti-CD40, since it gave the most consistently high percentages and numbers of both CD4 V β 3 and CD8 V β 3 T cells.

Once the timing of anti-CD40 injection was determined, the dose of anti-CD40 was titrated. One mg of anti-CD40 was injected into each mouse for the timing experiments. We tested whether lower doses would still be effective at preventing Ag-specific T cell deletion. Experiments were set up in B10.Br mice, which were injected with anti-CD40 two days before receiving SEA. Anti-CD40 was injected at 1 mg, 0.5 mg, 0.25 mg or 0.125 mg. An uninjected control mouse and a mouse receiving SEA only were also included. Seven days after SEA was injected, the LN and spleen T cells were isolated, counted and stained for CD4, CD8 and V β 3. T cell analysis was done by flow cytometry.

LN-dosing experiments are shown in Fig. 2.3, and spleen data are similar, but not shown. The resulting percentages show the deletion of CD4 V β 3 (Fig. 2.3A) and CD8 V β 3 (Fig. 2.3C) T cells in the LN upon injection of SEA alone. All doses of anti-CD40 were effective at preventing deletion, as shown by the percentages (Fig. 2.3, A and C) and numbers (Fig. 2.3, B and D). The highest dose tested (1 mg) was the most effective at generating high T cell numbers, but even an eightfold lower dose of anti-CD40 provided some T cell rescue.

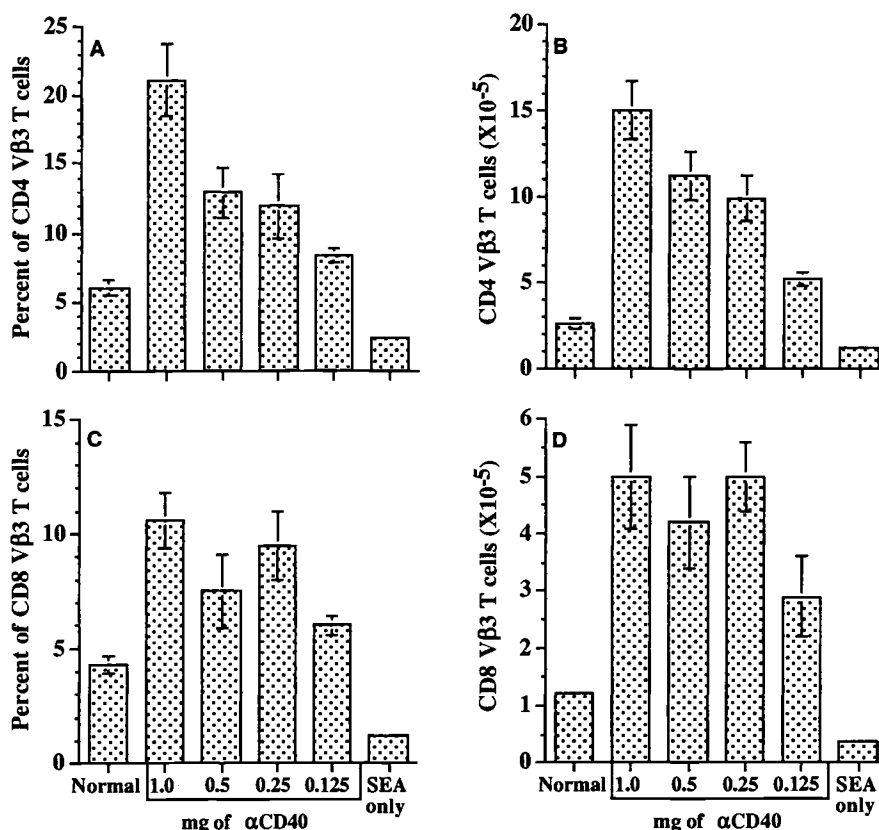


Figure 2.3: Low doses of anti-CD40 are effective at blocking Ag-specific T cell deletion. Five groups of mice were injected with 1.0 mg, 0.5 mg, 0.25 mg, 0.125 mg or 0 mg of anti-CD40, and then two days later, injected with 0.15 μ g of SEA. An uninjected control group was also set up. Seven days after SEA injection, LN T cells were purified, stained and enumerated for CD4 V β 3 (A, B) and CD8 V β 3 (C, D). Each bar represents four mice over two separate experiments combined. These data represent mean percentages, as determined by flow cytometry, and numbers \pm SEM. Comparable data were obtained from the spleens of these mice.

Both percentages and numbers show that the CD8 V β 3 T cells were rescued more effectively by lower doses of anti-CD40 than were CD4 V β 3 T cells. A dose of 0.25 mg yielded equivalent numbers of CD8 V β 3 T cells as the 1 mg dose ($5 \times 10^5 \pm 0.6$) (Fig. 2.3D). In contrast, CD4 V β 3 T cell deletion was less inhibited by

lower doses of anti-CD40 (Fig. 2.3B) but was still very effective. Based on these data, 0.25 mg of anti-CD40 was chosen as the standard dose in future experiments.

Activation of CD40 enhances the expansion and delays the deletion of SAg-specific T cells

Since anti-CD40 inhibited the deletion of Ag-specific T cells exposed to SEA (Figs. 2.1-2.3), we next investigated whether anti-CD40 affected the expansion and long-term deletion of V β 3 T cells by conducting a detailed time course. In this experiment, mice were injected with 0.25 mg of anti-CD40 or, as a control, 0.25 mg of rat IgG. Two days later, SEA was injected into each group. On days 2, 5, 7, 12 and 21 after SEA injection, LN and spleens from both groups of mice were obtained. T cells were purified from these tissues, counted, stained for CD4 V β 3 and CD8 V β 3, and analyzed by flow cytometry.

The control mice injected with SEA and rat IgG (squares) show some CD4 V β 3 T cell expansion (day 2), and significant deletion by day 5 (Fig. 2.4). Examination of numbers shows a small degree of expansion in the LN (Fig. 2.4C) but a greater than twofold increase in the spleen (Fig. 2.4D). The degree of expansion is quite variable depending on dose and batch to batch variation of SEA (our unpublished observations). After clonal expansion, T cell populations deleted quickly. By day 5, both percentages (Fig. 2.4, A and B) and numbers (Fig. 2.4, C and D) fell below normal and stayed there until day 21. The slight rise in T cell numbers at the end of the time course is most likely due to repopulation of deleted T cells from the thymus.

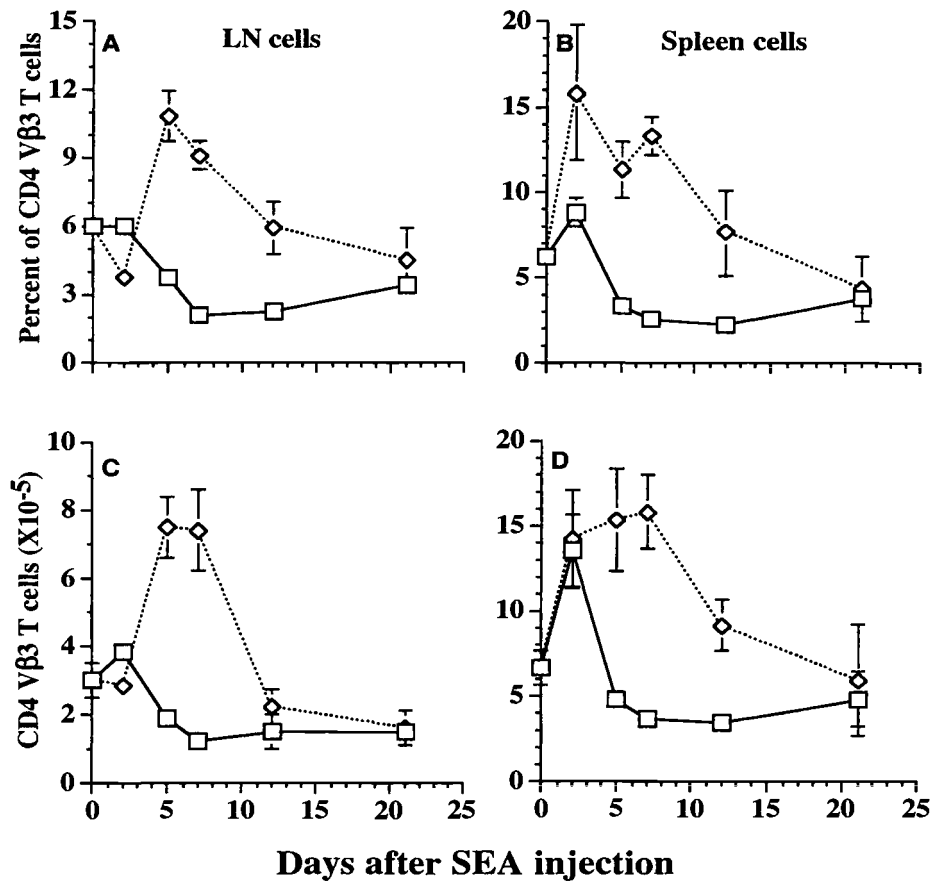


Figure 2.4: Time course of SEA-stimulated CD4 T cell deletion with and without CD40 activation. Mice were injected with 0.25 mg of anti-CD40 (◇) or rat IgG (□) two days before receiving 0.15 μg of SEA. T cells were purified after SEA injection on days 2, 5, 7, 12 and 21 from the LN (A, C) and spleen (B, D). T cells were stained for CD4 Vβ3 and analyzed by flow cytometry. Each point represents the mean percentages (A, B) and numbers (C, D) ± SEM from three mice from one representative experiment of two separate experiments.

The idea that CD40 activation enhances T cell clonal expansion and prevents their deletion was tested in mice injected with anti-CD40 and SEA (diamonds). The percentages of CD4 Vβ3 T cells show a small decrease in the LN (Fig. 2.4A) and a large increase in the spleen (Fig. 2.4B) to about three times

starting levels on day 2. By day 5, both tissues were showing T cell expansion to about three times control levels. T cell percentages stayed above the rat IgG control levels throughout the time course but did decline to control levels as day 21 approached. Based on numbers, the LN showed about a fourfold expansion of the CD4 V β 3 T cells by day 5, only to decline to near control levels by day 12 (Fig. 2.4C). Spleen numbers were not much greater than controls on day 2, but T cell deletion was greatly inhibited until day 21 (Fig. 2.4D).

The CD8 V β 3 time course results closely resembled the CD4 V β 3 data (Fig. 2.5). The percentages and numbers of CD8 V β 3 LN or spleen T cells from SEA/rat IgG-injected mice slightly increased on day 2, followed by deletion starting after day 2. SEA/anti-CD40-injected mice again showed increased expansion and delayed deletion. Both percentages (Fig. 2.5, A and B) and numbers (Fig. 2.5, C and D) showed greater than fourfold increases over the levels observed in SEA/rat IgG-injected mice on days 5 and 7, but came back down to control levels by day 21. It is important to note that, as a negative control, V β 14 T cells were also examined during this time course. There was little variation in V β 14 T cell populations when compared with uninjected mice (data not shown).

Two important conclusions can be made from the data presented thus far. First, CD40 activation enhances the clonal expansion of both CD4 and CD8 T cells in the presence of SAg. Second, the Ag-specific T cell deletion normally observed after clonal expansion is delayed when CD40 has been stimulated.

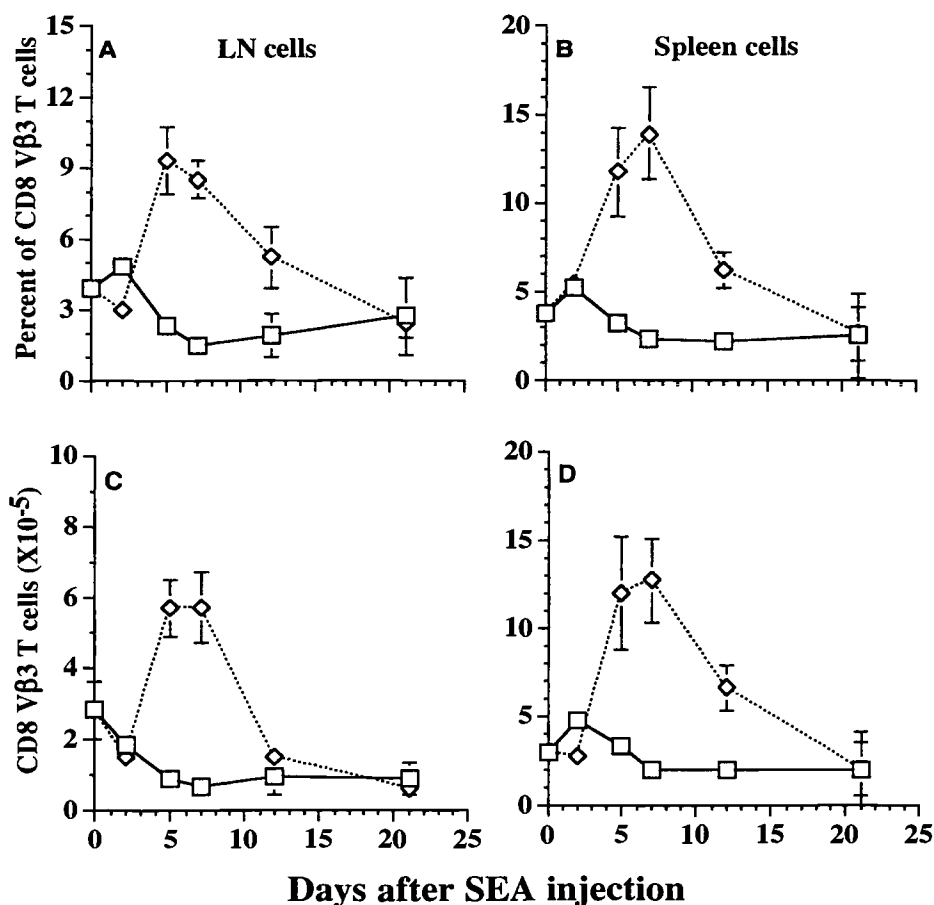


Figure 2.5: Time course of SEA-stimulated CD8 T cell deletion with and without CD40 activation. These data are from the experiments described in Fig. 2.4, except the cells were stained (A, B) and enumerated (C, D) for CD8 Vβ3. LN (A, C) and spleen (B, D) data are presented.

Stimulation of CD40 delays T cell death

In each of the previous experiments, it was shown that injection of anti-CD40 inhibited the Vβ3 T cell deletion characteristic of SEA stimulation. These data raise the possibility that CD40 activation delays the death of the SEA-stimulated T cells.

To determine whether T cell death was inhibited by CD40 activation, mice were treated as follows: injected with SEA alone, anti-CD40 alone, SEA and anti-CD40 or left as uninjected controls. On days 3 and 9 after SEA injection, the mesenteric LN and spleen from each mouse were fixed in PBS containing 4% paraformaldehyde and stained for apoptotic cells as described in Materials and Methods. Confocal microscopy was used to generate images of each stained tissue.

Fig. 2.6, A-D, shows LN results 3 days after SEA injection, while Fig. 2.6, E-H, shows the results from day 9. On day 3, SEA alone led to significant apoptosis (Fig. 2.6B), some of which was still observed 9 days later (Fig. 2.6F). Injection of anti-CD40 alone yielded low levels of apoptosis on both days 3 and 9 (Fig. 2.6, C and G, respectively). Injection of SEA and anti-CD40 led to low levels of death on day 3 (Fig. 2.6D) but enhanced apoptosis by day 9 (Fig. 2.6H). These data show that injection of anti-CD40 and SEA does delay cell death, but they do not indicate whether the dead cells are Ag-stimulated T cells or not.

To test whether the dying cells were Ag-stimulated T cells, the peripheral LNs from the mice used to generate the data in Fig. 2.6 were crushed, and the T cells were isolated and stained for CD4, V β 3, V β 14 and extracellular phosphatidylserine (PS). PS is displayed extracellularly on dying cells and can be detected by staining with its natural ligand, annexin V, and analyzed by flow cytometry (Fig. 2.7) (63).

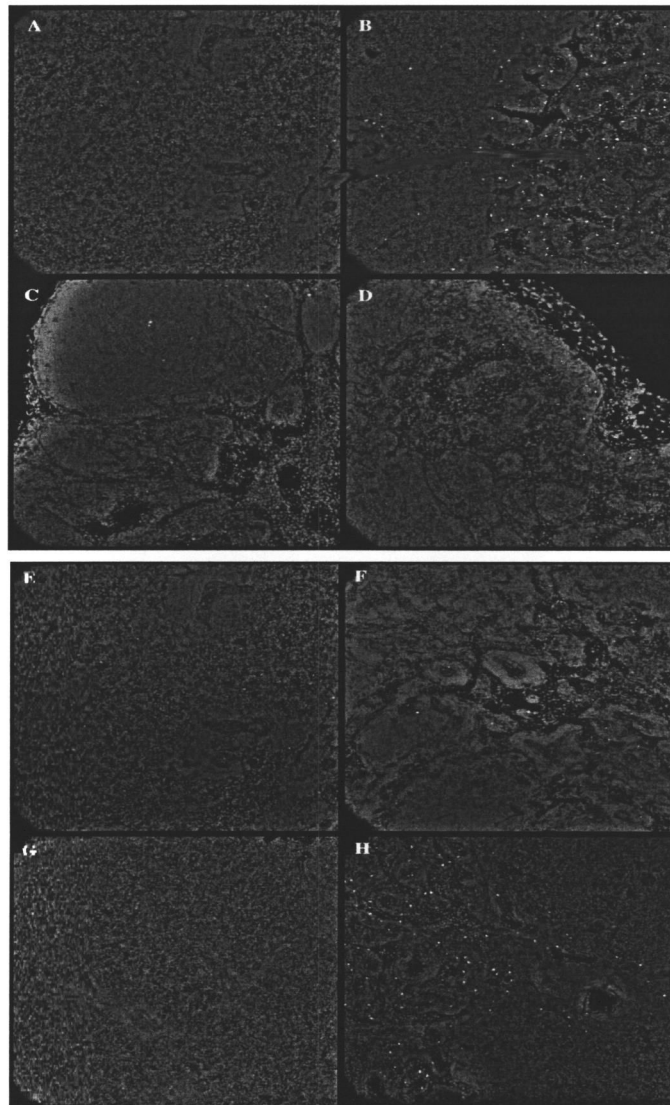


Figure 2.6: Apoptosis is delayed by CD40 activation in the presence of foreign Ag. Four B10.A mice were set up as follows. One mouse was left as an uninjected control (A, E). One mouse was injected with 0.30 μ g of SEA (B, F). One mouse was injected with 0.25 mg of anti-CD40 on day -2 (C, G). One mouse was injected with 0.25 mg of anti-CD40, and two days later injected with SEA (D, H). On days 3 and 9 after SEA injection, the mesenteric LN and spleen from each mouse were collected and fixed in 4% paraformaldehyde. The tissues were stained and examined as described in Materials and Methods. Images from the LN on day 3 (A-D) and day 9 (E-H) are shown here. Spleen images were taken and are comparable to the LN images, but are not shown. These data are similar to one other experiment.

On day 3, the percentage of CD4 V β 3 T cells with extracellular PS was $19.3 \pm 6.0\%$ in mice injected with SEA alone (Fig. 2.7A); thus, significant death was occurring at this early time point in comparison with uninjected animals ($8.5 \pm 0.5\%$). Mice injected with anti-CD40 alone, or with SEA and anti-CD40, remained about 2.5-fold lower ($6.8 \pm 2.7\%$ and $7.1 \pm 2.8\%$, respectively). On day 9, death had more than doubled in SEA-injected mice to $40.2 \pm 3.9\%$; however, it should be kept in mind that these mice contained far fewer V β 3 T cells on day 9 than on day 3 (see time course data in Figs. 2.4 and 2.5). Mice injected with anti-CD40 alone showed only a slight increase (Fig. 2.7A). The percentage of death in mice injected with SEA and anti-CD40 after 9 days ($39.1 \pm 3.3\%$) was nearly equal to that in mice injected with SEA alone, rising about sixfold from day 3. The day 9 mice treated with SEA and anti-CD40 also contained a greater number of V β 3 T cells than the SEA alone group (Figs. 2.4 and 2.5). As a control, CD4 V β 14 T cells were also examined for extracellular PS expression (Fig. 2.7B), and no major changes above normal percentages were observed in any of the groups.

These data suggest that SAg-stimulated T cells begin to die early after injection with SEA and will continue along that path for days afterward. Activation of CD4 delayed the death of those SAg-specific T cells.

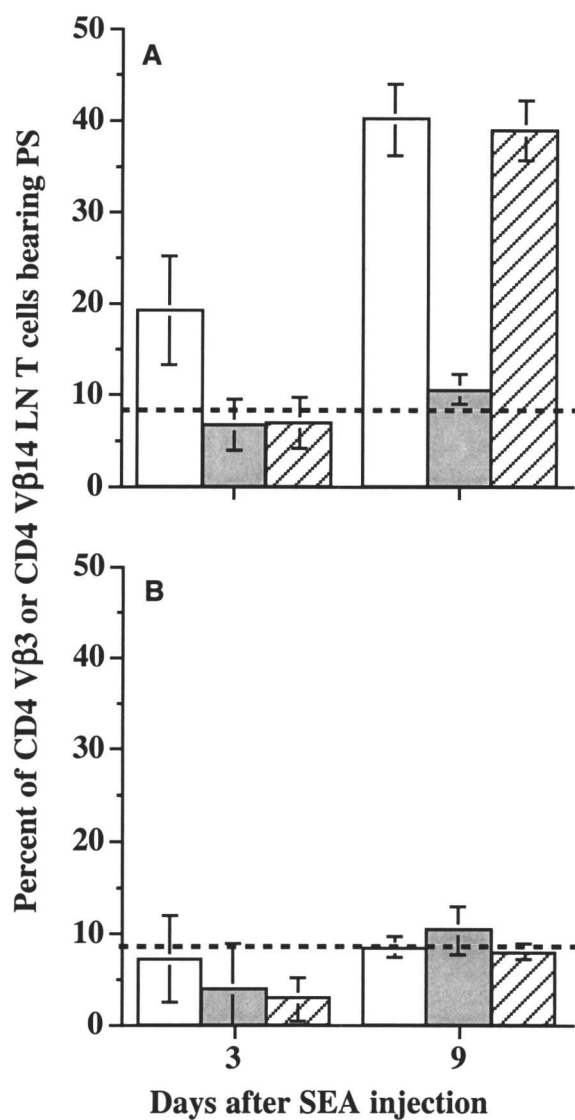


Figure 2.7: CD40 activation delays, but does not prevent Vβ3 T cell death. Four B10.A mice were set up as follows. One mouse was left as an untreated control (dotted line). One mouse was injected with 0.30 μg of SEA (open bar). One mouse was injected with 0.25 mg of anti-CD40 on day -2 (dark bar). One mouse was injected with anti-CD40 two days before receiving SEA (striped bar). LN T cells were isolated on days 3 and 9 after SEA injection. T cells were stained for CD4 Vβ3 and CD4 Vβ14 and were also stained with annexin V to detect extracellular phosphatidylserine (PS). Percentages of CD4 Vβ3 (A) and CD4 Vβ14 (B) T cells displaying PS were analyzed by flow cytometry. Data represent mean percentages ± SEM from four mice (three mice for untreated) over three combined experiments.

B7-1 and B7-2 play different roles in CD4 and CD8 T cell stimulation in the presence of SEA and CD40 activation

Since CD40 activation delayed SAg-specific T cell death, we sought to test the mechanism by which CD40 was acting. One possibility was that CD40 activation altered APCs. To examine this hypothesis, three separate experiments were performed in which one mouse was injected with anti-CD40 and another mouse was given rat IgG. Two days later, the LNs and spleens were isolated, and the cells were stained to identify B cells and macrophages. Expression of MHC class II, B7-1 and B7-2 on these cells was examined by flow cytometry.

Mean channel fluorescence (MCF) of MHC class II expression increased approximately fourfold on both macrophages and B cells obtained from the LN and spleen of mice injected with anti-CD40 compared with rat IgG (data not shown). Both B cells and macrophages also upregulated B7-1 and B7-2 in the spleen (Fig. 2.8) as well as the LN (data not shown) of mice treated with anti-CD40 compared with mice treated with rat IgG. Specifically, splenic MHC class II⁺ B220⁺ B cell MCF of B7-1 (16.7) and B7-2 (18.0) increased by more than 2.5-fold over control cells (6.6 and 5.5, respectively). MCF MHC class II⁺ F4/80⁺ macrophage expression of B7-1 (17.6) and B7-2 (22.0) in anti-CD40-treated mice increased by more than fivefold over control levels (3.5 and 3.9, respectively). These data are similar to those previously reported (64). Thus, one possibility was that CD40 activation was assisting T cell expansion by increasing expression of B7-1 and/or B7-2 on APC.

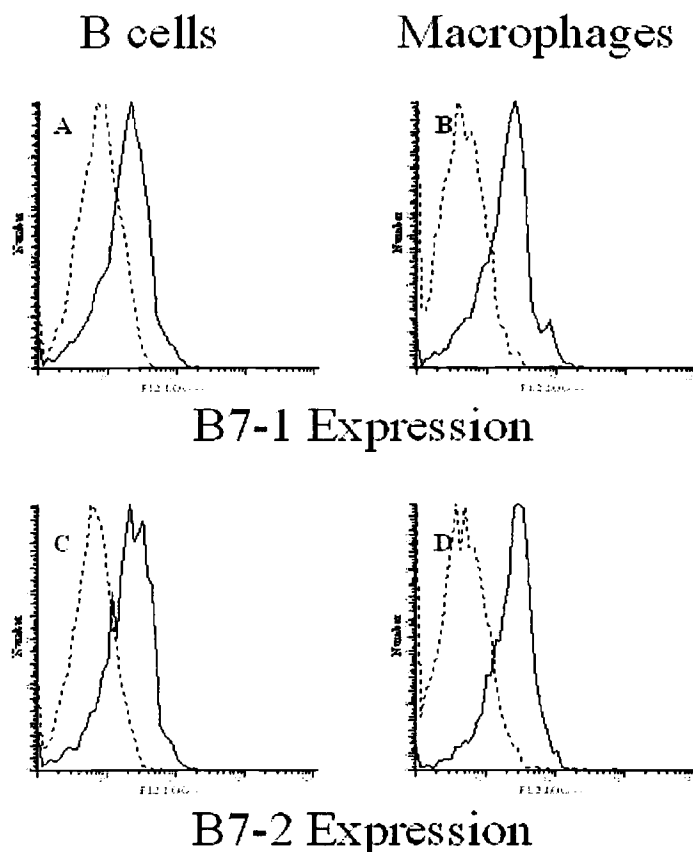


Figure 2.8: CD40 activation enhances expression of B7-1 and B7-2 on B cells and macrophages. These results represent one experiment of three conducted. In each, three B10.A mice were used. One mouse was left untreated, one mouse was injected i.p. with 0.25 mg of rat IgG (dotted line), and one mouse was injected with 0.25 mg of anti-CD40 (solid line). Two days later, LN and spleens were analyzed by three-color flow cytometry. MHC class II⁺ B220⁺ B cells were stained for B7-1 (A) and B7-2 (C) expression, as were MHC class II⁺ F4/80⁺ macrophages (B, D). Only spleen data are shown here. Uninjected mice were comparable to rat IgG injected mice (data not shown).

Based on Fig. 2.8 and previous reports (64, 65), we next tested the hypothesis that CD40 mediated T cell activation *in vivo* through B7. We first sought to inhibit the CD28-B7 interaction using CTLA4-Ig. One group of mice received SEA only; one received SEA, anti-CD40 and CTLA4-Ig; and another

group received SEA, anti-CD40 and the control chimeric Ab L6. Five days after SEA injection, T cells from the LN and spleen of each mouse were isolated, counted and stained for CD4 V β 3 and CD8 V β 3. The cells were analyzed by flow cytometry.

Mice injected with SEA alone showed the expected decrease in CD4 V β 3 and CD8 V β 3 expansion in the LN and spleen (Table 2.2). Injection of SEA, anti-CD40 and L6 led to significant expansion over normal levels in both CD4 and CD8 T cell populations in the LN and spleen, similar to that shown in Figs. 2.4 and 2.5. When CTLA4-Ig was injected with SEA and anti-CD40, however, CD4 V β 3 percentages were lowered in both tissues, although never to the level of mice injected with SEA alone. CD4 T cell populations in the spleen and LN decreased twofold, ($22.1 \pm 0.6\%$ to $11.1 \pm 0.6\%$, and $16.0 \pm 0.9\%$ to $7.9 \pm 1.2\%$, respectively). CD8 V β 3 percentages differed from the CD4 percentages in that they were only slightly inhibited by CTLA4-Ig injection. Spleen percentages decreased from $17.8 \pm 0.9\%$ to $15.1 \pm 1.4\%$, while the LN percentages dropped from $13.7 \pm 0.6\%$ to $11.7 \pm 1.1\%$ (Table 2.2).

Total numbers of CD4 and CD8 V β 3 T cells were also calculated (Table 2.3). The splenic CD4 V β 3 T cell numbers in SEA/anti-CD40/L6-injected mice ($30.6 \times 10^5 \pm 2.5$) dropped to uninjected levels ($8.9 \times 10^5 \pm 0.4$) when CTLA4-Ig was given. CD8 V β 3 T cell numbers did show more of a decline than the percentages did, especially in the spleen, falling from $22.3 \times 10^5 \pm 1.6$ in SEA/anti-CD40/L6-treated mice to $14.9 \times 10^5 \pm 1.7$ in mice treated with CTLA4-Ig. The

numbers of T cells, however, were still almost fourfold greater than uninjected or SEA-injected ($4.2 \times 10^5 \pm 0.3$) mice (Table 2.3).

Table 2.2: CTLA4-Ig inhibits SEA-specific CD4 T cell survival more than SEA-specific CD8 T cell survival in the presence of anti-CD40 and SEA*

Treatment	% of CD4 V β 3 T cells		% of CD8 V β 3 T cells	
	Spleen	LN	Spleen	LN
Uninjected	6.1 ± 0.3	5.5 ± 0.6	3.7 ± 0.1	3.8 ± 0.1
SEA	3.0 ± 0.6	3.3 ± 0.5	3.4 ± 0.3	2.7 ± 0.7
SEA/ α CD40/L6	22.1 ± 0.6	16.0 ± 0.9	17.8 ± 0.9	13.7 ± 0.6
SEA/ α CD40/CTLA4-Ig	11.1 ± 0.6	7.9 ± 1.2	15.1 ± 1.4	11.7 ± 1.1

* Twenty B10.A mice were set up as follows. Five mice were left as untreated controls. Three mice were given 0.15 μ g of SEA. Six mice were injected with 0.5 mg of anti-CD40 on day -2 and 0.5 mg of the control chimeric Ab L6 2 h before receiving an injection of SEA at time 0. The remaining six mice were injected with 0.5 mg of anti-CD40 on day -2 and 0.5 mg of CTLA4-Ig 2 h before SEA injection at time 0. Five days after SEA injection, T cells from the LN and spleen were isolated, stained for CD4 V β 3 and CD8 V β 3, and analyzed by flow cytometry. The data represent mean percentages \pm SEM from two combined experiments.

Table 2.3: Total number of SEA-reactive T cells that CTLA4-Ig inhibits in the presence of anti-CD40 and SEA*

Treatment	# of CD4 V β 3 T cells ($\times 10^5$)		# of CD8 V β 3 T cells ($\times 10^5$)	
	Spleen	LN	Spleen	LN
Uninjected	8.9 ± 0.4	2.3 ± 0.4	4.1 ± 0.1	1.3 ± 0.1
SEA	5.1 ± 0.9	1.2 ± 0.3	4.2 ± 0.3	0.9 ± 0.2
SEA/ α CD40/L6	30.6 ± 2.5	5.5 ± 0.1	22.3 ± 1.6	5.9 ± 0.4
SEA/ α CD40/CTLA4-Ig	8.7 ± 0.8	2.5 ± 0.4	14.9 ± 1.7	4.6 ± 0.7

* The data are from the experiment described in Table 2.2 and represent mean numbers ($\times 10^5$) \pm SEM from two combined experiments involving a total of 20 mice.

These data led us to hypothesize that costimulation through B7 was important for expansion, as others have shown (66-68); however, a role for B7-1 vs. B7-2 had yet to be discerned in this model. To this end we injected antagonistic Abs to B7-1 and B7-2. Six groups of mice were set up as follows: one group received no injection, one group received SEA alone, and one group received SEA and anti-CD40. The remaining three groups all received SEA and anti-CD40, but also received anti-B7-1, anti-B7-2 or both Abs, respectively. Five days after SEA injection, the LN and spleens were removed from each mouse. T cells were isolated, enumerated and stained for CD4, CD8 and V β 3. Analysis of the stained cells was done by flow cytometry.

Both percentages and numbers of CD4 V β 3 T cells showed the expected deletion of SEA-activated T cells and the expected enhanced expansion of those V β 3 T cells upon CD40 stimulation, both in the LN (Fig. 2.9, A and C) and spleen (Fig. 2.9, B and D). Injection of anti-B7-1 led to a modest drop in both percentages and numbers, while injection of anti-B7-2 led to a slightly greater decrease. When both anti-B7-1 and anti-B7-2 were injected, CD4 V β 3 T cell populations dropped below uninjected control levels, close to SEA-injected levels. For example, in the LN-uninjected mice there were $2.7 \times 10^5 \pm 0.2$ CD4 V β 3 T cells, while in mice treated with both Abs there were $2.0 \times 10^5 \pm 0.3$ T cells.

Perhaps the most interesting results were found with the CD8 V β 3 subpopulation (Fig. 2.10). The decrease and increase of CD8 percentages and

numbers upon SEA injection and SEA/anti-CD40 injection, respectively, were still noted in both the LN (Fig. 2.10, A and C) and spleen (Fig. 2.10, B and D).

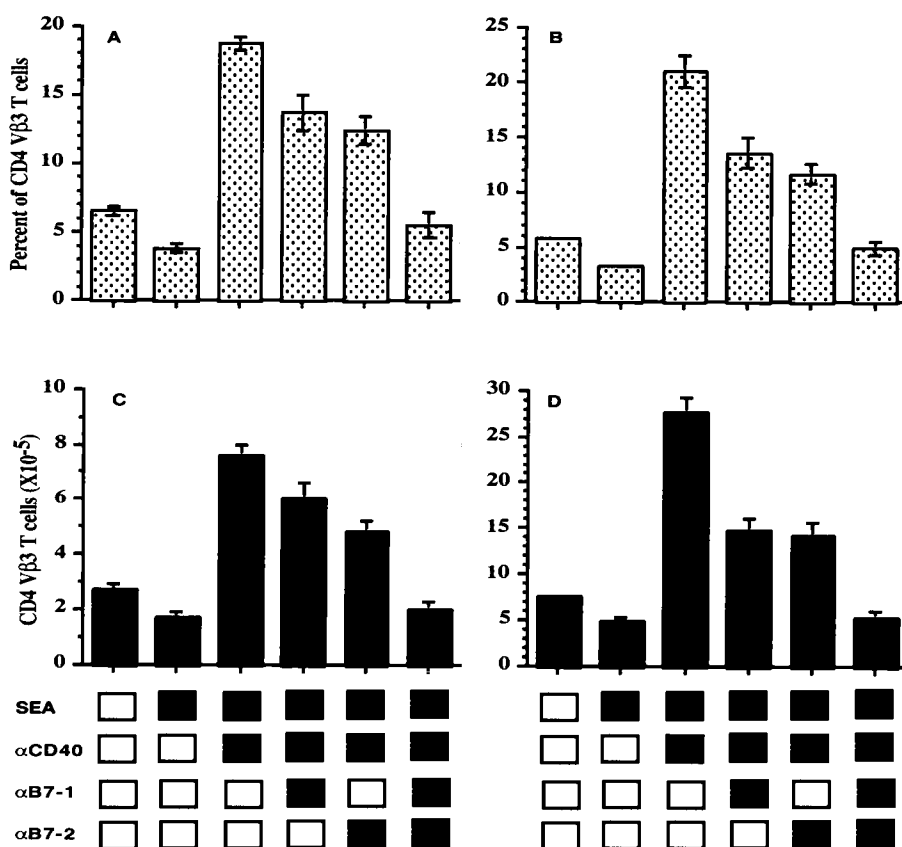


Figure 2.9: Neutralizing B7-1 and B7-2 inhibit Ag-specific CD4 T cell survival in the presence of CD40 activation. Six groups of B10.A mice were treated as follows: One group was left untreated; a second group was given 0.15 μ g of SEA at time 0; a third group was given 0.25 mg of anti-CD40 two days before receiving SEA at time 0; a fourth group was given anti-CD40 two days before, and 1 mg of anti-B7-1 2 h before, SEA; a fifth group was injected like the fourth, except 1 mg of anti-B7-2 was injected in the place of anti-B7-1; the last group was injected like the previous two, but simultaneously received 1 mg of both anti-B7-1 and anti-B7-2 2 h before SEA. Five days after SEA injection, LN (A, C) and spleen (B, D) T cells were isolated, counted and stained for CD4 Vβ3. Stained cells were analyzed by flow cytometry. Each bar represents the mean percentages and numbers \pm SEM from 5-10 mice over four separate experiments combined.

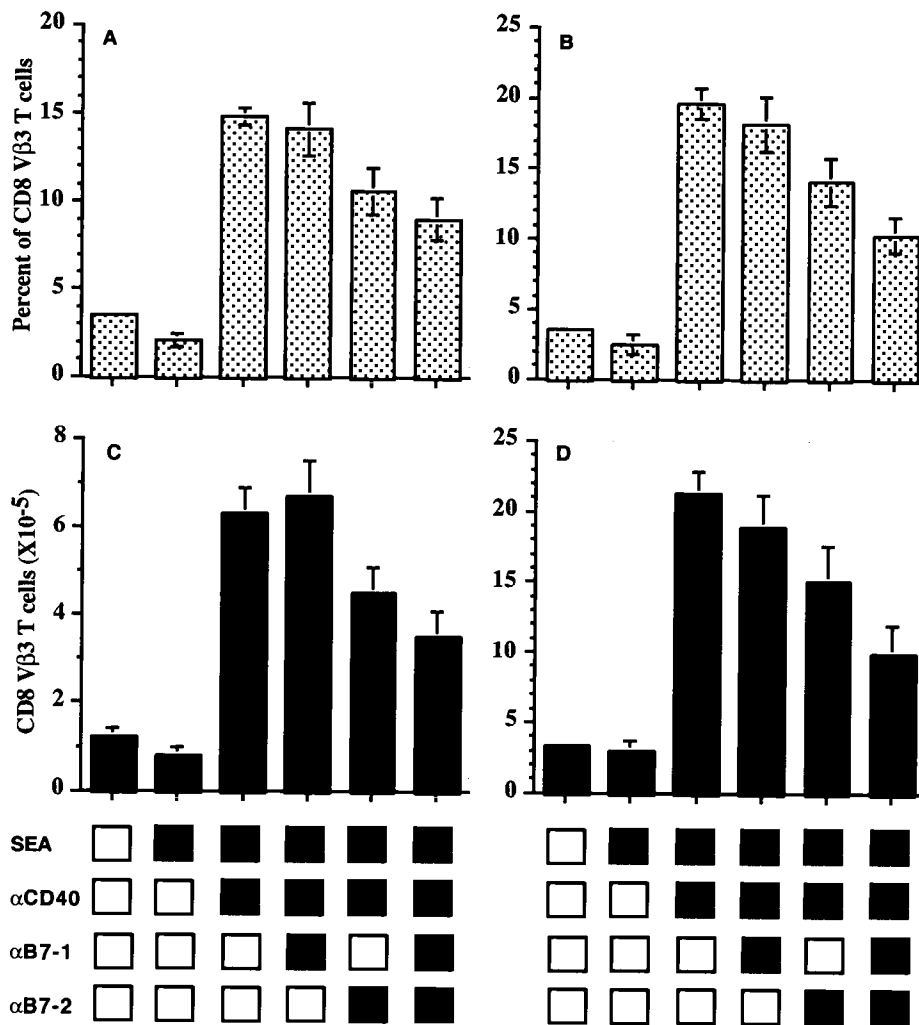


Figure 2.10: Neutralizing B7-2, but not B7-1, inhibits Ag-driven CD8 T cell expansion in the presence of CD40 activation. The T cells from Fig. 2.9 were stained and enumerated for CD8 V β 3. Flow cytometric data from the LN (A, C) and spleen (B, D) are shown here.

Injection of anti-B7-1 did little to inhibit anti-CD40-mediated T cell expansion. Anti-B7-2 consistently dropped the percentages and numbers, suggesting that it was the dominant molecule for CD8 V β 3 T cell clonal expansion.

These data show that B7-1 costimulation in the context of SAg and CD40 activation is less important for CD8 T cells than it is for CD4 T cells. Additionally, the increased drop in CD8 T cell expansion observed when blocking B7-1 and B7-2 may be due to the low levels of CD4 T cell help available.

DISCUSSION

In this study, we set out to understand how SAg-induced T cell immunity is affected by ligation of CD40. Our results showed that activation of CD40, in the presence of SAg, enhanced CD4 and CD8 SEA-specific T cell clonal expansion. We further showed that CD40 activation delays SAg-induced peripheral T cell death.

Initial timing experiments provided many important clues as to the mechanism by which CD40 activation enhances a T cell response. The timing data show that stimulation of CD40 before SAg exposure creates the conditions for an optimal T cell response to foreign SAg (Fig. 2.2). Conversely, CD40 activation does not enhance SAg-induced T cell clonal expansion when it occurs after SAg exposure. One explanation for the latter result is that the T cells have already interacted with SAg/MHC and, thus, APCs are unable to costimulate activated T cells outside the context of SAg *in vivo*. Therefore, it is likely that stimulating CD40 before Ag injection helps create a better costimulatory environment on APCs, as evidenced by the enhanced expression of B7-1 and B7-2 on B cells and macrophages (Fig. 2.8) as well as increased MHC class II expression (data not shown). These primed APCs can enhance T cell stimulation. We have, however,

recently found B cells to be unessential for the increased T cell expansion in this model, since a similar response occurs in B cell knockout mice (our unpublished observations).

Without stimulation of CD40, the SEA-activated T cells clonally expand to peak levels within two days and delete to below starting levels after about day 5 (Figs. 2.4 and 2.5). Activation through CD40 in the presence of SAg, however, enhances the clonal expansion observed on day 2 and delays the time it takes for the T cell populations to delete to control levels to about 21 days. Additionally, we show that low doses of anti-CD40 worked very well at enhancing clonal expansion and delaying SAg-specific T cell deletion (Fig. 2.3). Collectively, the results in this study show that acute activation of CD40 is sufficient to enhance T cell clonal expansion in peripheral and mucosal lymphoid tissue.

One of the most intriguing effects of CD40 activation was its ability to delay SAg-specific T cell deletion. We hypothesized that the observed deletion was due to death. Deletion during negative selection in the thymus has recently been shown to be due to apoptosis (69). Likewise, in the periphery, reports have shown that peptides presented by MHC promote apoptosis of TCR transgenic T cells (70).

Tissue sections from mice in which apoptotic cells were detected showed that there was indeed a greater level of apoptosis observed in SEA/anti-CD40-treated mice nine days after SEA injection (Fig. 2.6). Without anti-CD40, SEA induced death by 72 h in vivo. Because cell death occurred, it was determined

whether the dying cells were indeed SEA-specific T cells. We confirmed this by examining V β 3 T cell populations for extracellular PS expression by staining with annexin V (Fig. 2.7). Not only did CD40 activation enhance T cell clonal expansion, but additionally, the stimulated T cells were viable for longer periods of time. Ligation of CD40, however, does not keep the T cells alive indefinitely; it only delays death. B cells have also been reported to receive a “life” signal when CD40 is activated (55). Collectively, these data suggest that CD40 activation increases the lifespan of effector T cells by delaying their death.

Once the effects of CD40 activation on T cell responses to SA_g had been examined, a role for costimulation in this response was addressed. CD28 is a costimulatory molecule found on T cells that binds B7-1 and B7-2 molecules on APCs. CD28 knockout mice show several T cell deficiencies, including poor T cell proliferation and IL-2 production (71-73). Thus, the importance of CD28 ligation in the SEA/anti-CD40 model was studied.

Using CTLA4-Ig to block the ligation of CD28 by B7 molecules, we found a decrease in SEA-specific T cell expansion in vivo (Tables 2.2 and 2.3). This, taken along with the time course data, suggests that CD28 ligation is important for T cell expansion, but not for long-term survival, since the cells still go on to die even with CD28 ligation. Because CD8 T cell responses were less inhibited by CTLA4-Ig, it became interesting to examine the roles of B7-1 and B7-2 separately in this model to begin uncovering the costimulatory dependence of each T cell subpopulation.

B7-1 and B7-2 both bind CD28 and CTLA4 (6, 7, 10). Since B7-1 and B7-2 behave in a similar manner, extensive work has been conducted to determine what different functions B7-1 and B7-2 may have. To date, results have been contradictory. It has been held that ligation of B7-1 can skew CD4 T cell development in the Th1 direction, while ligation of B7-2 promotes Th2 development (74, 75). However, others have found no differential effects in T cell proliferation or cytokine production depending on the B7 molecule ligated (76, 77). Data showing contrasting effects between B7-1 and B7-2 stimulation in CD8 T cell development are also contradictory (76, 78, 79); however, Xu et. al. have found less of a dependence on B7-1 in CTL activation (80). Currently, B7-2 appears to be a more important molecule in that it is often found expressed earlier in an immune response (81, 82) and is expressed on murine memory CD4 T cells (83). These latter data suggest that memory cells may be able to costimulate other T cells, resulting in quicker responses to Ag. Although our data do not resolve these issues, they do provide in vivo evidence that CD8 T cells depend more on B7-2 for clonal expansion than B7-1.

Several groups have recently reported that CD40 activation can actually bypass the helper T cell requirement in CTL activation (49-51). Their in vivo and in vitro data have found that CTL killing activity was normally dependent on CD4 T cell function, yet this requirement for T cell help could be circumvented by CD40 ligation. Interestingly, we show that CD40 activation enhances CD8 T cell expansion and delays their subsequent death in vivo. Thus, it may be that more

CTLs are active when CD40 is activated, which will thus generate a greater CTL activity due to the higher numbers of CTLs. Additionally, our data suggest that one way CD40 activation may bypass the T helper cell requirement for CTL function is by keeping the activated CTL alive for longer periods of time so that a greater number of cellular targets can be destroyed. Ridge et al. further found that signaling via B7-1 or B7-2 was necessary, but not sufficient for CTL activity (49). We observed a minor requirement for CD28 ligation in driving CD8 T cell clonal expansion. It may be that the CD28-B7 interaction is less important for SAg-driven CD8 T cell clonal expansion than it is for actual killing activity.

An important question in immunology today is centered around identifying factors that are important in overriding tolerogenic responses. This is important not only as a basic immunological question, but also as a method to achieve better vaccination protocols and prevention of autoimmunity. The use of SAg to study central and peripheral tolerance is widely accepted. Injection of SAg into mice leads to clonal expansion of the reactive T cells followed by profound deletion (16). Here, it is shown that SEA does induce death and that this death can be delayed by coactivating CD40-bearing cells. Ultimately, the frequency of SEA-specific cells was not increased by day 21, regardless of whether anti-CD40 was injected with SEA (Figs. 2.4 and 2.5). The significance of these data is underscored when one considers that the CD40-bearing APCs were indeed well activated since they bore large quantities of B7 and MHC class II, well over that of mock-activated cells (Fig. 2.8 and data not shown). Thus, these data suggest that

APC activation is not enough to permanently override a tolerogenic response. The mechanistic data in this report show unequivocally that CD40 activation drives T cell activation through ligation of CD28. Therefore, the data show that CD28 ligation is not sufficient to force a long-term T cell response in this model. Hence, tolerance induction is the default pathway, and overriding this type of a response cannot be accomplished through Ag stimulation in conjunction with CD28 ligation.

For the most part these data are dissimilar to much of the data that have been garnered in vitro. For years it has been suggested that the difference between long-term immunity and tolerance is ligation of CD28 (84). Many laboratories have shown that Ag stimulation in the absence of CD28 ligation led to anergy, whereas TCR and CD28 stimulation broke this tolerogenic response (85, 86). These data are almost exclusively from in vitro studies and have been very difficult to test in vivo. Recent studies have shown that CD28 ligation occurred when SAg alone was injected into mice (66). Those studies raised several issues that were not addressed until now. First, it was unclear whether B7-1 and/or B7-2 was involved, and the response of CD8 T cells was not addressed in those studies. Moreover, it was unclear whether the amount of CD28 ligation was sufficient. This is an important point since it is known that APCs upregulate B7 after activation. Thus, activated APCs may be able to deliver a qualitatively and quantitatively different signal to cognate T cells. For example, it is possible that ligation of CD28 in response to SAg alone was minimal in the absence of APC activation. In the present study we tested this idea more directly by activating APCs with a potent

agonist mAb specific for CD40. Under these circumstances it is clear that APC activation was profound, and, based on the data shown in Figs. 2.4, 2.5, 2.9 and 2.10, CD28 was ligated significantly over that when SEA alone was injected. Perhaps surprisingly, this treatment was still unable to break tolerance even though clonal expansion was far greater when CD40 was activated. Therefore, CD28 ligation, be it minimal or presumably maximal, with TCR stimulation *in vivo* does not promote long-term T cell survival but only enhances expansion and delays subsequent death in this model.

These data raise the question of what can block T cell deletion. As shown previously, coinjection of bacterial LPS is capable of inhibiting Ag-induced deletion (87). This response was shown to occur independently of CD28 ligation but was profoundly dependent on TNF- α production (66, 87). In contrast, the CD40 mAb response shown here drives CD4 responses almost entirely through CD28 ligation. One possibility is that CD40 activation does not induce as much TNF- α as LPS. Also it is possible that a different pattern of cytokines is produced when comparing the two types of responses. Ultimately, it may be that the combination of TNF- α and CD40 activation may synergize to yield an optimal response. For example, CD40 may drive the clonal expansion phase, and TNF- α may prevent death by delivering a survival signal to the activated cells. These ideas are currently being tested by our laboratory. Nevertheless, these data suggest that vaccine development should rely not only on CD28 ligation or APC activation through CD40, but also on treatments that prime for long-term T cell survival.

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Chapter 3

Danger and OX40 Receptor Signaling Synergize to Enhance Memory T Cell Survival by Inhibiting Peripheral Deletion

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ABSTRACT FOR CHAPTER 3

This report defines a cell surface receptor (OX40) expressed on effector CD4 T cells, which when engaged in conjunction with a danger signal, rescues Ag-stimulated effector cells from activation-induced cell death in vivo. Specifically, three signals were necessary to promote optimal generation of long-lived CD4 T cell memory in vivo: Ag, a danger signal (LPS) and OX40 engagement. Mice treated with Ag or superantigen (SAg) alone produced very few SAg-specific T cells. OX40 ligation or LPS stimulation enhanced SAg-driven clonal expansion and the survival of responding T cells. However, when SAg was administered with a danger signal at the time of OX40 ligation, a synergistic effect was observed which led to a 60-fold increase in the number of long-lived, Ag-specific CD4 memory T cells. These data lay the foundation for the provision of increased numbers of memory T cells which should enhance the efficacy of vaccine strategies for infectious diseases, or cancer, while also providing a potential target (OX40) to limit the number of auto-Ag-specific memory T cells in autoimmune disease.

INTRODUCTION

A major paradigm in current immunological thought is the notion that Ag stimulation alone is not sufficient for the induction of potent and long-lasting immune responses. Support for this paradigm is revealed in studies that show two signals are necessary for T cell growth and cytokine production (1, 2). CD28 and its ligands, B7-1 and B7-2, have emerged as the second signal required for efficient activation of naïve T cells (3-7). Recently, it has been shown that CD28 ligation on T cells during TCR stimulation *in vivo* leads to significant clonal expansion; however, the initial expansion is followed by profound deletion (8). Therefore, it was hypothesized that other signals were necessary to generate and maintain long-lived memory CD4 T cells. The existence of other signals is further supported by the observations that B7 transgenic mice do not spontaneously develop autoimmune disease (9), and CD28 knockout mice are capable of clearing certain pathogenic infections (10).

The danger theory proposed by Matzinger (11) addresses this very complex and controversial issue of immune activation and memory T cell generation. Her hypothesis suggests that a stimulus that facilitates some type of biologic “damage” is critical for the induction of a long-lasting immune response. Furthermore, it is thought that the “danger signal” can promote autoimmune disease under the appropriate conditions, such as mice immunized with adjuvants and myelin components in experimental autoimmune encephalomyelitis (EAE) (12). Elements of the danger signal include factors that promote destruction of tissue and/or

necrotic death of cells (13). These are not the only situations that lead to damage, but they represent examples that can be found in nature. For example, it has been known for some time that bacterial LPS is very capable of promoting severe inflammation, leading to tissue destruction (14, 15). LPS can activate macrophages to produce large quantities of proinflammatory cytokines (TNF- α , IL-1 β and IL-6) that attract and stimulate other inflammatory cell types including T cells (16-18).

LPS can interfere with peripheral tolerance (19), and recent data show that Ag-induced peripheral T cell deletion can be tempered in the presence of LPS stimulation (20). The rescuing effect of LPS occurs independently of CD28 ligation, but is profoundly dependent on TNF- α production. Thus, it is likely that stimuli similar to LPS are danger signals and may be responsible for interfering with tolerance induction. Nevertheless, optimal T cell immunity in vivo is multifactorial and loss of one signal or receptor function may be compensated for by alternate ones.

Other endogenous signals have been reported to influence peripheral deletion, especially those involving members of the TNF receptor family such as Fas, CD40 and 4-1BB (21). OX40 is a member of the TNF receptor family that is expressed primarily on activated CD4⁺ T cells, which when engaged induces a potent costimulatory signal (22, 23). OX40⁺ T cells have been found preferentially within the inflammatory compartments of patients and rodents with autoimmune disease and cancer (24, 25). There is little or no expression of OX40 on T cells in

the periphery. Thus, it is possible that OX40 is exclusively expressed on Ag-stimulated T cells.

At the effector stage auto-Ag-specific T cells recognize Ag and produce large amounts of cytokines, leading to inflammation within a target organ (26). It has been hypothesized that a low percentage of the effector T cells that recognize Ag and produce cytokines will subsequently differentiate into memory T cells. Because, at this stage, the effector T cells become quite susceptible to activation-induced cell death (AICD) (27). Effector T cells are quite sensitive to OX40-specific costimulation as compared with naïve T cells, although the combination of B7 and OX40L signals delivered to naïve T cells was found to be synergistic (28). We hypothesized that a signal delivered through OX40 might inhibit AICD during Ag-specific stimulation of effector T cells, thereby increasing the number of effector T cells that survive and become memory cells. To test this hypothesis, we explored the expression of OX40 and the effects of OX40 engagement on effector T cells in a superantigen (SAg) model system, which is known to result in the deletion of SAg-specific effector T cells. The SAg staphylococcal enterotoxin A (SEA) system allows for convenient detection of SAg-specific T cells with an anti-V β 3 TCR Ab. We also examined effector T cell survival in an adoptive T cell transfer model in which peptide-induced deletion has been observed (29).

MATERIALS AND METHODS

Mice

B10.Br and BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or from the National Cancer Institute (Frederick, MD). DO11.10 transgenic mice were generously provided by Drs. Nancy Kerkvliet and Marc Jenkins (Oregon State University, Corvallis, OR and University of Minnesota, Minneapolis, MN, respectively) (30). All mice were maintained at Oregon State University under specific pathogen-free conditions in accordance with federal guidelines.

Reagents, Abs and flow cytometry

SEA and LPS were purchased from Sigma (St. Louis, MO) and administered to mice as i.p. injections. Chicken OVA (Sigma) was solubilized in balanced salt solution and administered i.p. without adjuvant.

T cells were purified by nylon wool fractionation as described previously (31). Flow cytometry staining was conducted as described previously (8). Briefly, cells were blocked for nonspecific binding and incubated with biotinylated anti-OX40 (32) for 30 min on ice. The cells were washed twice and incubated with anti-CD4-PE (PharMingen, San Diego, CA), anti-TCR V β 3-FITC mAb KJ25-607.7 (33), and Red 613-conjugated streptavidin (Life Technologies, Grand Island, NY). After several washes, the cells were analyzed on an Epics XL flow cytometer (Coulter Electronics, Miami, FL).

Experimental design

The experiment in Fig. 3.2 was set up as follows. One group was noninjected (day 0). The remaining groups received SEA and rat IgG (open circles); SEA and anti-OX40 (filled circles); SEA, LPS and rat IgG (open squares); and SEA, LPS and anti-OX40 (filled squares). On day 0, mice were given 0.15 μ g of SEA and immediately afterward, an injection of 25 μ g of anti-OX40 or rat IgG. On day +1, mice were given a second injection of anti-OX40 or rat IgG, followed immediately by 30 μ g of LPS. On day +2, mice were given a final injection of anti-OX40 or rat IgG. The percent and number of SEA-specific T cells were determined.

The experiments in Tables 3.1 and 3.2 were set up as follows: On days 0 and +2, 500 μ g of OVA in balanced salt solution was injected, followed immediately by injection of 50 μ g of anti-OX40. On days +1 and +3, anti-OX40 was injected, and, immediately after, 50 μ g of LPS was injected i.p. On day +4, a final injection of anti-OX40 was administered. On day 10 or day 62, T cells from lymph node (LN) and spleen were isolated and examined for OVA-specific T cells.

RESULTS

Detection of OX40 expression on Ag-specific T cells

Expression of OX40 by V β 3 T cells was examined after mice were stimulated with SEA. Mice were injected with LPS alone, SEA alone, or SEA and LPS, and T cells were analyzed at 12, 24, 48 and 72 h later. SEA induced OX40

expression on the SEA-specific CD4 V β 3 T cells which peaked at 12 h and declined at 24 and 48 h (Fig. 3.1 shows 48 h time point). Only a small percentage of SEA-specific CD8 T cells expressed OX40, and SEA-unreactive T cells (TCR V β 14) from the same mice showed no increase (data not shown). We also tested whether or not a danger signal alone would be capable of enhancing OX40 surface expression on T cells. Mice were injected with a high dose of LPS and T cells were analyzed as described above. In the absence of SAg stimulation, OX40 expression was not upregulated (data not shown and Fig. 3.1). These data show that TCR stimulation is sufficient to induce OX40 surface expression on SAg-stimulated CD4 T cells in vivo.

Next, we tested whether or not a danger signal in the presence of TCR stimulation in vivo could influence OX40 expression. Mice were injected with SEA and LPS, and after various times OX40 expression on V β 3 and V β 14 T cells was analyzed. It is shown that SEA and LPS increased OX40 expression on the SEA-specific CD4 T cells only (Fig. 3.1). The levels of OX40 on the SEA-specific T cells was significantly higher at 48 h than with either reagent alone (Fig. 3.1) and had prolonged expression compared with SEA (data not shown). As before, staining of V β 14 T cells showed no evidence of upregulation of OX40. Thus, we conclude that a danger signal and TCR stimulation synergized to enhance OX40 expression on SAg-specific CD4 T cells in vivo.

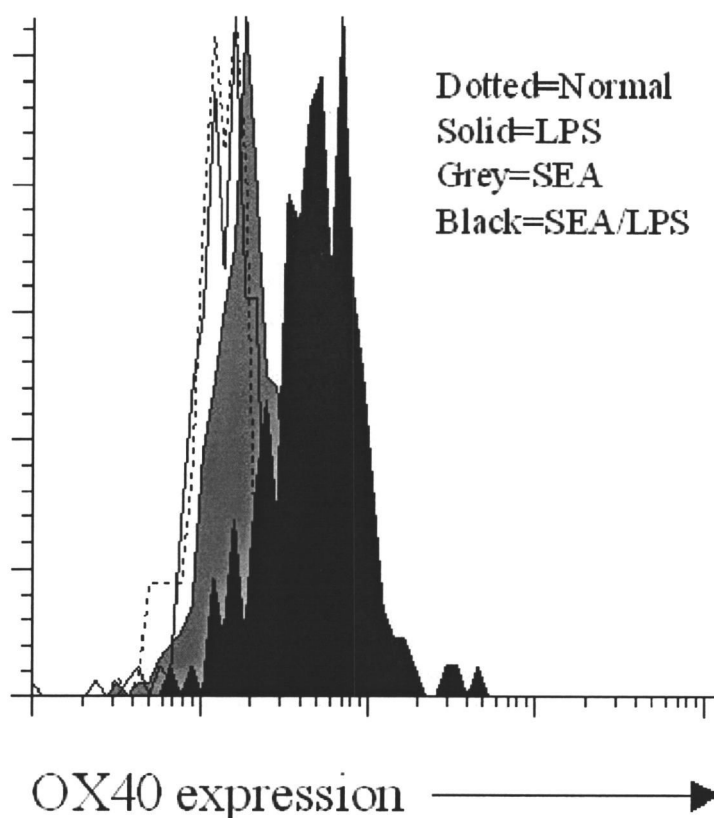


Figure 3.1: OX40 expression on SAg-stimulated T cells is enhanced in the presence of a danger signal. A total of 15 B10.A mice were divided into four groups. The first group was left noninjected. The second group received 0.15 μ g of SEA 12, 24, 36, 48, 60 or 72 h before analysis. The third group received 120 μ g of LPS i.p. 12, 24, 36 or 48 h before analysis. The last group received SEA 36, 48, 60 or 72 h before analysis, and an additional injection of LPS 24 h after the SEA injection. The LN T cells from each mouse were purified and stained with Abs against OX40, V β 3, and CD4 or CD8. Rat IgG staining was also done as a control. Analysis of stained cells was completed by flow cytometry. Histograms representing the expression of OX40 on CD4 V β 3 T cells are shown for mice injected with SEA 48 h and LPS 24 h before analysis. The other time points are not shown. Similar data were generated by staining for OX40 with a soluble OX40-ligand fusion protein.

Evaluating the effects of OX40 ligation on SAg-stimulated T cells during clonal expansion

The SAg model in mice has been used to study central and peripheral T cell tolerance. Early after SAg injection into mice, SAg-activated peripheral CD4 and CD8 T cells expand 2-to 5-fold during a 48 h period (34). This expansion is followed by profound deletion of those same T cells. These measurements are possible because unactivated (V β 14) and activated (V β 3) T cells can be detected over time by analyzing the respective cell populations by flow cytometry.

The hypothesis that ligation of OX40 on SEA-activated T cells will influence peripheral deletion of T cells was tested. Mice were not injected or injected with SEA with a control IgG (SEA); SEA with LPS and control IgG (SEA/LPS); SEA with anti-OX40 (SEA/OX40); or SEA with LPS and anti-OX40 (SEA/LPS/OX40). On days 2, 5, 7 and 12 after SEA injection, the percent and absolute number of LN and spleen CD4 and CD8 T cells were examined for TCR V β 3 expression (Fig. 3.2; LN data not shown). As expected, there was an initial expansion of V β 3⁺ spleen T cells in the SEA-injected mice by day 2. However, by day 5, the number of V β 3 CD4 cells was lower than in noninjected mice, suggesting that deletion of T cells had occurred. The V β 3 T cells remained low in number for the duration of the experiment. V β 3 T cells from mice injected with SEA/LPS or SEA/OX40 had expanded on day 2 to a greater level in the LN (data not shown) than that observed in mice with SEA alone. Nevertheless, for splenic T cells, both treatments resulted in some degree of T cell rescue compared with mice

injected with SEA alone, but there was still profound clonal deletion in all three groups (Fig. 3.2). For example, by day 12, 1.12×10^6 and 1.53×10^6 V β 3 splenic T cells were observed in SEA/OX40- and SEA/LPS-treated mice, respectively, compared with 0.19×10^6 splenic V β 3 T cells in the mice receiving SEA alone.

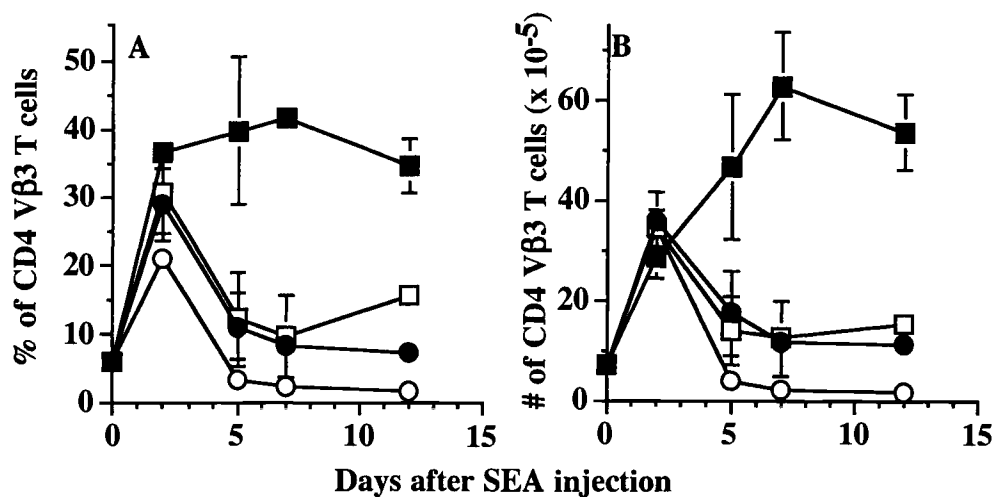


Figure 3.2: OX40 receptor ligation and a danger signal enhance SAg-stimulated splenic CD4 T cell growth and survival in vivo. A total of 52 B10.A mice were put into five groups as indicated in Materials and Methods. One group was noninjected (day 0). The remaining groups received SEA and rat IgG (○); SEA and anti-OX40 (●); SEA, LPS and rat IgG (□); and SEA, LPS and anti-OX40 (■). On days 2, 5, 7 and 12 after SEA injection, the spleen and LNs (data not shown) were removed from three mice per group. Purified T cells were counted, stained with Abs against CD4 and V β 3 and analyzed by flow cytometry. Each point represents the mean percentages (A) and numbers (B) \pm SEM from at least three mice from one representative experiment of four performed.

SAg plus the combination of danger and OX40 engagement, resulted in enhanced expansion and a marked inhibition of peripheral T cell deletion. Instead of the expected decrease in the splenic V β 3 T cells on day 5, we observed a 12-fold increase in the SEA/OX40/LPS-treated mice compared with SEA. This was true

for both percentage and absolute number of V β 3 T cells (Fig. 3.2, A and B, respectively). The V β 3 population continued to increase on day 7 (28.5-fold; Fig. 3.2B). There was also an increase in the CD8 V β 3 T cell population but not to the same degree as the CD4 T cells, and no significant changes were observed in a SEA-nonspecific V β 14 T cell population (data not shown). Collectively, these data show that in vivo engagement of OX40 in the presence of a danger signal block SAg-induced peripheral T cell deletion while promoting effector T cell expansion.

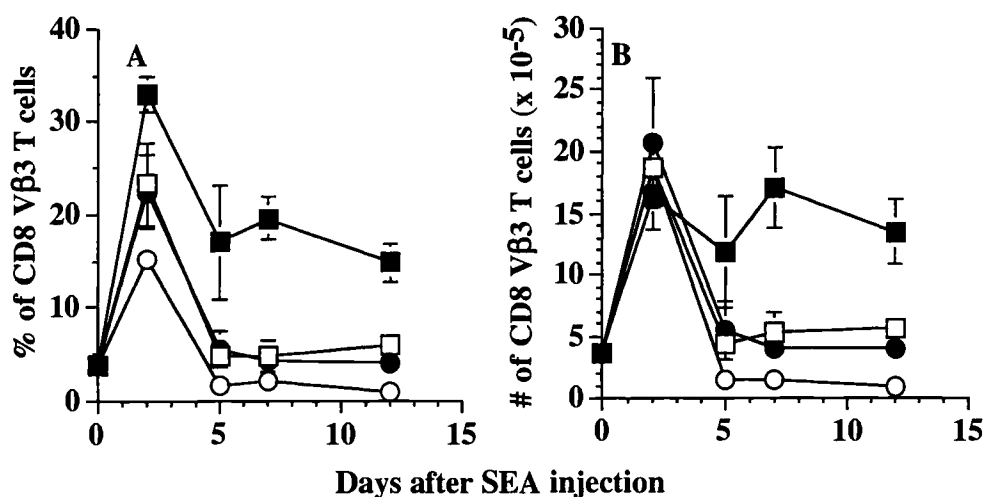


Figure 3.3: OX40 receptor ligation and a danger signal enhance SAg-stimulated splenic CD8 T cell growth and survival in vivo. These data are taken from the experiment shown in Fig. 3.2, except that cells were stained for CD8 V β 3 expression. Day 0 represents noninjected mice. The remaining groups received SEA and rat IgG (○); SEA and anti-OX40 (●); SEA, LPS and rat IgG (□); and SEA, LPS and anti-OX40 (■). Each point represents the mean percentage (A) and absolute number (B) \pm SEM from at least three mice from one representative experiment of four performed.

There also appeared to be rescue of CD8 T cells even though their level of OX40 expression was less compared with the CD4 T cells (Fig. 3.3). There was a

peak of expansion on day 2, both in terms of percentages and absolute numbers, but thereafter the response waned. Nevertheless, there was significant CD8 survival when animals were treated with all three signals, which was somewhat perplexing since there was little OX40 expression on the SEA-activated CD8 T cells (data not shown).

Three signals induce T cell memory development

To characterize the effects of OX40 engagement during presentation of peptide Ag, we examined the well-characterized model DO11.10 TCR transgenics for tracking peptide/MHC class II-activated CD4 T cells (29). T cells from the DO11.10 TCR transgenic mice recognize OVA in the context of Ia^d and the OVA-specific T cells can be detected with the anti-idiotypic Ab KJ1-26. DO11.10 TCR transgenic T cells were transferred into five groups of mice and thereafter treated with OVA, OVA with anti-OX40 (OVA/anti-OX40), OVA with LPS (OVA/LPS), or all three (OVA/anti-OX40/LPS) and compared with noninjected mice (no OVA). Seven days after Ag exposure, T cells were analyzed for the absolute number of DO11.10-bearing cells in the LN and spleen (Table 3.1).

Injection of OVA alone did not increase the number of Ag-specific T cells at day 7 postimmunization. Based on previous experiments, we believe that inspection of earlier time points would have shown an increase in the DO11.10 T cell population (data not shown). Coinjection of LPS and OVA did rescue some of the DO11.10 T cells from deletion compared with the OVA group, but this effect was minimal compared with the number of Ag-specific T cells on day 7 in the

Table 3.1: OX40 costimulation and danger promote optimal clonal expansion of Ag-specific T cells in vivo*

Treatment	# of CD4 KJ1-26 T cells (x 10 ⁵)	
	Spleen Cells	LN Cells
No OVA	1.34 ± 0.08	0.42 ± 0.04
OVA alone	1.26 ± 0.34	0.41 ± 0.01
OVA/anti-OX40	22.31 ± 6.82	2.10 ± 0.58
OVA/LPS	1.92 ± 0.19	0.50 ± 0.11
OVA/LPS/anti-OX40	42.63 ± 14.12	3.96 ± 1.15

* On day -1, 3.3 or 3.8 x 10⁶ CD4⁺ KJ1-26⁺ T cells from DO11.10 TCR transgenic mice were adoptively transferred into individual BALB/c mice that had been separated into five groups as described in Materials and Methods. The cells were counted and stained for CD4 and the OVA-specific TCR using the clonotypic mAb KJ1-26. Analysis was done using flow cytometry. The KJ1-26⁺ cells were transferred as bulk LN and spleen cells by i.v. injection and back-calculated for the number of DO11.10 T cells transferred. All other injections were i.p. The results represent the mean number of cells ± SEM from two experiments with a combined total of four mice per group.

OVA/anti-OX40 group. There was a 17.7-fold increase in the number of splenic KJ1-26⁺ T cells in the OVA/anti-OX40 mice compared with OVA alone.

Furthermore, there was an even greater increase of DO11.10 T cells in the OVA/LPS/anti-OX40 mice (33.8-fold over the OVA-alone mice). These observations held true for both splenic and LN T cells but the enhancement was greater for the spleen population. Although the data show that OX40 had a significant effect on T cell survival, there continues to be additional benefit when LPS and OX40 are combined during a peptide Ag-specific T cell response.

The final experiments were designed to examine the long-term effects on Ag-specific T cells that have been activated via OX40 engagement in the presence of a danger signal. The DO11.10 T cells were adoptively transferred into

thymectomized BALB/c recipients that were used to prevent peripheral T cell repopulation by the thymus. Mice were treated as described in Table 3.1 and tested for DO11.10-bearing T cells 62 days after exposure to Ag (Table 3.2). Our data show that Ag stimulation followed by OX40 engagement enhanced growth and long-term survival of the DO11.10 T cells. The addition of LPS further enhanced this effect. A 12.3-fold increase was observed with Ag and OX40 engagement as compared with Ag alone, and a striking 59.8-fold increase in long-term surviving T cells was observed when both anti-OX40 and LPS were used to stimulate the OVA-specific T cells.

Table 3.2: Optimal long-term memory T cell survival of Ag-activated CD4⁺ T cells is obtained when OX40 engagement occurs in a proinflammatory environment*

Treatment	# of CD4 KJ1-26 T cells (x 10 ⁴)	
	Spleen Cells	LN Cells
No OVA	2.62 ± 0.91	1.76 ± 0.17
OVA/IgG	3.21 ± 1.54	1.26 ± 0.59
OVA/anti-OX40	39.63 ± 20.25	7.63 ± 4.67
OVA/LPS/IgG	5.24 ± 0.19	1.88 ± 0.11
OVA/LPS/anti-OX40	191.85 ± 30.92	12.06 ± 2.23

* On day -1, 3.6 x 10⁶ DO11.10 T cells were injected into thymectomized BALB/c recipients. Five groups were organized and injected as described in Table 3.1 using 500 µg of OVA, 50 µg of anti-OX40 or rat IgG, and 40 µg of LPS. On day 62 after the initial OVA injection, the LN and spleen T cells were isolated. Cells were counted, stained with anti-CD4 and KJ1-26 Abs, and analyzed by flow cytometry. These data represent mean numbers ± SEM from one representative experiment of two involving three mice per group.

Phenotypic analysis of the OVA-specific T cells (KJ1-26⁺) in the OVA/LPS/anti-OX40 mice revealed that they were small, resting memory cells as assessed by low forward scatter and upregulation of CD44 expression (Fig. 3.4). In

contrast, the KJ1-26⁻ cells had a broad range of CD44 expression (4-fold lower in mean channel fluorescence) than that observed for the OVA-specific T cells.

Therefore, we conclude that the optimal development of long-lived memory T cells required three signals during effector T cell stimulation: Ag, OX40 costimulation, and danger (LPS).

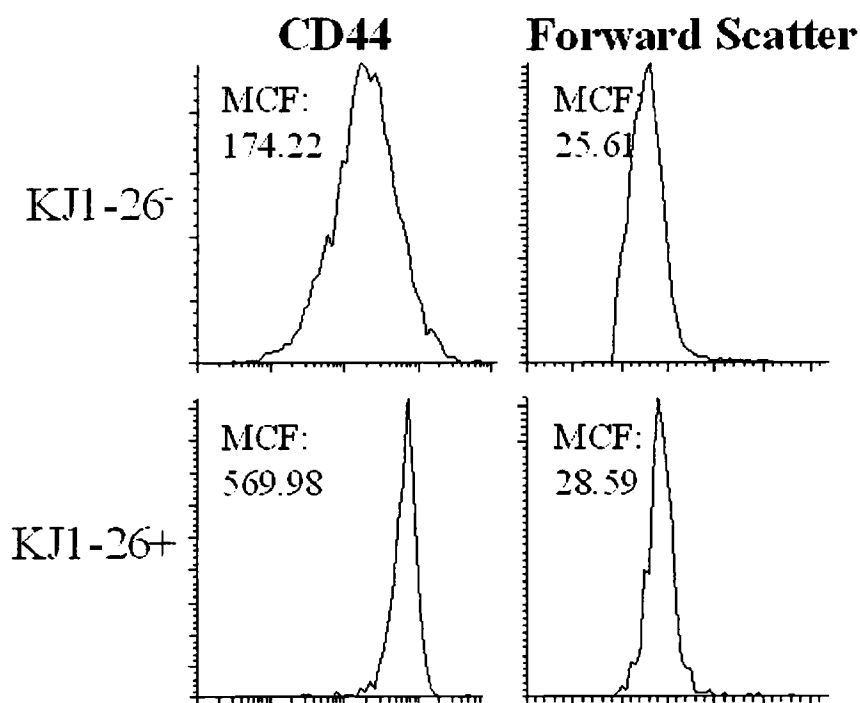


Figure 3.4: Surviving Ag-stimulated T cells express a memory phenotype in vivo. KJ1-26⁺ T cells from mice injected with OVA/LPS/anti-OX40 from the experiment described in Table 3.2 were stained for CD44 expression and examined by flow cytometry. Histograms representing CD44 expression and forward scatter are shown for the KJ1-26⁺ or KJ1-26⁻ T cell populations. Mean channel fluorescence (MCF) for each peak is listed. Data are representative of two separate experiments.

DISCUSSION

For years the mechanism of memory T cell acquisition has been vigorously studied and hotly debated (35, 36). Much emphasis has been dedicated to defining the phenotype, activation requirements, and lifespan of memory cells.

Nevertheless, these parameters are not very well defined as compared with the data gathered on B cells. In part this is due to the fact that naïve B cells are readily distinguished from memory cells by the type of surface Ig they express. Memory T cells do not change their Ag receptors nor does the affinity increase after antigenic challenge, which is in contrast to B cells. The data presented in this study are focused on the requirements necessary for optimal memory CD4 T cell generation.

Our data clearly show that two signals are better than one and that three signals are better than two. Nevertheless, what is key is that they are three different signals. Of course Ag is signal 1, signal 2 is a growth signal such as that observed with OX40, and signal 3 is a danger signal, which in this study is LPS. Although signals 2 and 3 are somewhat interchangeable, they seem to synergize when activated concomitantly during an antigenic response. For example, LPS can enhance growth at high doses (37), and OX40 can certainly increase survival without LPS (see Table 3.2); but when combined the greatest amount of long-term survival is observed. One important difference between costimulatory or growth signals and danger signals is that LPS alone can induce shock at very low doses, whereas high doses of anti-OX40 alone does not (our unpublished observations).

These data suggest that CD4 T cell memory development can be obtained by more than one set of parameters.

We show that optimal T cell memory acquisition requires three signals at refined doses, but nevertheless suboptimal survival was obtained without LPS or without OX40 stimulation (Fig. 3.3 and Table 3.2). Additionally, it is clear that LPS can synergize with CD40 stimulation to generate profound SAg-specific T cell survival; however, each signal individually with SAg is far less effective (our unpublished data). This latter model is totally dependent on CD28/B7 ligation for growth (8). Interestingly, two costimulatory signals through OX40 and CD40 (B7/CD28) were not sufficient to block peripheral deletion of SAg-stimulated T cells (data not shown), which strongly suggests that two costimulatory signals are not sufficient to substitute for one survival signal.

It is clear from our data that survival was not limited to CD4 T cells. For example we show that CD8 T cells can also be rescued from deletion even though OX40 expression was far less on activated CD8 T cells than that observed on activated CD4 T cells (Fig. 3.3). One possibility is that OX40 ligation and Ag stimulation prime CD4 T cells to secrete large quantities of cytokines which activate bystander CD8 T cells to survive. Alternatively, it is possible that the low levels of OX40 on the surfaces of CD8 T cells were in enough quantity to be ligated and thereby promote rescue. Nevertheless, this is a very complex problem that is currently being investigated.

A role for cytokines in this system is likely to be paramount to the rescuing process. It was previously shown that LPS could rescue T cells from SAg-induced deletion through the action of TNF- α and a minor role for IFN- γ (20). It is still unclear how these cytokines promote long-term survival, especially in light of the fact that TNF- α has also been implicated in driving T cell death (38). This also seems to be the case for CD95 which has been shown to be a very important death signal in a variety of systems (39), but has also been shown to promote growth in others (40). Therefore, it is likely that the cytokine environment influences activated T cells to respond with a survival or death response depending on the variety and balance of cytokines. This has been clearly observed in other systems including Th1 and Th2 skewing.

Proinflammatory cytokines are not the only cytokines that promote T cell survival. Several ligands specific to members of the common γ -chain family of receptors have also been implicated in mediating T cell survival (41, 42). Of these, IL-4, which is clearly not proinflammatory, can block Ag-induced death and spontaneous death of nonactivated "fresh" T cells. Oddly enough, TNF- α does not seem to block death in vitro of these same cell types (data not shown). These data argue that the mechanism of survival induction is different between TNF- α and IL-4. IL-4-induced T cell survival is definitely dependent on the common γ -chain for rescue (data not shown), whereas it is not clear whether TNF- α rescues Ag-activated T cells directly. For example, under the appropriate circumstances, activated T cells may bind TNF- α , which in turn may inhibit an activated death

program. Alternatively, it is possible that TNF- α induces other factors to block various death pathways.

Of particular interest in this regard is IL-6. IL-6 is involved in acute phase responses, B cell stimulation, T cell activation, hematopoiesis and many other functions (43). In particular, however, IL-6 has been shown to block spontaneous death on resting T cells (44). This result is consistent with the fact that IL6 $-/-$ mice have a diminished number of peripheral T cells (45). Therefore, it is possible that a cytokine like IL-6, which is induced by TNF- α , may mediate survival. Preliminary studies have shown that IL-6 does not block activation-induced death in vitro (data not shown), and others have shown that IL-6 administration in vivo only minimally affects T cell rescue from deletion (46). Once again, it may be that a mixture of cytokines in the right proportions can influence memory acquisition. Therefore, individually they are ineffective but in combination are substantially effective.

Other cytokines induced by TNF- α are also possibilities and include IL-1 β and IL-12. By itself, IL-1 β has been shown to block deletion (47), but it is not known whether this cytokine directly inhibits death by binding to T cells or inhibits deletion by an indirect method. Previous reports have shown that IL-12 can stimulate Th1 differentiation during a tolerogenic response but does not block deletion by itself (46, 48). Furthermore, it has been firmly established that OX40 stimulation potentiates cytokine production on effector T cells (27). Specifically, it has been shown that Th1 and Th2 cytokine production can be enhanced by cross-linking OX40 on Ag-stimulated T cells (23, 49). Finally, it is clear that resolving

the survival mechanism in vivo will certainly involve cytokines and chemokines, surface receptors, non-T cells, and possibly factors from the endocrine system.

These data show that peptide-specific T cells are very responsive to OX40 ligation in combination with a danger signal, suggesting that this treatment may significantly improve the efficacy of vaccines designed to activate T cells. A major limiting factor in vaccine development has largely been the identification of a practical adjuvant that is efficient. Because most adjuvants would be too toxic for human use, due to massive inflammatory reactions, it is likely that a more refined targeted treatment will be necessary. The OX40 protein is an excellent target because it is expressed only on recently activated Ag-specific cells, which are primarily found at the site of inflammation (24, 50). Perhaps the danger mechanism described in this study (i.e. upregulation of OX40 expression on Ag-activated T cells by LPS) helps explain the positive effects adjuvants exert on activated T cells and may lead to more defined approaches for vaccination protocols.

Collectively, these data suggest that costimulation in the absence of danger can lead to Ag-dependent clonal expansion, but does not elicit the same magnitude of long-term T cell survival when compared with the same response elicited in the presence of a danger signal. These data may help explain why B7 transgenic mice do not develop autoimmune diseases spontaneously, and why the same mice crossed with TNF- α transgenic mice develop autoimmune disease (9).

Additionally, it is widely accepted that TNF- α is an important effector molecule in

the development of EAE (51). Recently, it has also been shown that OX40 and OX40-ligand expression is found on T cells and activated macrophages, respectively, within the inflamed tissue of rodents with clinical signs of EAE (our unpublished data). Thus, it is possible that inflammatory cytokines like TNF- α promote the appropriate activation of autoreactive memory T cells via OX40 upregulation.

Our data support a new model of T cell activation that incorporates three definable signals. In this study, we suggest that one set of optimal conditions for long-term survival of Ag-specific T cells requires three signals, which include an antigenic stimulus, OX40 stimulation, and activation by LPS.

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Chapter 4

Memory T Cell Development Does Not Require Proinflammatory Cytokines or NF- κ B Activation, but is Sensitive to Cyclosporin A

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ABSTRACT FOR CHAPTER 4

The requirements for inducing memory T cell development are poorly understood. Although two signals (antigen and costimulation) are necessary to drive optimal T cell clonal expansion, few memory T cells remain after the response wanes. Inclusion of adjuvant treatment, however, can greatly enhance T cell responses and memory development. The adjuvant lipopolysaccharide (LPS) is very potent at generating increased numbers of long-lived antigen-specific T cells, but its mechanism of action is not fully understood. When combined with two-signal stimulation, LPS greatly enhances T cell survival. This survival is not dependent on the cytokines TNF- α , IL-1 β , IL-6 and IFN- γ . Additionally, the transcription factor NF- κ B, although important for inflammatory and LPS signaling, is surprisingly necessary for short-term survival, but not long-term survival. The immunosuppressant cyclosporin A (CsA) did prevent survival, an observation that may perhaps help to narrow the search for the mechanism by which LPS keeps T cells alive.

INTRODUCTION

One of the most significant concepts in current immunological thought is the two-signal hypothesis first proposed by Bretscher and Cohn (1). After modifications by others, it is generally believed that specific recognition of antigen (Ag)/MHC complexes by the T cell receptor (TCR) is not enough to drive a productive and long-lasting T cell response (2, 3). Only concurrent delivery of a costimulatory signal to the T cell will facilitate rapid clonal expansion of the Ag-specific T cell population and differentiation of those cells into effectors (4-6). Although CD28 is the most well studied costimulatory molecule, ligation of many other cell surface molecules such as CD40, OX40 and 4-1BB has been described as promoting enhanced T cell clonal expansion in vivo (7-9). Once the Ag has been cleared, the majority of the responding T cells undergo apoptosis, leaving behind a small cohort of memory T cells that can respond much faster and more potently upon Ag reexposure (10, 11).

Although the two-signal hypothesis effectively describes T cell activation, it does not fully address how long-lived memory T cells develop and stay alive. It is certain that memory T cell responses can develop after two-signal stimulation (8, 12), but it is not yet clear what specifically directs the T cells into this surviving state. A variety of poorly understood compounds known as adjuvants can enhance an immune response and may be central to uncovering how T cells survive.

Lipopolysaccharide (LPS) is well known for having adjuvant properties. LPS is a component of Gram-negative bacterial cell walls that induces potent

inflammatory responses (13). Binding of LPS to CD14 or toll-like receptors on cells triggers the secretion of reactive oxygen species and inflammatory cytokines such as TNF- α , IL-6 and IL-1 β (14, 15). Additionally, LPS can activate the transcription factor NF- κ B, a transcription factor that is well documented in its ability to trigger cellular proliferation and cytokine secretion among many other effects (16, 17).

LPS was previously shown to promote the long-term survival of T cells stimulated with the superantigen (SAg) staphylococcal enterotoxin A (SEA) (18). This survival effect could develop in the absence of CD28 signaling and was at least partially dependent or partially substituted by proinflammatory cytokines like TNF- α , IL-1 β , and IFN- γ (19).

Subsequent work with LPS found that although it promoted significant levels of T cell survival, its effects could synergize with costimulation through OX40 in the presence of either SEA or ovalbumin as the Ag (8). Delivery of these three signals not only enhanced T cell expansion compared to delivery of two signals in the form of Ag and costimulation, but it significantly enhanced the number of T cells expressing a memory phenotype for at least two months. Thus, delivery of an adjuvant such as LPS in the context of the two-signal model can potentiate T cell clonal expansion, but even more importantly, can drive the long-term survival of those responding cells.

In this paper, we sought to better understand the mechanism of how LPS was working in a three-signal model of T cell stimulation involving SAg (signal

1), CD40 stimulation (signal 2), and LPS (signal 3). SAgS like SEA have proven very useful for studying the clonal expansion and tolerance of T cells in vivo (20, 21). Their specificity for T cells possessing particular β -chains makes it easy to track the T cells responding to the SAg in vivo (22). CD40 is a member of the TNF receptor (TNFR) family that is found primarily on APCs (23-25). Signaling via CD40 can enhance the activation and survival of APCs during an immune response (26-28). SEA and CD40 stimulation together enhance T cell clonal expansion in a CD28-dependent manner, but the majority of the Ag-specific T cells are still deleted afterwards (7).

As expected, LPS stimulation in conjunction with SEA and CD40 stimulation markedly enhanced T cell survival, much like that observed with OX40. Additionally, we unexpectedly found that proinflammatory cytokines such as TNF- α , IL-1 β , IL-6 and IFN- γ are not necessary for inducing T cell survival, and in fact, may be somewhat detrimental to the response. Additionally, the transcription factor NF- κ B, known to be very important for LPS, cytokine and other signaling pathways is also not required for long-term T cell survival in vivo. The enhanced generation of long-lived T cells in this model, however, is sensitive to cyclosporin A (CsA) treatment. CsA is a potent immunosuppressant that most strongly affects T cells via inhibition of calcineurin (29, 30). The result of this inhibition is a block in the activity of transcription factors such as NFAT (31-33). Ultimately, the inhibition observed in this model might help to elucidate the

mechanism of how three-signal stimulation can drive optimal T cell survival and lend a clue in constructing a basic blueprint for vaccine development.

MATERIALS AND METHODS

Mice

B10.A mice were purchased from the National Cancer Institute (Frederick, MD). C57BL/6, TNFR1 KO, TNFR2 KO, TNFR1/II double KO (34), and B6, D2-TgN(LCK-NFKBIA)5Dwb mice (35) (hereafter referred to as I κ B Tg mice) were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in an animal facility at Oregon State University under specific pathogen-free conditions in accordance with federal guidelines. All mice were between 6 and 12 weeks of age.

Reagents, mAbs, and flow cytometry

SEA, SEB, LPS and rat IgG were purchased from Sigma (St. Louis, MO) and administered to mice as intraperitoneal (i.p.) injections in balanced salt solution (BSS) or phosphate buffered saline (PBS). The recombinant human IL-1Ra was a gift from Amgen Corp. (Thousand Oaks, CA).

The anti-CD40 mAb producing hybridoma FGK45.5 was a kind gift from Dr. A. Rolink (Basel Institute, Switzerland) (36). The anti-IFN- γ producing hybridoma XMG1.2 (37) was obtained from ATCC (Manassas, VA). The anti-TNF- α producing hybridoma MP6-XT22 (38) and the anti-IL-6 producing

hybridoma MP5-20F3.11 (39) were both obtained from DNAX (Palo Alto, CA) via ATCC. Supernatants from each of the above hybridomas were purified over protein G agarose (Life Technologies, Grand Island, NY) and dialyzed against PBS for injection.

For flow cytometric staining, antibodies purchased from BD PharMingen (San Diego, CA) were used: anti-CD4 was conjugated to either PE or APC, anti-CD8 was conjugated to either PE or APC and anti-TCR V β 8 was conjugated to FITC. The anti-TCR V β 3 mAb KJ25-607.7 (40), was purified from hybridoma supernatant over protein G agarose (Life Technologies,) and conjugated to FITC as described previously (41). Briefly, purified antibody was dialyzed against 0.1M NaHCO₃, pH 9.4 to 9.6. Protein concentration was adjusted to 1 mg/ml and incubated with FITC-Celite (Sigma) for 30 min at room temperature. Free celite was removed by centrifugation and the antibody was dialyzed against PBS for use.

Injection schedule

All injections were i.p. The injection of the SAgS SEA and SEB was considered day 0. Injection of anti-CD40 mAb was done two days prior to SAg injection. LPS was always injected 24 hours after the SAgS (day +1). The anti-OX40 mAb was injected 24 and 36 hours after SAg injection. For the cytokine blocking experiments in figures 4.7 and 4.8 we used neutralizing mAbs and other reagents including anti-TNF- α , IL-1Ra, anti-IL-6, anti-IFN- γ or rat IgG. These reagents were injected 20 and 30 hours after SEA.

Cell processing and flow cytometry

Spleens and peripheral lymph nodes (inguinal, axillary, and bronchial) were teased through nylon mesh sieves (Falcon, BD PharMingen) and red blood cells lysed with ammonium chloride. After washing, cells were counted with a Z1 particle counter (Beckman Coulter, Miami, FL) and spleen cells were further purified over nylon wool as described previously (42). Briefly, 3-cc syringes were filled with 0.12 to 0.15 g of washed and brushed nylon wool. The columns were prepared with warm BSS 5% FBS, after which the cells were loaded in a 0.5 ml volume and incubated for 30 min at 37 °C. After draining 0.5 ml away, the columns were incubated an additional 30 min, followed by elution with BSS 5% FBS.

For two- and three-color staining, cells were incubated on ice with the primary Abs in the presence of 5% normal mouse serum, culture supernatant from hybridoma cells producing an anti-mouse Fc receptor mAb, 2.4.G2 (43), and 10 µg/ml human γ -globulin (Sigma) to block nonspecific binding. After a 30 min incubation on ice in staining buffer (BSS, 3% FBS, 0.1% sodium azide) with primary Abs, the cells were washed twice and analyzed by flow cytometry, or, if a secondary reagent was necessary, the incubation and wash procedures were repeated. Flow cytometry was conducted on a Becton Dickinson FACSCaliber flow cytometer, and the data analyzed using CellQuest software (BD PharMingen).

Bromodeoxyuridine (BrdU) staining

Mice were injected with 60 μ g of SEB, 0.20 mg of anti-CD40, and 10 μ g of LPS at the times described above. Additionally, the mice were injected with 1 mg of BrdU (Sigma) dissolved in PBS on days 0, 1 and 2. On day 3, T cells from the peripheral LNs and spleens from the treated mice were isolated, stained with biotin-conjugated anti-TCR V β 8 mAb and then with PE-conjugated streptavidin (BD PharMingen). The cells were then stained with a modified BrdU staining protocol (44). Briefly, the cells were dehydrated and fixed in ice cold 95% ethanol, then fixed in BSS containing 1% paraformaldehyde and 0.01% tween-20. Next, cellular DNA was lightly digested with 50 Kunitz units of DNase I (Sigma) and the cells stained with anti-BrdU-FITC (BD PharMingen).

In vitro proliferation

Mice were injected with 0.30 μ g of SEA, 0.25 mg of anti-CD40 mAb and 10 μ g of LPS as described above. Ten days after SEA injection, spleen cells were isolated for culture. Cells from each in vivo treatment group were plated in triplicate at 5.0×10^5 , 2.5×10^5 , 1.25×10^5 and 0.63×10^5 cells per well in complete tumor media (CTM). CTM consists of minimal essential medium with FBS, amino acids, salts and antibiotics. SEA was added to the wells at a concentration of 1 μ g/ml. The cultures were left for 72 hours with 1 μ Ci of 3 H-thymidine (ICN, Costa Mesa, CA) being added for the last eight hours. Incorporation of 3 H-thymidine

was measured on a 1450 Microbeta Trilux Scintillation Counter (Wallac, Turku, Finland).

Reverse transcriptase PCR

B10.A mice were injected with 0.25 mg of anti-CD40, 0.30 µg of SEA, and 10 µg of LPS as described. At the time points corresponding to 0.5, 1.5 and 6 hours after LPS injection (or 24.5, 25.5, and 30 hours after SEA injection), peripheral LNs and spleens were removed and total RNA was prepared from the cell suspensions using RNAwiz according to the manufacturer's suggested protocol (Ambion, Austin, TX). Synthesis of cDNA was performed with 1 µg of RNA using the Reverse Transcriptase System (Promega, Madison, WI) and accompanying protocol. Aliquots of cDNA were amplified with the GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT). Amplification conditions consisted of a 4 min denaturation step at 94 °C, followed by 30 cycles of amplification (95 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min) and a final extension at 72 °C for 7 min. PCR products were electrophoretically separated on a 1% agarose gel containing ethidium bromide. The polycompetitor plasmid PQRS (45), which contains cDNA sequences for each cytokine amplified, was used as a positive control. The sequences of the primers are as follows: HPRT, 5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3' and 5'-GAG GGT AGG CTG GCC TAT AGG CT-3'; TNF- α , 5'-GTT CTA TGG CCC AGA CCC TCA CA-3' and 5'-TAC CAG GGT TTG AGC TCA GC-3'; IL-1 β , 5'-AAG CTC TCC ACC TCA ATG GAC AG-3' and 5'-CTC AAA CTC CAC TTT GCT CTT GA-3'; IL-2, 5'-

TCC ACT TCA AGC TCT ACA G-3' and 5'-GAG TCA AAT CCA GAA CAT GCC-3'; IL-6, 5'-CCT CTG GTC TTC TGG AGT ACC AT-3' and 5'-GGC ATA ACG CAC TAG GTT TGC CG-3'; IL-15, 5'-ACT GAC AGT GAC TTT CAT CCC A-3' and 5'-GTG CTG CCT CTG AGC AGC AGG-3'; IFN- γ , 5'-CAT TGA AAG CCT AGA AAG TCT G-3' and 5'-CTC ATG AAT GCA TCC TTT TTC G-3'. All primers were made by the Central Services Laboratory at Oregon State University, Corvallis, OR.

Sub-cellular fractionation

Combined LN and spleen cells from one C57BL/6 and four I κ B transgenic mice were cultured overnight at a concentration of 10×10^6 cells/ml in CTM (minimal essential medium, FBS, amino acids, salts and antibiotics) in the presence of 2 μ g/ml ConA. Additionally, one naïve C57BL/6 mouse was cultured at the same concentration but without ConA. T cells were purified over nylon wool to between 50 and 90% purity.

Nuclear and cytoplasmic extracts were prepared as described (46). Briefly, cell pellets were resuspended in sucrose buffer (0.32 M sucrose, 3 mM CaCl₂, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM Mg Acetate, 1 mM DTT, 0.5 mM PMSF) with 0.5% (vol/vol) IGEPAL by gentle pipetting and centrifuged. To the cytoplasmic fraction, 0.22 volumes of 5X cytoplasmic extraction buffer (0.15 M HEPES, 0.7 M KCl, 0.015 M MgCl₂) was added. The cytoplasmic fraction was then centrifuged at 15,000 rpm in a microcentrifuge, the supernatant was transferred to a fresh tube containing 25% (vol/vol) glycerol and stored at -80 °C.

The nuclei were washed twice in sucrose buffer without IGEPAL. Nuclei were then resuspended in low salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM MgCl_2 , 0.02 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) and then one volume of high salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM KCl, 0.2 mM EDTA 1% IGEPAL, 0.5 mM DTT, 0.5 mM PMSF) was carefully added in 1/4 increments. Nuclei were incubated on ice for 30 minutes, diluted 1:2.5 with diluent (25 mM HEPES, 25% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) and centrifuged at 15,000 rpm in a microcentrifuge at 4 °C. Nuclear lysates were stored at -80 °C.

DNA binding assay

Electrophoretic mobility shift assays (EMSAs) were used to assess sequence specific binding of DC2.4 nuclear NF- κ B/Rel to DNA (46). Briefly, a synthetic 20-bp consensus κ B-RE probe (upper strand 5'-GAT CGG CAG GGG AAT TCC CC-3' and lower strand 5'-GAT CGG GGA ATT CCC CTG CC-3') was labeled with [α - 32 P]dATP using Klenow fragment (Invitrogen, Carlsbad, CA) and used for DNA binding assays. Nuclear extracts were prepared as described above. Samples (5 μ g) were incubated with binding buffer (12 mM HEPES, pH 7.3; 4 mM Tris-HCl, pH 7.5; 100 mM KCl; 1mM EDTA; 20 mM DTT; 1 mg/ml bovine serum albumin), 4 μ g poly dI-dC (Amersham Pharmacia, Piscataway, NJ) and 100,000 cpm of 32 P-labeled κ B-RE for 20 minutes at room temperature. Anti-RelA, anti-RelB, anti-c-rel, anti-p50 and anti-p52 was added to the reaction mixture according to manufacturer's instructions (Santa Cruz Biotech, Santa Cruz, CA) and

incubated for 10 minutes at room temperature. Samples were analyzed on a 5% polyacrylamide gel in 0.5% TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) and visualized by autoradiography.

Immunoblotting

Cytoplasmic extracts were subjected to SDS-PAGE as described (47).

Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol using a Genie Electrobloetter (Idea Scientific, Inc., Minneapolis, MN). Membranes were blocked overnight at 4 °C in TBS (25 mM Tris, pH 7.4, 150 mM NaCl) containing 5% nonfat dry milk (NFDM). Antibodies were diluted in TBS containing 1% NFDM and the membranes were incubated with primary antibodies for at least 1.5 hours at room temperature. Anti-I κ B α IgG (Santa Cruz Biotech) and HRP-conjugated secondary antibodies, donkey anti-rabbit IgG (Amersham Pharmacia) were used according to the manufacturer's instructions. Following each antibody treatment, blots were washed in TBS containing 0.05% Tween-20. Antibody complexes were visualized with chemiluminescent reagents (Pierce, Rockford, IL).

RESULTS

LPS and anti-CD40 synergize to enhance Ag-specific T cell survival

Our previous work with CD40 stimulation found that agonistic anti-CD40 mAb treatment could enhance SEA-mediated T cell expansion *in vivo*; however, it

could not keep the responding T cells alive for very long (7). After two to three weeks, Ag-specific T cell numbers declined to levels observed in mice treated with SEA alone. This death process was not prevented, only delayed.

A very similar trend was observed during treatment with an anti-OX40 agonistic mAb (8). OX40 stimulation enhanced Ag-specific T cell expansion, and much like CD40 stimulation, could only yield weak long-term survival. However, injection of LPS into mice treated with both Ag and anti-OX40 mAb dramatically enhanced T cell survival beyond two months.

With these observations, we hypothesized that LPS injection would prevent the death of T cells from mice treated with SEA and anti-CD40 mAb. Thus, mice were injected with the anti-CD40 agonistic mAb, SEA and LPS. At various time points after Ag treatment, T cell populations were examined in the lymphoid tissues. Fig. 4.1 is a time course showing the clonal expansion and survival of splenic CD4 V β 3 (Fig. 4.1, A and C) and CD8 V β 3 (Fig. 4.1, B and D) T cells stimulated by a combination of SEA, anti-CD40 mAb and LPS.

In mice treated with SEA alone, CD4 V β 3 T cell percentages (Fig. 4.1A) and numbers (Fig. 4.1C) declined dramatically on day 5 below their starting levels. Co-injection of either anti-CD40 mAb or LPS with SEA produced increased percentages and numbers of T cells on day 5, with LPS producing more potent T cell expansion and short-term survival than CD40 (CD28-mediated) stimulation. By day 14, however, T cells from these SEA/anti-CD40 or SEA/LPS treated mice

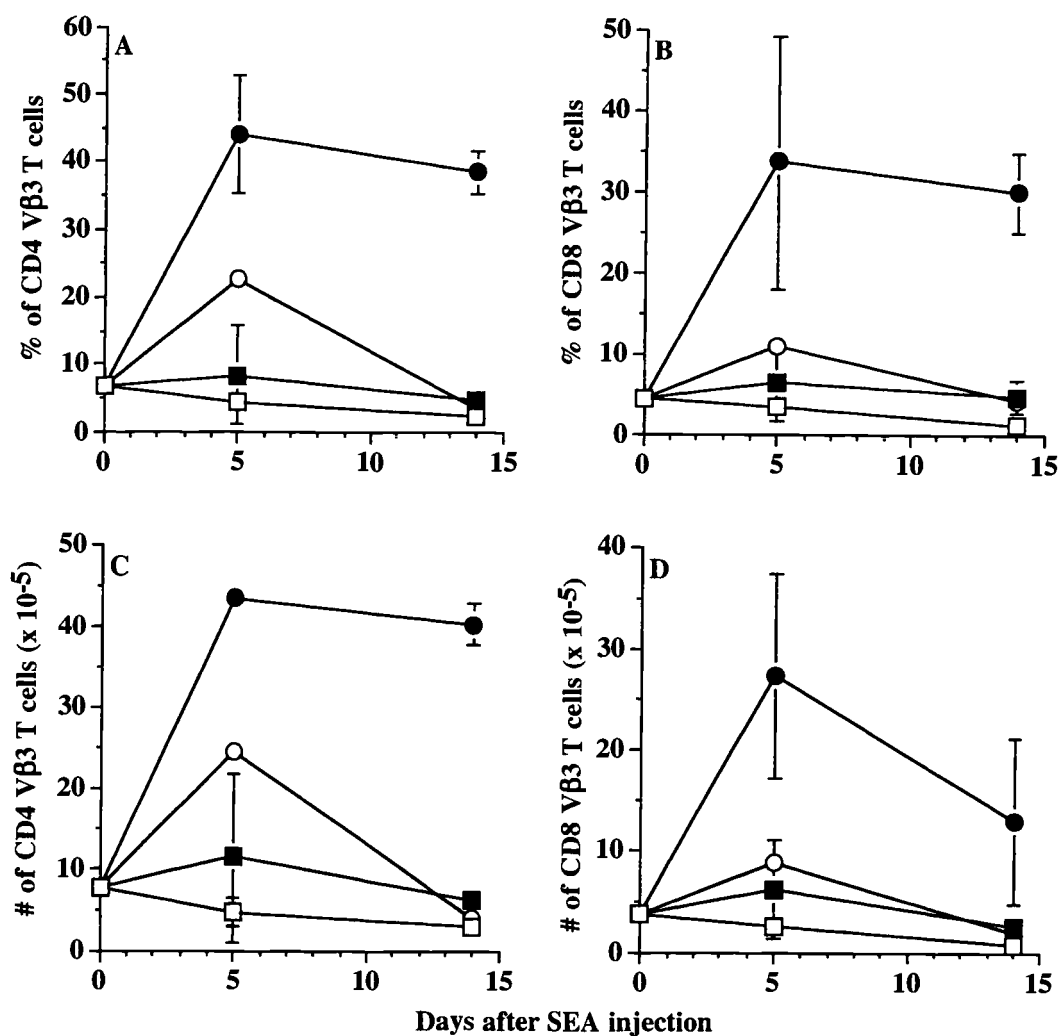


Figure 4.1: The combined effect of antigen, costimulation and LPS enhances Ag-specific CD4 T cell expansion and survival. A total of 17 B10.A mice were divided into four groups: SEA alone (□), SEA/anti-CD40 (■), SEA/LPS (○) and SEA/anti-CD40/LPS (●). Mice were injected with 0.25 mg of anti-CD40, 0.30 μ g of SEA and 10 μ g of LPS. On days 5 and 14 after SEA injection, LN and spleen cells were isolated, counted and stained as described in materials and methods. Mean percentages (A, B) and numbers (C, D) \pm SEM of splenic CD4 and CD8 T cells expressing V β 3 are shown from two mice in each group. Day 0 data is derived from uninjected mice. Data is one representative experiment out of four performed.

had slightly increased numbers of V β 3 T cells compared to those mice treated with SEA alone (Fig. 4.1, C and D). Based on percentages of CD4 V β 3 T cells on day 14, mice treated with SEA/LPS had 1.5-fold more splenic Ag-specific T cells ($3.40 \pm 0.49\%$), and SEA/anti-CD40 treated mice had 2-fold more splenic Ag-specific T cells ($4.70 \pm 0.25\%$) than SEA treated mice ($2.31 \pm 0.12\%$). Similar differences were observed when examining T cell numbers. Thus, a very low level of T cell survival is produced with SEA/anti-CD40 or SEA/LPS treatment.

What is most striking about the results is that a combination of the three signals (SEA, anti-CD40 mAb and LPS) sent many more T cells into a survival state. This effect is observed by examining both percentages and numbers. V β 3 T cells from mice stimulated by the three signals clonally expanded almost 10-fold by day 5, and had declined slightly in number after two weeks. On day 14, CD4 V β 3 percentages were increased by 16-fold over SEA alone to $38.46 \pm 3.08\%$, while the absolute numbers were increased by nearly 13-fold to $40.27 \times 10^5 \pm 2.69$.

An identical response to that described above was observed in the LN (not shown), as well as in the Ag-specific CD8 T cell population (Fig. 4.1, B and D). In mice injected with SEA, anti-CD40 mAb and LPS, splenic CD8 V β 3 T cells clonally expanded to a much greater level than any other treatment on day 5 and then began to gradually decline in both percentage (Fig. 4.1B) and number (Fig. 4.1D). By day 14, there was a 26-fold increase in the percentage of CD8 V β 3 T cells in three-signal treated mice ($29.90 \pm 4.86\%$) versus SEA treated mice ($1.13 \pm 0.02\%$). In contrast, SEA/LPS and SEA/anti-CD40 treated mice only produced a 4-

fold increase in Ag-specific T cell accumulation. These surviving T cells are functional, as evidenced by in vitro restimulation experiments (Fig. 4.2). Thus, both Ag-specific CD4 and CD8 T cell populations can be signaled to survive for long periods by the combined action of signal 1 (SAg), signal 2 (costimulation) and signal 3 (LPS).

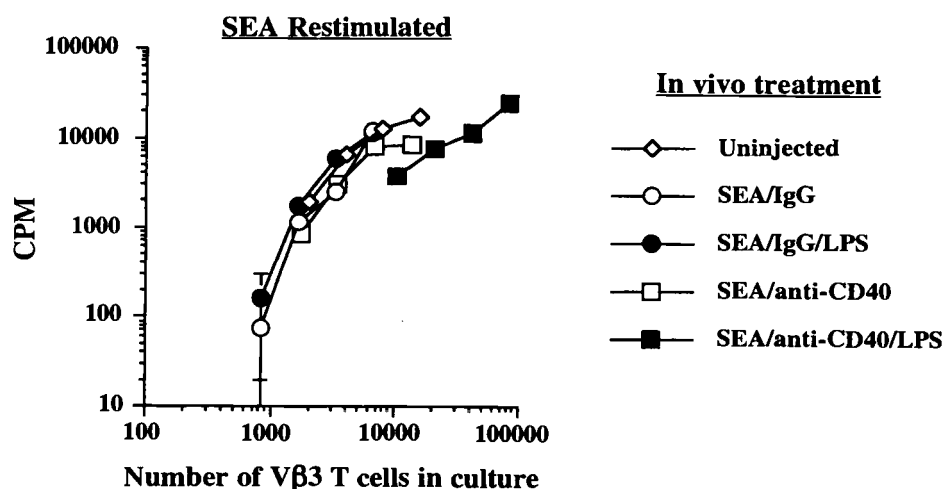


Figure 4.2: Three-signal stimulated T cells are not anergic. Mice were injected with the treatments shown on the right, and restimulated with SEA as described in materials and methods. Incorporation of ^3H -thymidine during the last 8 hours of culture is shown \pm SEM. Data represents one of five comparable experiments.

Three-signal mediated T cell survival is not a result of enhanced costimulation

Stimulation of APCs through CD40 causes an increase in the expression of both B7 molecules as well as MHC class II (7, 48). In addition, LPS stimulation can also enhance B7 expression and prolong OX40 expression on activated T cells (8, 49). We hypothesized that since LPS can synergize with both CD40 and OX40

stimulation to promote survival, its mechanism of action may involve combined signaling via CD28 and OX40 on the Ag-specific cells.

To test this idea, we injected mice with a triple combination of SEA, anti-CD40 mAb and anti-OX40 mAb. The time course of this experiment is shown in Fig. 4.3, and the same scale as in Fig. 4.1 is used to allow a direct comparison between the two figures.

The percentages of both CD4 (Fig. 4.3A) and CD8 (Fig. 4.3B) T cells expressing V β 3 behaved in a similar manner. Treatment of mice with SEA and anti-OX40 mAb enhanced the percentage of Ag-specific T cells in the spleen above that observed with SEA alone on day 3, but on subsequent days, the difference between the two treatments was minimal. SEA and anti-CD40 mAb treatment enhanced the CD4 V β 3 and CD8 V β 3 percentages in the spleen at the peak of expansion by 4- to 5-fold above that observed with SEA alone. By days 7 and 12, however, there was less than a 2-fold increase in the percentage of T cells remaining. When SEA, anti-CD40 mAb and anti-OX40 mAb were all injected, the observed response was very similar to injecting SEA and anti-CD40 mAb. Ag-specific T cell expansion was enhanced 4- to 5-fold on day 3, but following day 3, little survival was observed. There was a slightly larger percentage of Ag-specific T cells remaining on day 12 after the combined treatment (about 4-fold above SEA alone), but not as many as was consistently observed with LPS (Fig. 4.1). Thus, costimulation via CD28 and OX40 can effect small levels of T cell survival, but

when combined with signals produced upon LPS injection, the survival effect is greatly potentiated.

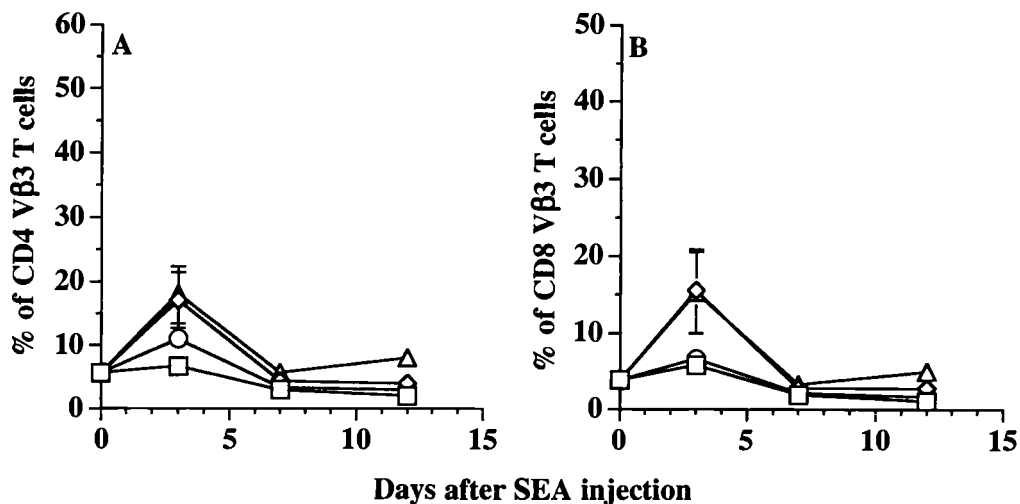


Figure 4.3: Enhanced costimulation cannot substitute for LPS in promoting T cell survival. B10.A mice were divided into four groups: SEA/IgG (□), SEA/anti-CD40 (◇), SEA/anti-OX40 (○) and SEA/anti-CD40/anti-OX40 (Δ). Mice were injected with 0.25 mg of anti-CD40 or rat IgG on day -2, and 0.15 μg of SEA on Day 0. At 24 and 36 hours after SEA injection, 50 μg of anti-OX40 or rat IgG was injected. On days 3, 7 and 12 after SEA injection, LNs and spleens from each group of mice were removed and cells prepared as described in materials and methods. Mean percentages ± SEM of (A) CD4 Vβ3 and (B) CD8 Vβ3 T cells are shown. Day 0 data is derived from uninjected mice. These data represent a combination of two separate experiments totaling 5 mice in each group.

Multiple costimulatory signals do not further enhance LPS-mediated survival

Since the survival response observed during three-signal stimulation does not appear to be a sum of many costimulatory signals being delivered, the next focus of study was whether the LPS response could be enhanced further. Since LPS can synergize with either CD40 or OX40 stimulation to improve Ag-specific T

cell survival, it was necessary to examine whether combining all of these signals could enhance the amount of surviving T cells.

Mice were injected with a combination of SEA, LPS, and the agonistic anti-CD40 and anti-OX40 mAbs. After 10 days, the T cell populations were examined. In mice injected with SEA, anti-CD40 and LPS, $18.96 \pm 2.86\%$ of the CD4 T cells (Fig. 4.4A) and $8.54 \pm 1.49\%$ of the CD8 T cells (Fig. 4.4B) were Ag-specific. A slightly larger percentage was observed in mice injected with SEA, anti-OX40 and LPS: $19.50 \pm 3.81\%$ CD4 V β 3 and $9.26 \pm 2.23\%$ CD8 V β 3 T cells. What was most interesting was that in mice treated with both mAbs, SEA and LPS, little enhancement of T cell survival was observed, increasing to $24.29 \pm 4.27\%$ in the CD4 V β 3 population and $11.56 \pm 3.17\%$ in the CD8 V β 3 population. In these experiments, we used 2.5-times less anti-CD40 mAb to conserve on reagent and thus the magnitude of the response is lessened compared to Fig. 4.1. Increasing the LPS dose to 15 μ g did not further enhance T cell survival, yielding slightly lower percentages and numbers of Ag-specific T cells than observed with 10 μ g (data not shown). Again, the numbers of Ag-specific T cells as well as the LN cells both behaved in a similar fashion (data not shown). Thus, costimulation of T cells via either CD28 or OX40 in the presence of LPS can produce a substantial population of surviving Ag-specific T cells that cannot be significantly augmented by additional costimulation through these receptors.

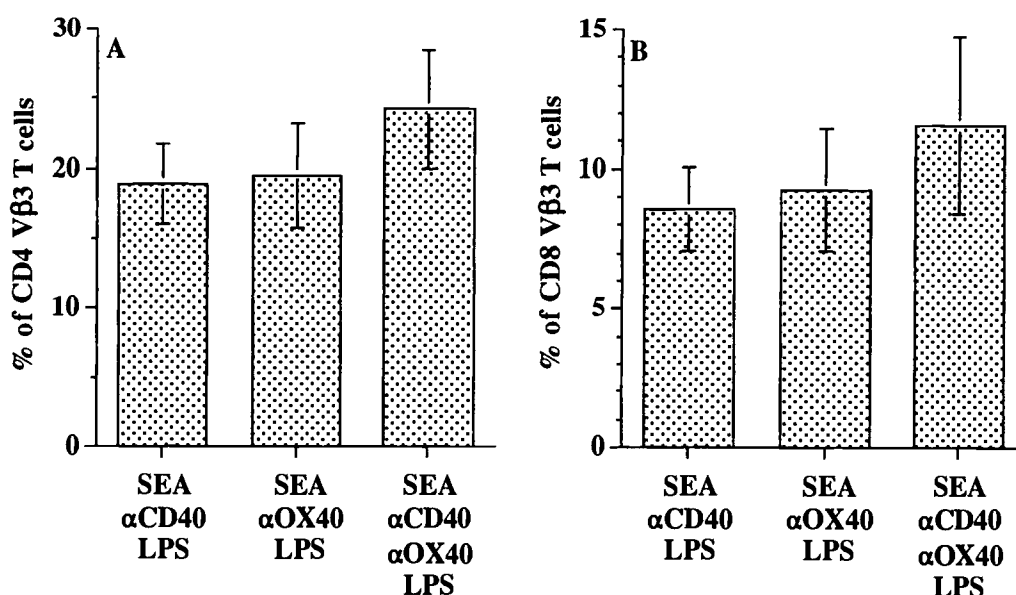


Figure 4.4: Combined CD40 (CD28-mediated) and OX40 costimulation does not further enhance LPS-mediated T cell survival. B10.A mice were injected with 0.10 mg of anti-CD40 on day -2 and 0.30 μ g of SEA on day 0. An LPS injection of 10 μ g was given 24 hours after SEA, and 25 μ g of anti-OX40 was injected 24 and 36 hours after SEA. On day 10, LN and spleen cells were counted, purified and stained for flow cytometric analysis. Mean percentages \pm SEM of (A) CD4 Vβ3 and (B) CD8 Vβ3 T cells are shown. These data represent a combination of two separate experiments totaling six mice in each group.

Proinflammatory cytokines are not necessary for T cell survival in the presence of anti-CD40 and LPS

LPS stimulation of APCs can trigger the production of many proinflammatory cytokines. To begin to understand what LPS was doing to promote survival in our model, mRNA expression of various cytokines was examined at early time points after LPS injection by rt-PCR (Fig. 4.5). The goal was to identify candidate cytokines that may be contributing to the survival response.

exact same time. IL-2 was also examined, but was not seen in any treated or untreated mouse (data not shown). All reactions were normalized to HPRT, which allowed for semi-quantitative comparisons to be made. Thus, production of many proinflammatory cytokines was enhanced by LPS treatment in this model. The next step was to determine if any of these cytokines could induce T cell survival.

Previous work with mice injected with SEA and LPS showed that TNF- α can substitute to a small degree for LPS in keeping T cells alive (19). The survival effect was not as potent as that observed with LPS injection, but it was still significant. This result, coupled with the fact that TNF- α message expression was increased by LPS injection in the three-signal model system, suggested that TNF- α might be at least partially responsible for long-term T cell survival.

To investigate the importance of this cytokine in the three-signal model system, mice deficient in either or both TNFRs were used (34). Because the knockout mice are on a C57BL/6 background, we used SEB instead of SEA to avoid the effect of endogenous mouse mammary tumor viruses on V β 3 T cells. Each mouse strain was injected with SEB and anti-CD40 mAb with or without LPS, and the lymphoid tissues examined on day 10 for Ag-specific T cell survival. Fig. 4.6 shows the percentage of CD4 V β 8 Ag-specific T cells in the spleens of the treated mice. By day 10, all strains receiving a particular treatment had similar percentages of CD4 V β 8 T cells, cells which are specific for SEB. In mice not treated with LPS, the percentages were generally very close, with little variation between mice. However, in mice injected with LPS, there was quite a bit of

variation. Some mice responded very well, accumulating a large percentage of Ag-specific T cells, while others showed little T cell survival.

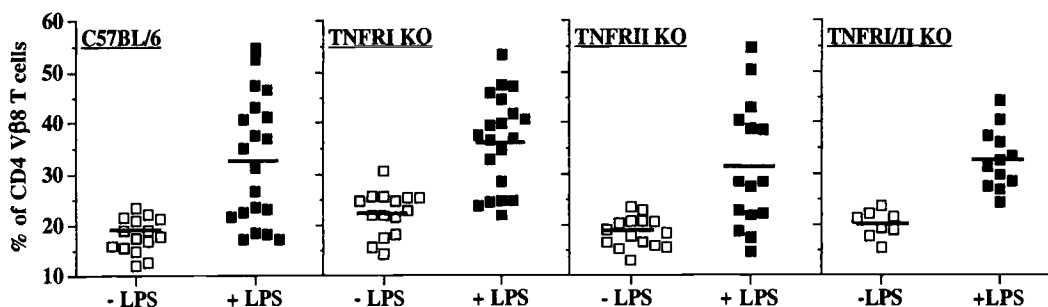


Figure 4.6: TNF- α signaling is not necessary for T cell survival. C57BL/6 mice, or mice deficient in TNFR1, TNFR2 or both TNF receptors, were injected with 0.20 mg of anti-CD40, 60 μ g of SEB and 10 μ g of LPS as described in materials and methods. Ten days after SEB injection, the T cell populations from each strain were analyzed. Each box represents the percentage of CD4 V β 8 T cells in the spleen from each mouse used. The bars represent mean values. The graphs represent pooled data over a total of eight experiments.

In C57BL/6 mice treated with SEB and anti-CD40 stimulation, CD4 V β 8 T cells declined to $18.00 \pm 0.92\%$ after ten days. When those mice were given LPS, the percentage of Ag-specific T cells rose to $32.69 \pm 2.85\%$; an 85% increase. Mice deficient in TNFR1 had $22.30 \pm 1.18\%$ CD4 V β 8 T cells in the spleen in the absence of LPS treatment, but a 63% increase to $36.34 \pm 2.13\%$ was observed when LPS was injected. T cells from TNFR2 knockout mice increased by 69% upon LPS treatment, rising from $18.35 \pm 0.79\%$ CD4 V β 8 T cells without LPS to $31.13 \pm 3.30\%$ with LPS.

In the absence of one TNFR, there remained the possibility that the other receptor was still delivering a survival signal due to redundancy of function between the two receptors. Thus, mice deficient in both TNFRs were treated with

three signals as in the other strains. Mice receiving SEB and anti-CD40 mAb had a CD4 V β 8 T cell percentage on day 10 of $20.03 \pm 1.00\%$, but when injected with LPS, the percentage rose 63% to $32.65 \pm 1.79\%$, similar to that observed in the other strains. Overall, these data suggest that signaling through TNFRI and TNFRII is dispensable for long term T cell survival in this model.

The observation that T cell survival can still occur in the absence of TNFR signaling does not mean that such signaling cannot contribute to survival. Other proinflammatory cytokines may be contributing a survival signal and thus substituting for TNFR signaling. To get at the issue of whether other cytokines were delivering the signals necessary to keep activated T cells alive, a combination of cytokines were blocked in vivo during SAg, anti-CD40 mAb and LPS stimulation.

B10.A mice were injected with SEA, anti-CD40 mAb and LPS as usual, but before and after LPS injection, IL-1Ra, anti-TNF- α mAb and anti-IL-6 mAb were all injected to try to neutralize as much activity of these three cytokines as possible. Since the rt-PCR data showed that IFN- γ expression was increased after LPS injection (Fig. 4.5), another group was also set up in which IFN- γ was blocked with a mAb to investigate what effect this cytokine had on survival. The percentages (Fig. 4.7A) and numbers (Fig. 4.7B) of CD4 V β 3 T cells ten days after SEA injection are shown.

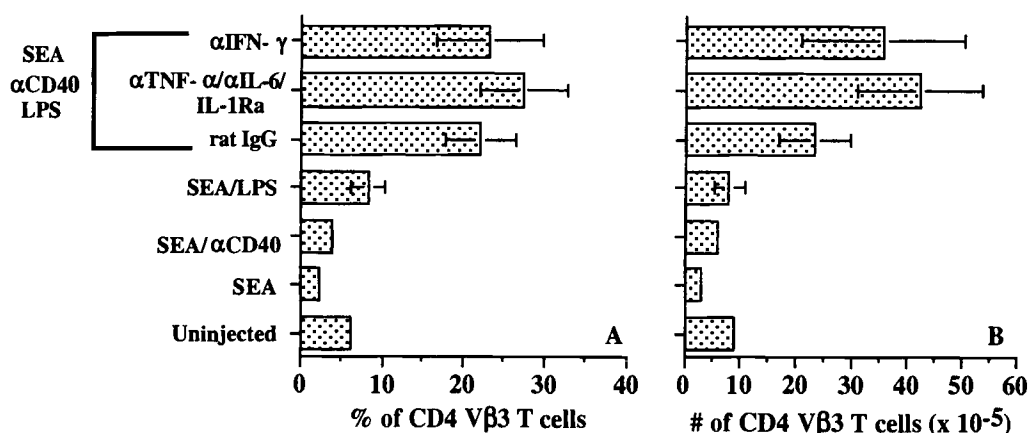


Figure 4.7: Blockade of proinflammatory cytokines does not adversely affect long-term CD4 Vβ3 T cell survival. B10.A mice were injected with 0.25 mg of anti-CD40, 0.30 μg of SEA and 10 μg of LPS as before. Additionally, 1.5 mg of anti-TNF-α, 1.5 mg of IL-1Ra, 0.75 mg of anti-IL-6, 1 mg of anti-IFN-γ or 3.75 mg of rat IgG were injected four hours before and six hours after LPS injection. Ten days after antigen injection, LN and spleen cells were prepared as described in materials and methods. These data show the percentages (A) and numbers (B) ± SEM of CD4 Vβ3 T cells in the spleen. The graphs represent pooled data from three separate experiments with a total of nine mice in each group.

SEA treatment led to the expected decline in percentages dropping from 6% CD4 Vβ3 T cell population in uninjected mice to a little over 2% by day 10. SEA/anti-CD40 mAb and SEA/LPS treatments both produced small increases in T cell percentages in comparison to SEA alone, again showing that two signals can generate some long-term T cell survival. Mice treated with SEA/anti-CD40/LPS and the control IgG had a large increase in percentage of Ag-specific T cells to $22.12 \pm 4.32\%$. What was most interesting, however, was that when IL-1β, TNF-α and IL-6 signaling were all blocked, the percentage of surviving T cells was not affected, and actually increased slightly to $27.42 \pm 5.34\%$. Even blocking IFN-γ

did not decrease survival, yielding $23.25 \pm 6.56\%$ CD4 V β 3 T cells after 10 days in vivo.

A much better enhancement of T cell survival after cytokine neutralization was observed based on CD4 V β 3 T cell numbers (Fig. 4.7B). Mice receiving three-signal stimulation and the control rat IgG had $23.28 \times 10^5 \pm 6.31$ Ag-specific T cells remaining after ten days. Blocking TNF- α , IL-1 β and IL-6 increased the numbers of surviving T cells to $42.40 \times 10^5 \pm 11.2$, while IFN- γ inhibition increased the numbers to $35.83 \times 10^5 \pm 14.62$.

CD8 V β 3 T cells responded a bit differently to the cytokine blocking studies than the CD4 V β 3 T cells (Fig. 4.8). Both percentages (Fig. 4.8A) and numbers (Fig. 4.8B) of CD8 T cells expressing V β 3 in the spleen were increased poorly, if at all, by inhibition of proinflammatory cytokines. This could represent a difference in activation/survival requirements between CD4 and CD8 T cells. However, what is identical between both kinds of T cells is that blocking TNF- α , IL-1 β , IL-6 and IFN- γ did not adversely affect survival.

Similar results were observed when IL-1 β and IL-6 were inhibited in TNFR double knockout mice (data not shown). No adverse effects on long-term T cell survival were observed in this model system, and some slight increases in surviving T cell numbers were observed. Thus, from these data it appears that the proinflammatory cytokines TNF- α , IL-1 β , IL-6 and IFN- γ , are not necessary for long-term T cell survival, and in fact may actually be somewhat detrimental,

possibly playing a role in attenuating an Ag-specific CD4 T cell response to Ag, strong costimulation and LPS treatment.

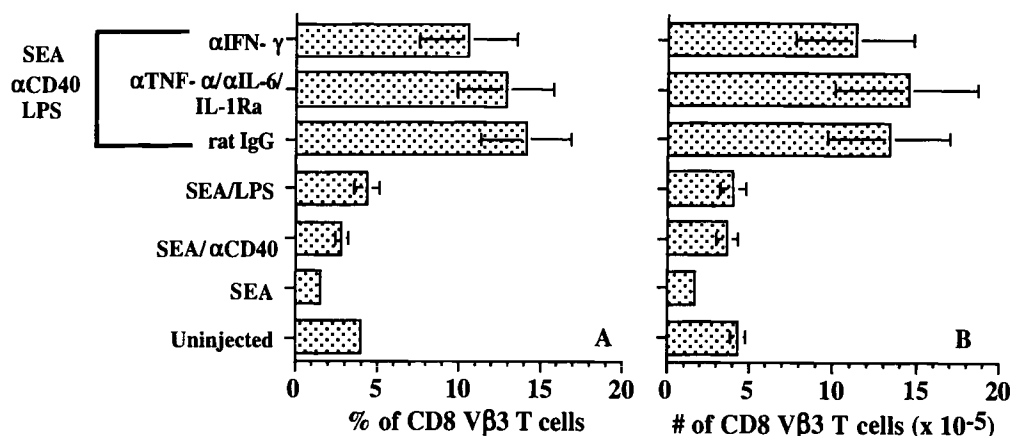


Figure 4.8: Inhibition of proinflammatory cytokines does not prevent CD8 Vβ3 T cell survival. Data taken from the experiment in Fig. 4.7, but showing percentages (A) and numbers (B) \pm SEM of CD8 Vβ3 T cells in the spleen ten days after antigen injection. Again, each bar represents a total of nine mice used over three separate experiments.

NF-κB is dispensable for long-term T cell survival

The transcription factor NF-κB is very important for inflammatory responses as well as T cell proliferation and survival (16, 17, 50-52). Many inflammatory cytokines, as well as LPS, activate transcriptional activity of this molecule (53-56). To examine the role of NF-κB in T cell survival in vivo, mice transgenic for a mutant IκB-α molecule that cannot be degraded were used (35). Thus, these mice have reduced NF-κB binding to DNA response elements and thus were used to examine how effective T cell survival is in the presence of reduced NF-κB activity.

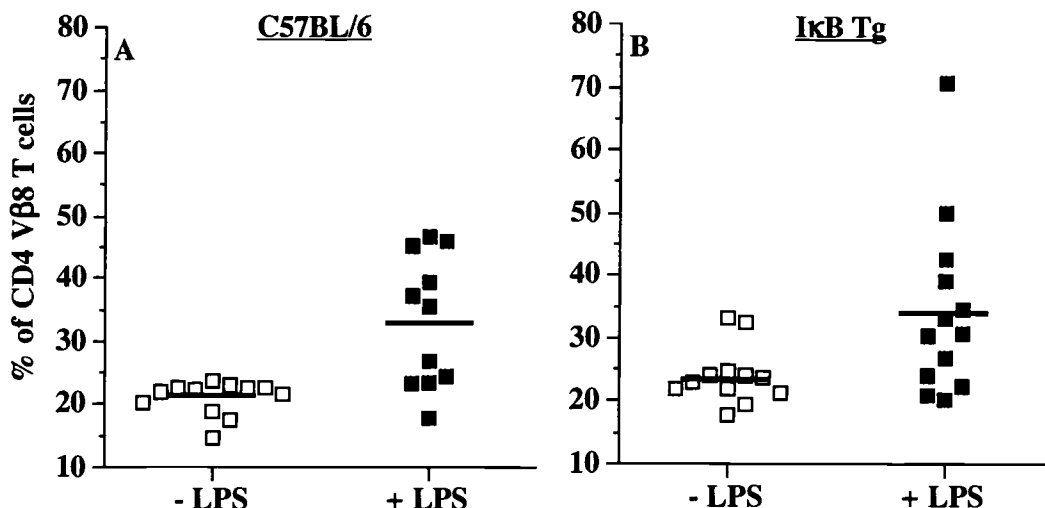


Figure 4.9: The transcription factor NF- κ B is not necessary for T cell survival. C57BL/6 (A) or I κ B Tg (B) mice were injected with 0.2 mg of anti-CD40, 60 μ g of SEB and 10 μ g of LPS as described in materials and methods. Ten days after SEB injection, the CD4 V β 8 T cell populations were analyzed by flow cytometry. Each box represents the percentage of CD4 V β 8 T cells in the spleen for each individual mouse used. The lines in the graphs signify the mean value from all data points for each treatment. Graphs represent pooled data from six separate experiments.

As shown in Fig. 4.9, the percentage of splenic CD4 V β 8 T cells in C57BL/6 mice given SEB and anti-CD40 mAb without LPS was $20.91 \pm 0.83\%$ and rose to $33.26 \pm 3.35\%$ when LPS was injected. A very similar increase was observed in the transgenic mice whose T cell populations increased from 23.91 ± 1.39 without LPS to 34.17 ± 4.04 with LPS. These numbers correspond to a 59% difference between treatments in C57BL/6 mice and a 43% difference in the transgenic mice. A similar trend was observed in the absolute numbers of CD4 V β 8 T cells, which differed by 32% in the C57BL/6 mice, and 22% in the transgenic mice (data not shown). The transgenic mice injected with LPS had larger mean percentages and numbers of T cells than C57BL/6 mice, and quite a few

individual mice that had much higher levels of T cells than observed in control strains. One transgenic mouse in particular had over 70% of its CD4 T cells bearing V β 8 in the spleen ten days after Ag injection. This is in contrast to C57BL/6 mice, which never got above 50%.

CD8 T cell data was also collected in these experiments (data not shown). Although the percentages of Ag-specific CD8 T cells increased upon LPS treatment in a comparable manner between the two strains, the absolute numbers of T cells were severely limited in the transgenic mice (data not shown). This is due to the developmental deficiencies in the CD8 T cell population in these mice that have been described previously (35). Within this limited population, however, CD8 V β 8 T cells in the spleen did increase slightly upon LPS treatment. Lymph node percentages and numbers of CD4 V β 8 and CD8 V β 8 T cells also behaved similar to the spleen except lymph node numbers in the transgenic mice actually decreased by about 40% on day 10 when LPS was given (data not shown).

Expression of I κ B- α was consistent in all of the transgenic mice, so the survival cannot be due to poor transgene expression (Fig. 4.10A). Additionally, electrophoretic mobility shift assays (EMSAs) that were performed showed no correlation between NF- κ B DNA binding activity and survival (Fig. 4.10B). Overall, the data presented here surprisingly suggests that NF- κ B is not necessary for long-term T cell survival.

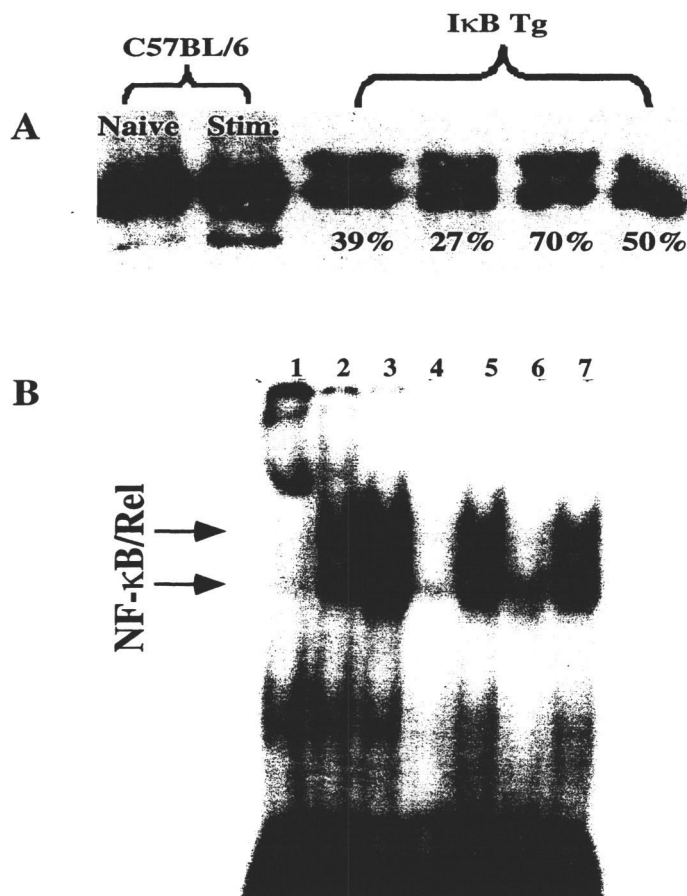


Figure 4.10: Enhanced survival is not due to poor NF- κ B inhibition. LN and spleen cells from C57BL/6 or from four I κ B Tg mice injected with SEA/anti-CD40 mAb/LPS in vivo were restimulated with ConA overnight. Additionally, cells from an uninjected C57BL/6 mouse were cultured without stimulation overnight. Cytoplasmic and nuclear extracts were prepared from purified T cell populations as described in materials and methods. (A) A western blot was performed on the cytoplasmic extracts to detect I κ B α protein. Percentages correspond to the approximate percentage of CD4 T cells expressing V β 8 shown in Fig. 4.9 for each mouse examined. (B) Nuclear extracts were subjected to an EMSA to detect NF- κ B DNA-binding activity. Lane 1 shows a supershift from naïve cells incubated with antibodies against multiple NF- κ B family members (see materials and methods). Lanes 2 and 3 are from naïve and three-signal stimulated C57BL/6 cells, respectively, as labeled in (A). Lanes 4 through 7 are from I κ B Tg mice treated with three-signals in vivo and correspond to the mice with 39%, 27%, 70% and 50% CD4 V β 8 T cells, respectively, as labeled in (A).

LPS can circumvent proliferation defects in NF- κ B deficient T cells

Since LPS-induced long-term T cell survival can still occur in NF- κ B deficient T cells, it was important to investigate whether T cell proliferation was deficient and if LPS could somehow overcome that deficiency. Thus, transgenic or C57BL/6 mice were injected with different combinations of Ag, anti-CD40 mAb and LPS and given BrdU injections for three days. After this treatment, The percentages and numbers of Ag-specific T cells incorporating BrdU were evaluated (Fig. 4.11).

Both C57BL/6 and transgenic mice had similar percentages of V β 8 T cells incorporating BrdU in the LN on day 3 (Fig. 4.11, A and C). Based on percentages or rate of BrdU incorporation, no major proliferation defect was apparent. SEB treatment caused about 40% of the responding T cells to take up BrdU in both normal and deficient mice. Three-signal treatment only slightly enhanced the incorporation of BrdU to around 50% of the V β 8 population.

When the absolute numbers of V β 8 T cells taking up BrdU was examined, however, a major defect was observed (Fig. 4.11, B and D). C57BL/6 mice had similar numbers of V β 8 T cells incorporating BrdU regardless of whether SEB alone or three-signals was delivered. The number of NF- κ B deficient T cells taking up BrdU was dramatically reduced in SEB treated mice, strongly supporting the notion that NF- κ B activity promotes short-term survival. However, co-injection of anti-CD40 mAb and LPS with SEB increased the numbers by 4.5-fold, suggesting

that such stimulation enhances short-term survival and accumulation of Ag-specific T cells in the LN even in the absence of NF- κ B.

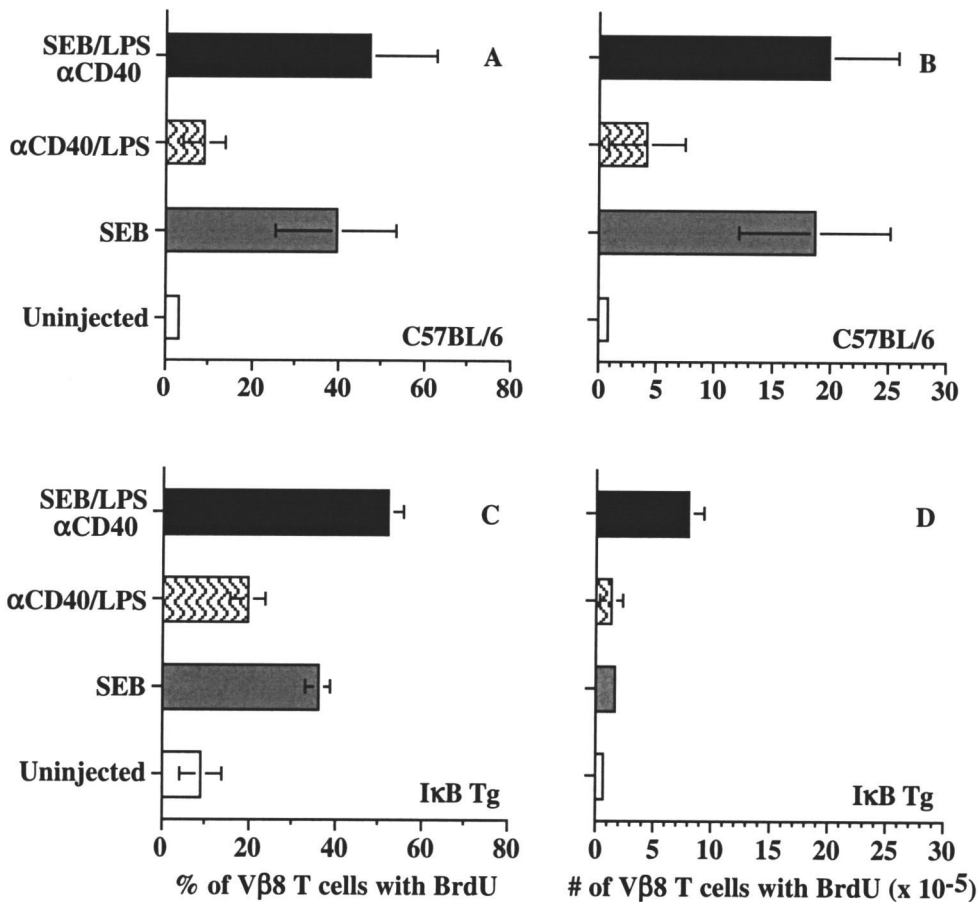


Figure 4.11: NF- κ B is important for T cell proliferation and short-term survival. C57BL/6 (A, B) and I κ B Tg (C, D) mice were injected with SEB, anti-CD40, LPS and BrdU as described in materials and methods. On day 3 after SEB injection, Ag-specific V β 8 T cells were analyzed for BrdU incorporation by flow cytometry. These data represent the mean percentage (A, C) and number (B, D) \pm SEM of V β 8 T cells incorporating BrdU in the lymph node. Bars represent a total of three to five mice combined from three separate experiments.

Results from the spleen showed that SEB treated mice had reduced numbers of BrdU incorporating Ag-specific T cells, but were somewhat comparable to C57BL/6 mice (data not shown). Co-injection of anti-CD40 and LPS only slightly

enhanced the BrdU incorporation. Thus, in the absence of effective NF- κ B activation, T cell proliferation is affected, but LPS can still serve to enhance proliferation and both short- and long-term survival of Ag-specific cells.

T cell survival is susceptible to cyclosporin A inhibition

Since neither proinflammatory cytokines or NF- κ B activation are required for T cell survival, some source of inhibition remained to be found. The immunosuppressive drug cyclosporin A (CsA) has been shown to enhance SAg-induced T cell deletion (57) and is widely used to prevent transplant rejection (30). To investigate whether anti-CD40 and LPS stimulation could prevent this deletion, B10.A mice were injected with SEA, anti-CD40 mAb and LPS in the continued presence of CsA or vehicle. Ten days after SEA administration, percentages of CD4 (Fig. 4.12A) and CD8 (Fig. 4.12B) T cells expressing V β 3 were analyzed.

In the spleens of mice treated with vehicle alone, the percentage of CD4 V β 3 T cells was $17.36 \pm 0.07\%$ and the percentage of CD8 V β 3 T cells was $9.15 \pm 1.09\%$. When CsA was injected, however, the CD4 V β 3 percentage dropped to $3.97 \pm 0.35\%$ and the CD8 V β 3 percentage dropped to $2.88 \pm 0.10\%$, values that are well below the normal value found in uninjected mice. Thus, CsA was found to be very potent at suppressing three-signal mediated T cell survival. Such a decline is uncharacteristic of three-signal stimulation and is observed most commonly in mice treated with SAg alone or with just two of the three signals. Thus, the mechanism by which T cell survival is induced in this model may rely more on

calcium mobilization and possibly activation of the transcription factor NFAT (29, 30) as opposed to NF- κ B activation.

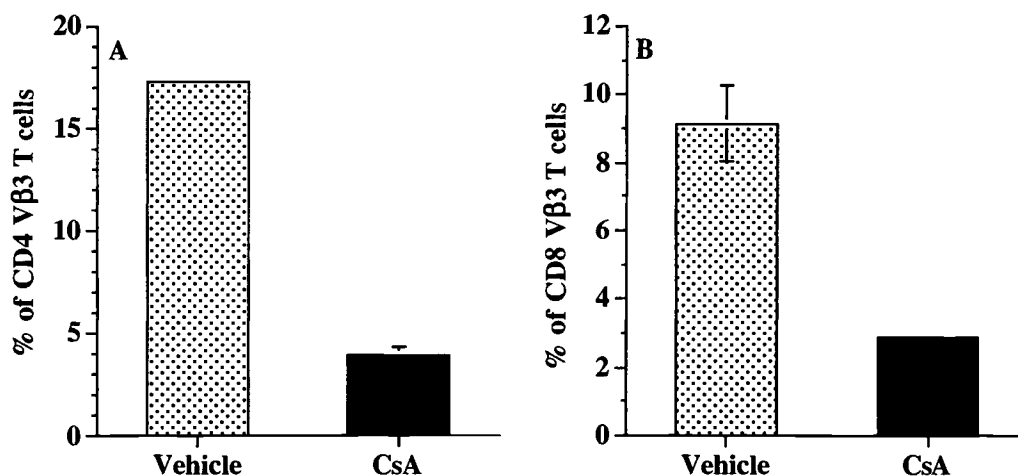


Figure 4.12: Cyclosporin A treatment inhibits three-signal-mediated T cell survival. Four B10.A mice were injected with 0.25 mg of anti-CD40 mAb, 0.30 μ g of SEA and 10 μ g of LPS as described. On days -3 to +9, 1 mg of CsA was injected with olive oil as the vehicle. Ten days after antigen injection, the LNs and spleens were removed from mice injected with CsA (dark bars) or vehicle (light bars) and analyzed by flow cytometry. The data represent mean percentages \pm SEM of (A) CD4 V β 3 and (B) CD8 V β 3 T cells in the spleen on day 10.

DISCUSSION

Our data clearly show that LPS potentiates long-term Ag-specific T cell survival. The initial reports of this phenomenon described a 3- to 4-fold increase in CD4 V β 3 T cells after two weeks in mice injected with SEA/LPS compared to those given SEA alone (18). Fig. 4.1 shows that SEA/LPS, as well as SEA/anti-CD40 mAb treatment both yielded between 2- and 5-fold increases in surviving CD4 and CD8 T cells bearing V β 3. Additionally, this three-signal treatment was not generating anergic cells, since they could be effectively restimulated in vitro

(Fig. 4.2). Thus, delivery of two signals is effective at enhancing T cell expansion and promoting small levels of long-term T cell survival. However, to generate optimal long-term immunity, three signals are necessary.

The process of using a combination of Ag, costimulation and inflammation to produce an optimal T cell response is something that has been described in three different in vivo model systems. The SAg model has been used to show that either OX40 or CD40 stimulation can dramatically augment a T cell response to SEA and LPS (8). OX40 is found primarily on activated CD4 T cells while CD40 is primarily on APCs. Direct T cell costimulation via OX40 mAb, or indirect stimulation via CD40 mAb-induced upregulation of B7 on APCs can both synergize with LPS to enhance survival. Additionally, the DO11.10 adoptive transfer model was also used to show that OX40 and LPS synergize to enhance T cell survival in response to ovalbumin protein injection (8). This last experiment was carried out as far as two months where a 60-fold increase in surviving T cells was observed compared to ovalbumin injection alone. Thus, there is no question that delivery of three separate signals is involved in optimal T cell immunity.

The focus of this paper sought to better understand the mechanism by which LPS promotes long-term T cell survival. LPS on its own can enhance B7 expression on APCs and prolong OX40 expression on activated T cells (8, 49). Since LPS could synergize with CD40 or OX40 to enhance T cell survival, it was initially reasoned that LPS might be merely a summation of various costimulatory signals such as these. Thus, we set out to deliver OX40 and CD40 costimulatory

signals in the place of LPS to see if they could synergize to effect similar levels of surviving T cells. Fig. 4.3 shows that combined signaling via CD40 and OX40 cannot yield the same level of survival as what is observed in SEA/anti-CD40/LPS treated mice. The percentages of Ag-specific T cells observed in Fig. 4.3 are increased 3- to 5-fold above SEA treated mice when both costimulatory signals are delivered, a response similar to what is observed when only two signals are given (Fig. 4.1). Thus, there appears to be something more to LPS in the context of the three-signal model.

The survival response produced in the wake of three-signal stimulation *in vivo* is very potent. To see if there was some limit to the level of T cell survival that could be generated, we injected mice with SEA, LPS, and the agonistic mAbs against OX40 and CD40 (Fig. 4.4). There was a slight increase in the percentage of CD4 V β 3 and CD8 V β 3 T cells when both costimulatory signals were provided compared to only one; however, this increase is not biologically significant. Thus, these data show that the synergism of LPS with costimulation is not recapitulated with OX40 and CD28 ligation. Specifically, these data show that signal 3 cannot be substituted with a different costimulatory signal. Nevertheless, it is possible that other costimulatory signals not tested here can substitute for LPS.

In initially investigating how this survival response is produced, proinflammatory cytokines were examined. Cytokine analysis by rt-PCR confirmed the LPS-dependent increase in expression of many cytokines such as TNF- α , IL-1 β , IL-6 and IFN- γ (Fig. 4.5). TNF- α was the first cytokine studied

because previous work found that blocking TNF- α signaling in vivo with a mAb inhibited T cell survival in response to SEA/LPS injection (18). Additionally, subsequent work found that injection of TNF- α in the place of LPS could promote T cell survival following SEA injection (19). However, the percentage of Ag-specific T cells surviving after SEA/TNF- α treatment was only about one-third of that observed with injection of SEA and LPS. The fact that TNF- α could not completely substitute for LPS in enhancing survival implied that other cytokines might also contribute to the overall survival response. Thus, while some decrease in T cell survival might be expected in the absence of TNFR signaling, it may not be completely abolished.

What is most apparent and perhaps surprising about the TNFR KO mouse data shown in Fig. 4.6 is that neither TNFR is required for effective T cell survival resulting from three-signal stimulation. This appears at odds with the results from SEA/LPS treated mice. It seems that TNF- α does have survival-inducing capabilities during weak antigenic signaling and is an important component of LPS-mediated T cell survival following SEA stimulation. However, when a strong costimulatory signal is delivered via CD40 (and ultimately through CD28), the survival effects of TNF- α are negated.

In the absence of TNFR signaling, other cytokines may be filling in. Earlier work suggested that IL-1 β and IFN- γ could also contribute to the survival response (18, 19). Thus, combinations of these proinflammatory cytokines were blocked in

the presence of three-signal stimulation to examine whether signals from multiple cytokines were delivering the survival stimulus.

TNF- α , IL-1 β and IL-6 were all blocked in the presence of SEA, anti-CD40 and LPS. Again, the inhibition of these three cytokines did not block T cell survival, and actually slightly enhanced it in the Ag-specific CD4 T cell population (Fig. 4.7B). Furthermore, the survival response was also unaffected or slightly enhanced by a blockade of IFN- γ . Thus, there appears to be no significant role of many important inflammatory cytokines in T cell survival induced by three-signal stimulation.

To verify that the cytokines were being inhibited by the injected antibodies, serum samples were taken from the injected mice one day after the LPS and antibody injections and ELISAs were performed using anti-rat IgG antibodies in a sandwich ELISA. In the control rat IgG group, quantities of rat IgG were detected in the serum that exceeded the maximum detectable amount (data not shown). In the anti-TNF- α , anti-IL-6 and IL-1Ra treated groups there was slightly less antibody detected, but still large amounts remained in the serum. The anti-IFN- γ treated mice had variable quantities of serum rat IgG. Overall, the ELISA data suggest that there were still large amounts of rat IgG antibodies present in the serum over 24 hours after LPS treatment. Although this does not prove effectiveness and specific binding, any cytokines that were produced could have been bound up very effectively.

The fact that blocking the above cytokines somewhat enhanced Ag-specific T cell accumulation in the LN and spleen suggests that they may help to shut down a T cell response. If this is the case, the survival signal that is generated is very potent, and can circumvent many strong death signals (TNF, TRAIL, Fas) for the long-term benefit of the cell. Still, another possibility is that in the presence of these cytokines, the Ag-specific T cells migrate out into the peripheral tissues more effectively, while in their absence, the T cells accumulate in the secondary lymphoid tissues to a greater degree, possibly signifying a slower migration response or activation process. It is becoming increasingly apparent that activated/memory cells are found quite readily in peripheral nonlymphoid tissues, and the above mentioned cytokines may be enhancing T cell migration (58, 59).

Despite the fact that these cytokines did not inhibit long-term survival, there still remains the possibility that other cytokines play a role in the response such as IFN- α/β and IL-15. IFN- α/β has been shown to be very effective at keeping activated T cells alive in vitro (60), and IL-15, a downstream cytokine triggered by IFN- α/β , has been shown to be a major player in LPS-induced T cell responses (61, 62). IL-15 was not investigated in this work as mRNA expression levels were not enhanced dramatically over the experiments conducted. Since IL-15 expression is not increased much it is unlikely that IFN- α/β is involved since expression of the cytokines are linked to each other. Still, either of these cytokines may be candidates for further in vivo investigation.

Since proinflammatory cytokines are not involved in survival, another possible source was the transcription factor NF- κ B. NF- κ B is an important molecule in T cell responses and is activated by signals from LPS and many inflammatory cytokines (50, 53). Activation of NF- κ B has been shown by many groups to inhibit apoptosis (54-56, 63). Additionally, NF- κ B is responsible for transcribing many survival genes such as A1, A20 and Bcl-x_L (64, 65). Thus, the hypothesis was tested as to whether a deficiency in NF- κ B signaling would alter three-signal induced T cell survival.

To get at this issue, mice were used that were transgenic for a trans-dominant form of the NF- κ B repressor I κ B- α that cannot be degraded (35). This mutant repressor was under the control of the lck promoter so expression of I κ B- α , and thus inhibition of NF- κ B, was restricted only to the T cell population. These mice and similar strains were shown to be deficient in T cell proliferation and IL-2 secretion in vitro (66-68). Additionally, the T cells were found to be more prone to apoptosis in vitro after activation. Most of the studies done with these mice have been in vitro. Very little is known about how these defective peripheral T cells respond in vivo.

Injecting the transgenic mice with SEB, anti-CD40 mAb and LPS surprisingly still produced effective survival that was comparable to that observed in C57BL/6 mice (Fig. 4.9). The transgenic mice had slightly larger average percentages and numbers of T cells than C57BL/6 mice whether or not they received LPS. Additionally, it is known that activated NF- κ B-deficient cells are

inclined toward apoptosis in vitro. We show here that upon exposure to three-signals, the NF- κ B-deficient T cells can still generate the capacity to survive for long periods of time, but that this survival is dependent on signals 2 and 3.

Although western blots found that I κ B- α was expressed in equivalent amounts in all transgenic mice examined (Fig. 4.10A), this mutation does not completely inhibit NF- κ B activity. Thus, the possibility remains that some small level of transcription by NF- κ B was still occurring in the transgenic cells that transcribed the survival factor. When EMSAs were performed, although there was detectable NF- κ B DNA-binding activity in cells from the transgenic mice, no correlation was found between NF- κ B activity and the level of T cell survival in vivo (Fig. 4.10B). Thus, NF- κ B does not appear to be important for survival of Ag-specific T cells.

The NF- κ B data is further supported by the cytokine data. In the experiments where cytokines were blocked, there would be presumably less NF- κ B activation. Both TNF- α and IL-1 β activate NF- κ B, and in the absence of signals through the receptors of these cytokines, less overall NF- κ B transcriptional activity would be occurring. In this situation, long-term T cell survival still occurs. Thus, the role of NF- κ B in T cell survival appears to be dispensable.

A recent study from the Kappler and Marrack laboratory suggested that adjuvants such as LPS may be causing T cell survival by inducing Bcl-3 which assists NF- κ B transcription (69). Such transcription was suggested to produce some protein responsible for survival. Our data suggest that NF- κ B may not be

necessary for long-term T cell survival, but we cannot rule out the possibility that NF- κ B activity can produce a survival factor. Since Bcl-3 plays a role in NF- κ B transcription and T cell responses, it may be that NF- κ B is most significant in the early phases of a T cell response for short-term survival.

Proliferation, as measured by BrdU incorporation, is not affected significantly by NF- κ B inhibition based on T cell percentages, but when the absolute numbers of T cells are examined, a dramatic decline in T cells incorporating BrdU is observed after SEB injection (Fig. 4.11). Thus, the Ag-specific T cells are dividing normally, but they cannot effectively accumulate in the lymphoid tissues without NF- κ B signaling. This is where Bcl-3 may be playing a role.

Again however, a combination of Ag, costimulation and LPS could circumvent some of the deficiencies in the response. Injection of these three-signals enhanced the numbers of Ag-specific T cells incorporating BrdU on day 3 by about 4-fold. Numbers were not restored to levels observed in C57BL/6 mice, but they were enhanced, implying that this three-signal stimulation can override some of the inhibition of short-term T cell survival that results from NF- κ B inhibition.

In the absence of NF- κ B signaling, other transcription factors may be responsible for producing the survival factor in T cells. Interestingly, the T cells in these mutant I κ B- α transgenic mice are also deficient in the *in vitro* activation of NFAT, AP-1, cJun and JunB, even after costimulation via CD28 (66). The potency

of the survival factor is again shown here, in that a combined defect in many important T cell transcription factors is still circumvented by delivery of three signals.

To determine just how real these multiple transcription factor defects were in vivo, inhibition of calcineurin-regulated transcription factors was conducted using CsA (29). Surprisingly, T cell survival was completely inhibited by daily treatment with CsA (Fig. 4.12). Two days after injection of SEA alone, T cell expansion in CsA treated mice was abolished (data not shown), in agreement with other studies (57). Thus, it remains to be seen whether the inhibition of T cell survival in this three-signal model was due to the activation of an irreversible death pathway that occurred during the 24 hours between SEA administration and LPS treatment, or whether direct inhibition of the LPS-induced survival signal was involved. Thus, the timing of the CsA injection should be investigated to uncover whether survival is still inhibited when CsA is administered during or after LPS injection. This is especially true since many of the inhibitory effects of CsA only work within a few hours of Ag treatment (29, 32). Such studies will help to elucidate which part of the three-signal stimulus is susceptible to CsA inhibition.

If CsA can specifically inhibit the survival signal, the search for the mechanism of LPS-induced T cell survival would be narrowed down. Although CsA does have somewhat broad effects resulting from its interaction with calcineurin, its primary effects are observed on T cells. CsA inhibits important steps in calcium-mediated signaling pathways like that induced by TCR

stimulation, something cytokine and LPS receptors have not currently been demonstrated to use (70). Thus, it would seem obvious that long-term survival would depend on some calcium-mediated activation signal being delivered to the T cell, but it may also be that the LPS-induced survival signal is also dependent on some calcium-dependent pathway.

The mechanism of memory induction in this three-signal model surprisingly can occur without proinflammatory cytokines and NF- κ B activity, but absolutely requires calcineurin activation to be generated. The transcription factors NFAT and AP-1 are both inhibited by CsA, thus these could be the transcription factors necessary for production of the survival signal (71). NF- κ B has also been shown to be affected by CsA as well (72). Maybe global inhibition of transcription factors is needed to shut off survival. Completely elucidating this survival pathway will certainly require further investigation of many important transcription factors.

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Chapter 5

Effects of Three-signal Stimulation on Early T Cell Clonal Expansion

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INTRODUCTION

T cell activation occurs when the T cell receptor (TCR) recognizes foreign proteins that have been degraded and presented by molecules of the major histocompatibility complex (MHC) on the surface of antigen presenting cells (APCs) (1). Not all antigens must be processed in this way to stimulate T cells. Superantigens (SAGs) are proteins that bind to class II MHC and the TCR and stimulate T cells based on the variable β -chain they express in their TCR (2, 3). SAGs have proven useful for developing in vivo models for studying memory T cell development (4, 5). Despite this long-term effect, little is known about the early phases of SAg-mediated T cell stimulation. This early phase of SAg-mediated T cell stimulation is interesting and may hold keys to understanding what signals are driving memory T cell differentiation.

SAg injection is a well-established method of generating in vivo T cell tolerance (6). After injection of staphylococcal enterotoxin B (SEB) into mice, the antigen-specific V β 8-expressing T cells proliferate to peak levels after two days and then most of the responding cells die. The cells that remain are rendered anergic, or unresponsive to further stimulation. Thus clonal expansion is followed by clonal deletion, suggesting that tolerance can occur after a strong immune response (7). This is qualitatively identical to peptide injection into mice, demonstrating the similarity between SAg and Ag responses (8, 9).

What is also interesting about the SAg response is that prior to clonal expansion, during the first 24 hours after SAg exposure, the responding T cell

population actually declines in number (10). This decline correlates with a transient period of hyperreactivity followed by prolonged hyporeactivity (10, 11). If the responding T cells are removed from the mouse at various time points and restimulated in vitro, the cells hyperproliferate if removed during the first hour after SEB injection, but become increasingly unresponsive to restimulation if removed greater than two hours after treatment. Additionally, the early decline in antigen-specific T cells appears to correlate with the disappearance of SEB from the mice. SEB is only present in biologically active quantities for about 24 hours before the kidneys have removed the majority of it (12). Thus, as the SAg disappears, the T cell response begins to develop.

SAg treatment generally leads to massive T cell deletion, but the coadministration of costimulation and adjuvant treatment can enhance the expansion of the responding T cells and prevent their deletion (5). In the work presented here, the effects of such three-signal stimulation on the early phase of a T cell response to the SAgS SEA and SEB was investigated. We found that the decline in T cell populations was actually prolonged when three signals were delivered, despite this treatment favoring optimal T cell clonal expansion and survival. This delayed accumulation was accompanied by an increase in high affinity IL-2 receptor (CD25) expression on the responding T cells, yet no definitive role for IL-2 in this process was proven. TNF- α , however, does appear to be partially responsible for some of this delayed early accumulation of Ag-specific T cells responding to three-signal stimulation.

MATERIALS AND METHODS

Mice

B10.A mice were obtained from the National Cancer Institute (Frederick, MD). C57BL/6, TNFRI KO and TNFRII KO (13) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in an animal facility at Oregon State University under specific pathogen-free conditions in accordance with federal guidelines. All mice were between 6 and 12 weeks of age.

Reagents, mAbs and flow cytometry

SEA, SEB, LPS and rat IgG were purchased from Sigma (St. Louis, MO). IL-2 was purchased from InterGen (Purchase, NY). All reagents were administered to mice as intraperitoneal (i.p.) injections in balanced salt solution (BSS) or phosphate buffered saline (PBS).

The anti-CD40 mAb producing hybridoma FGK45.5 was a kind gift from Dr. A. Rolink (Basel Institute, Switzerland) (14). The anti-TNF- α producing hybridoma MP6-XT22 (15) was obtained from DNAX (Palo Alto, CA). Supernatants from each of the above hybridomas were purified over protein G agarose (Life Technologies, Grand Island, NY) and dialyzed against PBS for injection.

For flow cytometric staining, the anti-TCR V β 3 mAb KJ25-607.7 (16) was purified from hybridoma supernatant over protein G agarose (Life Technologies) and conjugated to FITC and biotin using standard techniques (17). The remaining

Abs used for staining were purchased from BD PharMingen (San Diego, CA): anti-CD4 was conjugated to either PE or APC, anti-CD8 was conjugated to either PE or APC, anti-TCR V β 8 was conjugated to FITC, anti-CD25 or the corresponding control rat IgG was conjugated to PE and anti-Fas was conjugated to PE. The anti-OX40 antibody was a kind gift from Dr. Andrew Weinberg (Earle A. Chiles Research Institute, Portland, OR). The antibody was conjugated to biotin by us and incubated with the secondary reagent streptavidin-PE (BD PharMingen) for flow cytometric analysis.

Injection schedule

All injections were performed i.p. Injection of 0.30 μ g of SEA and 60 μ g of SEB was considered day 0. Injection of 0.10 mg of anti-CD40 mAb was done two days prior to SAg injection. LPS was always injected 24 hours after the SAg (day +1) in a dose of 10 μ g.

Cell processing and flow cytometry

Spleens and peripheral lymph nodes (inguinal, axillary, and bronchial) were teased through nylon mesh sieves (Falcon, BD PharMingen) and red blood cells lysed with ammonium chloride. After washing, cells were counted with a Z1 particle counter (Beckman Coulter, Miami, FL) and spleen cells were further purified over nylon wool as previously described (18). Briefly, 3-cc syringes were filled with 0.12 to 0.15 g of washed and brushed nylon wool. The columns were prepared with warm BSS 5% fetal bovine serum (FBS), after which approximately

10^7 cells were loaded in a 0.5 ml volume and incubated for 30 min at 37 °C. After draining 0.5 ml away, the columns were incubated an additional 30 min, followed by elution with BSS 5% FBS.

For two- and three-color staining, cells were incubated on ice with the primary Abs in the presence of 5% normal mouse serum, culture supernatant from hybridoma cells producing the 2.4.G2 anti-mouse Fc receptor mAb (19), and 10 µg/ml human γ-globulin (Sigma) to block nonspecific binding. After a 30 min incubation on ice in staining buffer (BSS, 3% FBS, 0.1% sodium azide) with primary Abs, the cells were washed twice and analyzed by flow cytometry, or, if a secondary reagent was necessary, the incubation and wash procedures were repeated. Flow cytometry was conducted on a Becton Dickinson FACSCaliber flow cytometer, and the data analyzed using CellQuest software (BD PharMingen).

Intracellular staining

For the surface and intracellular staining in Table 5.1, LN and spleen cells were initially stained for 30 min with an anti-Vβ3 mAb conjugated to biotin on ice as described above. After washing unbound mAb away, cells were further incubated with PE-conjugated streptavidin and either APC-conjugated anti-CD4 or anti-CD8. After this 30 min incubation, the cells were again washed and fixed for 20 min at room temperature with 2% paraformaldehyde in BSS. After washing twice in staining buffer, the cells were permeabilized by washing with staining buffer containing 0.5% saponin and 1% bovine serum albumin. Another incubation step was performed for 30 min at room temperature using FITC-conjugated anti-

V β 3. The cells were then washed twice with the permeabilization buffer and then twice with staining buffer to allow membrane closure.

In vitro IL-2 restimulation assay

Mice were injected with SEA, anti-CD40 mAb and LPS as described above. At 24, 30 or 36 hours after SEA injection, LN and spleen cells were isolated for culture. Cells from each in vivo treatment group were plated at 250,000 cells per well in complete tumor media (CTM). CTM consists of minimal essential medium with FBS, amino acids, salts and antibiotics. IL-2 was added to the wells at concentrations of 100, 33.3, 11.1, 3.7, 1.23, 0.41, 0.14 or 0 U/ml. The cultures were left for 72 hours with 1 μ Ci of 3 H-thymidine (ICN, Costa Mesa, CA) being added for the last eight hours. Incorporation of 3 H-thymidine was measured on a 1450 Microbeta Trilux Scintillation Counter (Wallac, Turku, Finland).

Bromodeoxyuridine (BrdU) staining

Mice were injected with 0.30 μ g of SEA, 0.10 mg of anti-CD40 mAb, and 10 μ g of LPS at the times described above. Additionally, the mice were injected with 1 mg of BrdU (Sigma) dissolved in PBS 24 and 30 hours after SEA. After 36 hours post-SEA injection, T cells from the peripheral LNs and spleens from the treated mice were isolated, stained with biotin-conjugated anti-TCR V β 8 mAb and then with PE-conjugated streptavidin. The cells were then stained with a modified BrdU staining protocol (20). Briefly, the cells were dehydrated and fixed in ice cold 95% ethanol, then fixed in BSS containing 1% paraformaldehyde and 0.01%

tween-20. Next, cellular DNA was lightly digested with 50 Kunitz units of DNase I (Sigma) and the cells stained with anti-BrdU-FITC (BD PharMingen).

RESULTS

Three-signal stimulation delays early T cell accumulation in peripheral lymphoid tissues

Early T cell responses in this model were originally examined in an attempt to uncover mechanisms that generate long-term T cell survival. During SAg-mediated stimulation of T cells, the Ag-specific T cell population initially declines for about 18-24 hours before clonally expanding to peak numbers (Fig. 5.1) (10). This decline has been attributed to both apoptosis and TCR internalization (10, 21). Interestingly, when stronger T cell stimulation was delivered, either via costimulation or via inflammatory effects resulting from LPS, the Ag-specific T cells took longer to accumulate in the LN and spleen. The most curious effect was observed with three-signal stimulation (SEA, anti-CD40 mAb and LPS). When mice were treated in this manner, the responding T cell populations failed to increase much above their lowest value even after 36 hours.

Fig. 5.1 shows that the percentage of CD4 (Fig. 5.1, A and B) and CD8 (Fig. 5.1, C and D) T cells in the spleen expressing the Ag-specific V β 3 TCR declined during the first 18 hours. CD4 V β 3 T cell populations began at about 6% and fell to 2% during that time, while CD8 V β 3 T cell populations declined from 4% to less than 1%. After this decline, however, the population increased

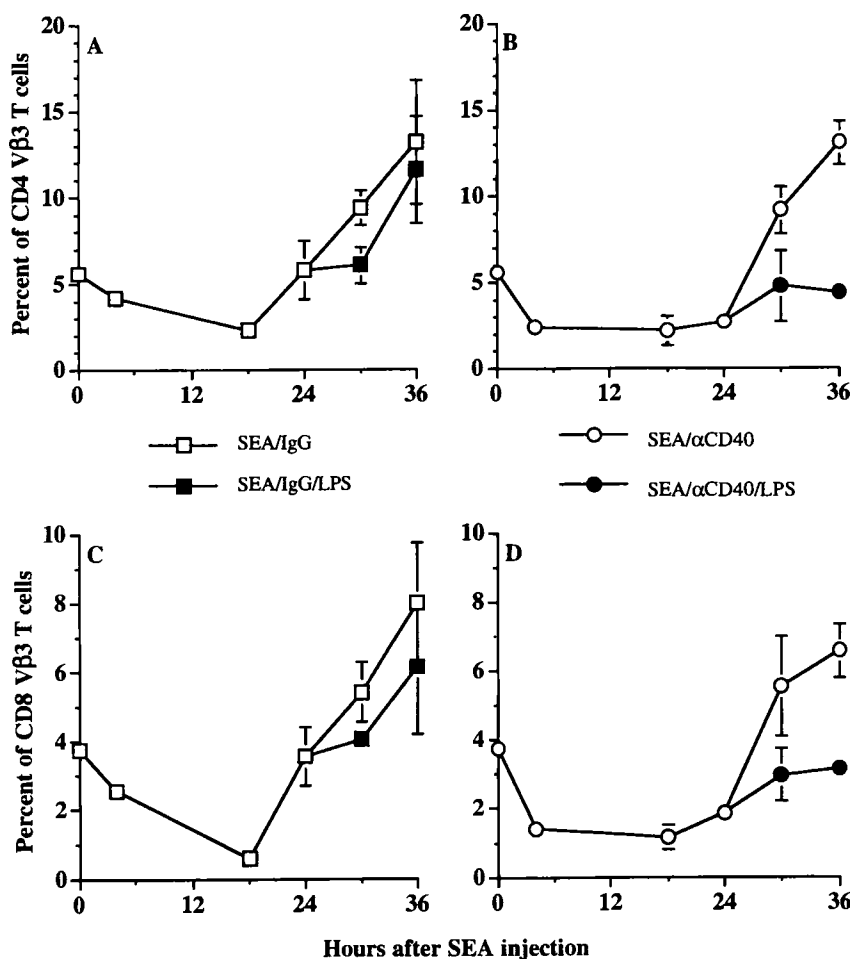


Figure 5.1: Optimal T cell stimulation inhibits the early accumulation of Ag-specific T cells. A total of 45 B10.A mice were placed into four groups: SEA/rat IgG (□), SEA/rat IgG/LPS (■), SEA/anti-CD40 mAb (○) and SEA/anti-CD40 mAb/LPS (●). Doses of 0.30 μ g of SEA, 0.10 mg of anti-CD40 mAb and 10 μ g of LPS were injected as described in materials and methods. At hours 6, 18, 24, 30 and 36 after SEA injection, LN and spleen cells were isolated from three mice per group and stained for CD4, CD8 and V β 3 expression. Graphs represent the mean percentages \pm SEM of splenic CD4 V β 3 (A, B) and CD8 V β 3 (C, D) T cells from three mice per group. Time zero data are derived from uninjected mice. Data are one representative experiment out of six performed.

dramatically over the next 18 hours to near-peak percentages that are typically reached between 48-72 hours. Administration of LPS at hour 24, a treatment that

generates enhanced T cell memory, partially stunted the growth of the SEA-reactive T cells for about six hours, after which, they resumed their clonal expansion (Fig. 5.1, A and C).

In mice that had been pretreated with a CD40 agonistic mAb prior to receiving SEA, the expansion of the V β 3 bearing T cells did not occur until after hour 24, the time of LPS injection (Fig. 5.1, B and D). If no LPS was injected into these mice, the Ag-specific T cells accumulated by 36 hours post-Ag. If, however, LPS was administered, this three-signal stimulation completely inhibited the growth of the CD4 and CD8 V β 3 populations out to 36 hours, allowing the population to grow from around 2% to only 4%.

This early growth inhibition was very interesting since the Ag-specific T cells in mice treated with three-signals reproducibly clonally expand to greater numbers and survive long-term (5). It was curious that such a response would be preceded by such a profound and maintained deletion phase.

To determine what was causing the deletion phase, initially TCR internalization was examined. Internalization of the TCR is a standard occurrence resulting from antigenic stimulation (21). Because Ag-specific T cells are analyzed by surface receptor mAb staining for flow cytometry, the absence of the TCR can make detection of these T cells difficult. To determine if the surface V β 3 was being internalized, CD4 and CD8 T cells were stained for surface V β 3 and then permeabilized and stained intracellularly with anti-V β 3 mAb conjugated to a different fluorochrome. The percentage change between the surface and internal

Table 5.1: Both surface and intracellular V β 3 expression decrease after T cell activation*

Lymph Node CD4	Uninjected	SEA/αCD40	SEA/αCD40/LPS
% Surface Vβ3	5.86	0.55	1.57
% Internal Vβ3	3.83	1.07	1.74
% Change	-34.6	94.6	10.8

CD8	Uninjected	SEA/αCD40	SEA/αCD40/LPS
% Surface Vβ3	3.81	0.40	0.68
% Internal Vβ3	2.47	0.47	0.62
% Change	-35.2	17.5	-8.8

Spleen CD4	Uninjected	SEA/αCD40	SEA/αCD40/LPS
% Surface Vβ3	5.73	1.37	1.33
% Internal Vβ3	3.17	1.22	1.01
% Change	-44.7	-10.9	-24.1

CD8	Uninjected	SEA/αCD40	SEA/αCD40/LPS
% Surface Vβ3	3.70	0.97	0.44
% Internal Vβ3	2.15	0.78	0.31
% Change	-41.9	-19.6	-29.5

* B10.A mice were injected as in materials and methods. At 30 hours after SEA injection, LN and spleen T cells were stained for CD4 and CD8, as well as for both surface and intracellular V β 3 as described in materials and methods. Data show the percentage of cells that are positive for surface and intracellular V β 3 in uninjected, SEA/anti-CD40 mAb treated and SEA/anti-CD40/LPS treated mice. The percent change represents the relative change of intracellular versus surface V β 3 levels: $[(\text{internal \%} / \text{surface \%}) - 1] \times 100$. Data are representative of one out of two experiments.

V β 3 expression in the LN and spleen is shown in Table 5.1. Both surface and intracellular V β 3 declined in two- or three-signal treated mice compared to the expression levels observed with uninjected mice. No significant increase was

observed in intracellular V β 3 expression compared to surface expression. From these data, it did not appear that the V β 3 was merely being internalized and maintained. If it was being internalized, then it was also being degraded and thus very little could be detected.

Total surface TCR expression was also examined by an anti-TCR mAb (data not shown). Little overall decline in TCR surface expression was observed in treated mice, and in some cases, two-signal treated mice had less TCR staining than three-signal treated mice. Thus, little additional information could be gained from looking at total TCR expression on T cells.

Another possible explanation for the observed growth inhibition was cell death, so apoptosis was examined in two- or three-signal treated mice. Ag-specific T cells were stained with annexin V, a molecule that detects phosphatidylserine expressed on dying cells (22, 23). Additionally, the TdT-mediated dUTP nick-end labeling (TUNEL) method was also used to detect degraded DNA in dying cells (24). Neither of these methods could reliably detect dying cells (data not shown). Detection was made difficult by the fact that there are so few Ag-specific T cells to analyze at these early time points. The inflammatory environment following LPS treatment is killing some cells, but whether this is the complete cause of the prolonged deletion phase observed in the three-signal treated mice is still unclear.

In another attempt at explaining what is occurring during these initial 36 hours, proliferation was measured by BrdU incorporation (Fig. 5.2). Interestingly, the percentage of V β 3 T cells that incorporated BrdU during a pulse between 24

and 36 hours after SEA was identical whether or not the mice received LPS (Fig. 5.2A). The striking difference between the two treatments was observed when the absolute number of V β 3 T cells incorporating BrdU was examined. Only about half as many V β 3 T cells took up BrdU when three signals were given compared to two signals (Fig. 5.2B). Thus, during three-signal stimulation, the Ag-specific T cells can at least initiate DNA replication very effectively, but they do not accumulate to the same degree as observed with non-LPS treatment.

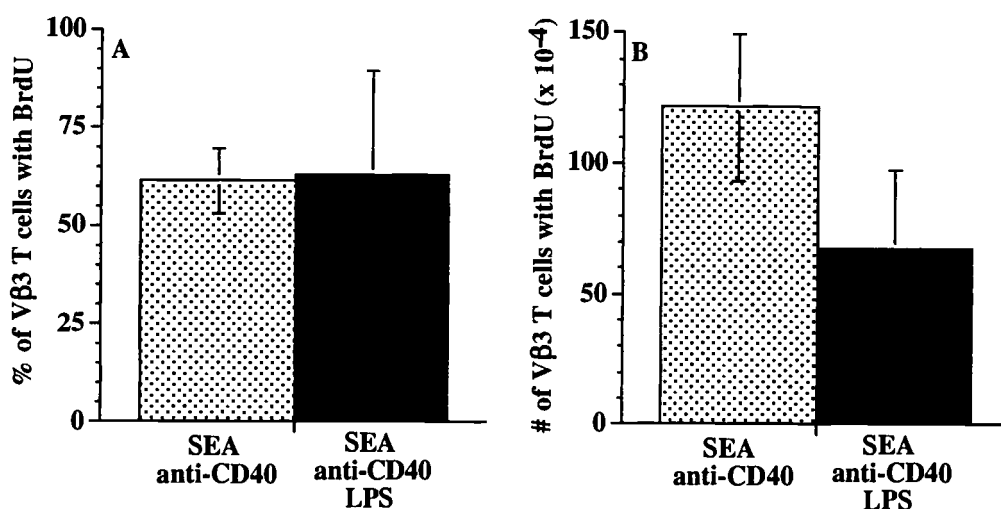


Figure 5.2: Three-signal treated T cells incorporate BrdU normally, but do not accumulate in peripheral lymphoid tissues. B10.A mice were injected with SEA and anti-CD40 mAb (stippled bars) or SEA, anti-CD40 mAb and LPS (black bars), and T cells analyzed from three mice per group as described in materials and methods. Graphs represent the percentage (A) \pm SEM and number (B) \pm SEM of splenic V β 3 T cells incorporating BrdU during a 12 hour pulse between 24 and 36 hours after SEA injection. Data are one representative experiment out of four performed.

When the V β 3-negative cells were examined for BrdU incorporation, between 15 and 25% of them were incorporating BrdU (data not shown). This is a

significant number of cells, and could mean that there are some dividing Ag-specific T cells that have internalized and degraded their TCR, but which cannot be detected by flow cytometry. However, LPS has been shown to promote non-specific T cell proliferation (25). Thus, the observed V β 3-negative cell proliferation could also be a result of other T cells or even B cells responding to the LPS. Overall, it is clear that the cells are “trying” to divide, but are not capable of accumulating.

LPS-stimulated Ag-specific T cells can respond to IL-2

During the analysis of the early expansion phase of the Ag-specific T cells, expression of the high affinity IL-2R α -chain (CD25) was also examined (Fig. 5.3). Prior to stimulation, very few of the Ag-specific T cells expressed CD25. Six hours after SEA stimulation, almost 100% of CD4 V β 3 and CD8 V β 3 T cells expressed CD25 (Fig. 5.3, A and C). This percentage declined dramatically by 24 hours and plateaued during the next 12 hours at around 25%. LPS injection 24 hours after SEA caused a transient increase in the percentage of Ag-specific T cells expressing CD25. By 30 hours, about 75% expression was observed, which declined by 36 hours to levels close to that observed with SEA alone. When SEA and anti-CD40 mAb were injected, the percentage of Ag-specific T cells expressing CD25 again plateaued at 24 hours, and still reached the same 25% level (Fig. 5.3, B and D). If LPS was injected, however, CD25 became increasingly expressed over the next 12 hours, becoming expressed on nearly 100% of all Ag-specific T cells. Thus, LPS stimulation can enhance and maintain the expression of the high-affinity

IL-2R on the surface of Ag-specific T cells, and this is further accentuated after CD40 stimulation.

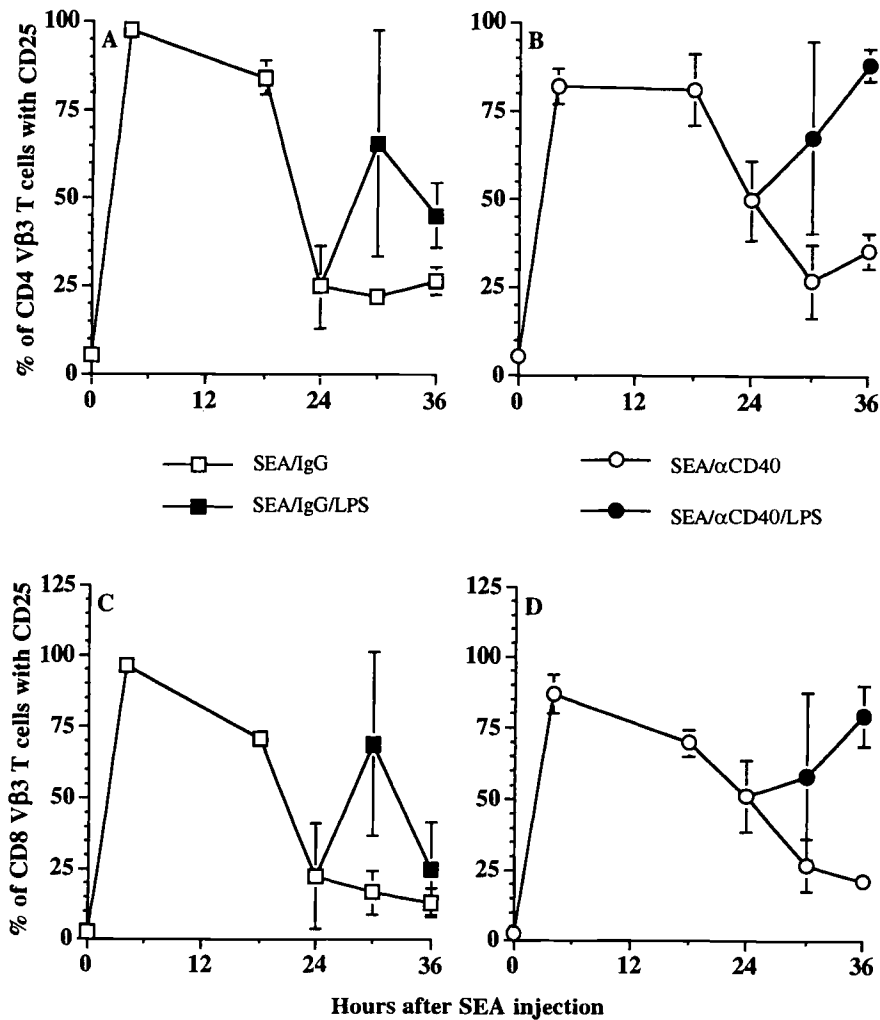


Figure 5.3: CD25 expression is enhanced by LPS, but maintained only when three signals are delivered. B10.A mice were injected and T cells analyzed as described in Fig. 5.1 and materials and methods. At six-hour increments after SEA injection, CD25 expression on CD4 Vβ3 T cells was examined by flow cytometry for the groups: SEA/rat IgG (□), SEA/rat IgG/LPS (■), SEA/anti-CD40 mAb (○) and SEA/anti-CD40 mAb/LPS (●). Data represent the percentages \pm SEM from three mice per group of splenic CD4 Vβ3 (A, B) and CD8 Vβ3 (C, D) T cells that are positive for CD25 expression. Time zero data are derived from uninjected mice. Data are one representative experiment out of six performed.

5.4A). After 30 hours in vivo, T cells from mice treated with or without LPS again responded well to IL-2 treatment (Fig. 5.4B), a trend that was observed again at 36 hours (Fig. 5.4C). At both 30 and 36 hours, T cells from three-signal treated mice proliferated better than T cells from two-signal treated mice in response to IL-2. In vivo, these three-signal exposed T cells did not increase in number early on despite increasing CD25 expression (compare Fig. 5.1 to Fig. 5.3). Based on the in vitro data in Fig. 5.4, these T cells can respond to IL-2, and thus the IL-2R is functional.

To investigate whether the Ag-specific T cells were responding to IL-2 in vivo, mice were treated with three signals and given two injections of IL-2. IL-2 injection did little to affect early T cell expansion at 36 hours (Fig. 5.5A) and survival on day 7 (Fig. 5.5B). IL-2 is a cytokine important for T cell proliferation, but one that can also prime T cells for death (26, 27). Neither enhanced T cell proliferation nor apoptosis was observed with IL-2 injection. This is curious since these T cells can respond to IL-2 in vitro. There also remained the possibility that no IL-2 was being produced in vivo. Using intracellular staining of Ag-specific T cells, no IL-2 could be detected within the Ag-specific T cell population (data not shown). These activated T cells did not appear to be secreting any IL-2 in vivo, however, some unidentified non-T cell was observed to be producing IL-2.

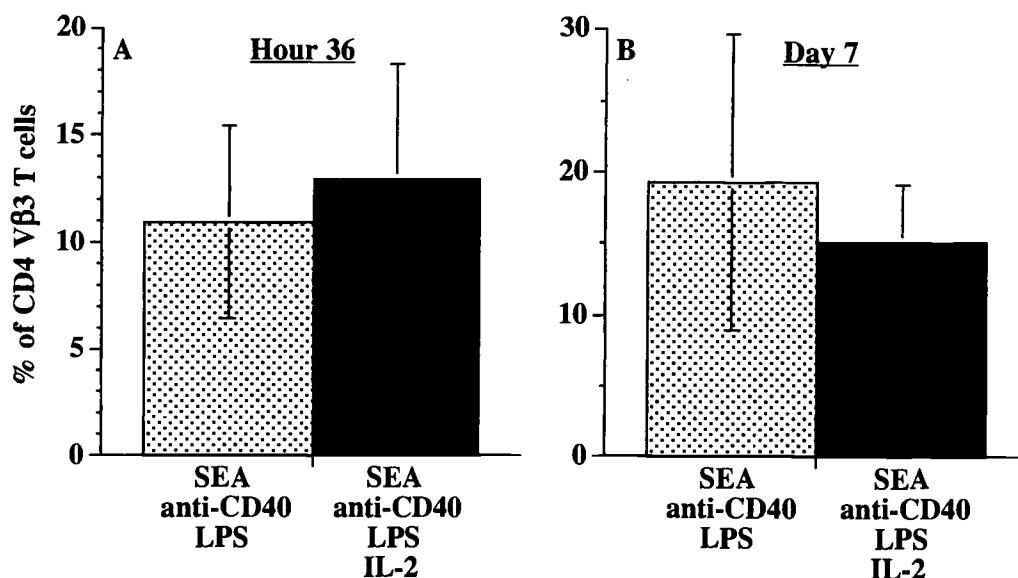


Figure 5.5: IL-2 injection does not affect early expansion and long-term survival of Ag-specific T cells in vivo. B10.A mice were injected with 0.10 mg of anti-CD40 mAb, 0.30 μ g of SEA and 10 μ g of LPS. At 24 and 30 hours after SEA injection, 1.25 μ g of IL-2 was injected i.p. Cells were analyzed as described in materials and methods. Data represent the mean percentages \pm SEM of splenic CD4 V β 3 T cells at 36 hours (A) and seven days (B) after SEA injection. Data depict three mice per group in one representative experiment out of five performed.

TNF- α is partially responsible for stunting early T cell clonal expansion, but is unnecessary for long-term survival

TNF- α is one of the primary mediators of LPS-induced inflammatory responses and a contributor to long-term survival (4, 28). To examine whether this cytokine was affecting the early expansion of the T cells, a blocking antibody was injected in the context of three-signal stimulation (Fig. 5.6A).

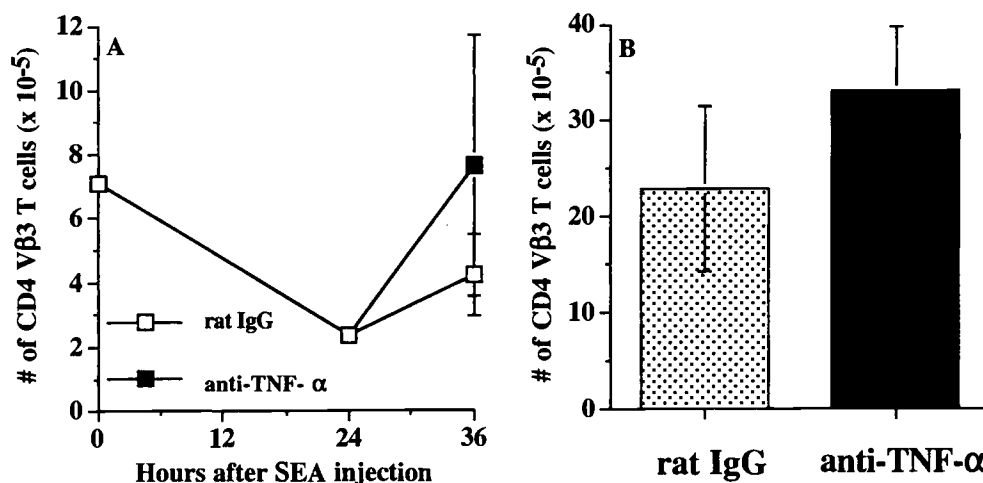


Figure 5.6: Blockade of TNF- α enhances both early T cell expansion and long-term survival. B10.A mice were injected with 0.10 mg of anti-CD40 mAb, 0.30 μ g of SEA and 12 μ g of LPS. At 24 hours post-SEA, 1 mg of an antagonistic anti-TNF- α mAb or rat IgG control mAb was injected i.p. T cells were prepared and analyzed as described from three mice per group. Graphs represent the absolute numbers \pm SEM of CD4 V β 3 T cells in the spleen over a 36-hour time course (A) or after seven days (B). Data are one representative experiment out of four performed.

These data indicate that blocking TNF- α enhanced the accumulation of T cells in the peripheral lymphoid tissues at 36 hours after SEA injection. This quicker early expansion mimicked what was observed with weaker injection regimens, such as with one or two signals, that lead to poor long-term survival. Thus, it seemed reasonable to expect the responding T cells in these mice to die off more readily as opposed to surviving. This is not what was observed, however. When the cells were examined seven days after SEA injection, the mice treated with anti-TNF- α actually had slightly elevated numbers of Ag-specific T cells

compared to those receiving three-signal stimulation alone (Fig. 5.6B). This is similar to other data reported earlier (Fig. 4.7 and 4.8).

To better examine the role of TNF- α in early T cell expansion, mice deficient for TNFRI or TNFRII were used (Fig. 5.7). Injection of these mice with two or three signals allowed a partial examination of the effects of TNFR signaling.

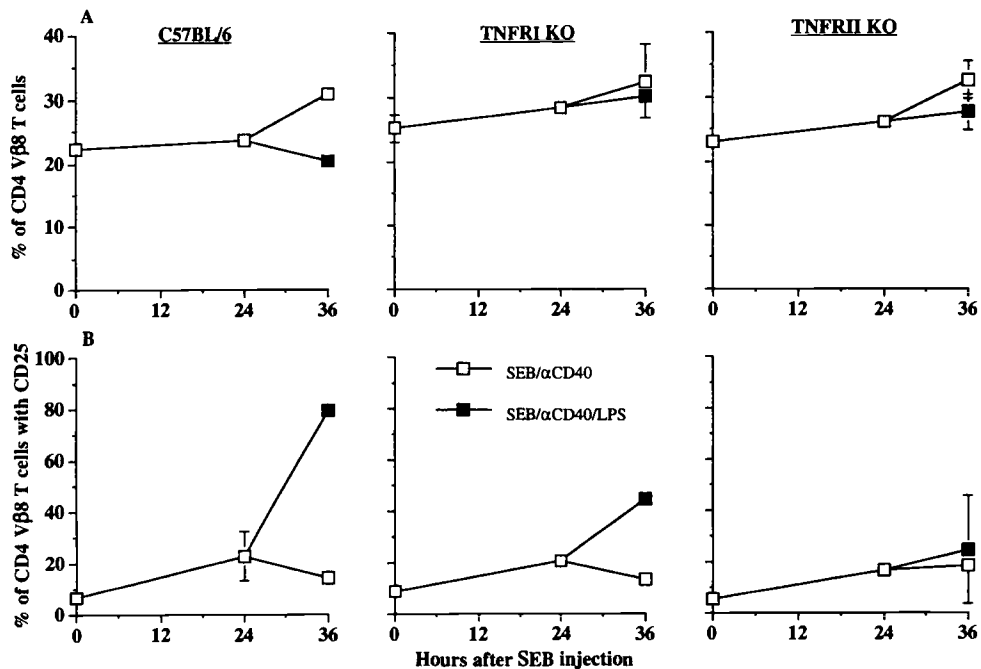


Figure 5.7: Lack of TNFRI or TNFRII signaling prevents some of the early T cell responses observed in normal mice. C57BL/6, TNFRI KO or TNFRII KO mice were injected with 0.20 mg of anti-CD40 mAb, 60 μ g of SEB and 10 μ g of LPS. At 24 and 36 hours after SEB injection, the splenic percentages \pm SEM of CD4 V β 8 T cells (A) and CD4 V β 8 T cells expressing CD25 (B) were determined by flow cytometry. Graphs represent data from three mice in each group: SEB/anti-CD40 mAb (\square) and SEB/anti-CD40 mAb/LPS (\blacksquare). Data is one experiment out of three performed.

Fig. 5.7A shows that the early increase in the percentage of Ag-specific T cells in the spleen at 36 hours was inhibited in control C57BL/6 mice when LPS

was injected. In the absence of either TNFR, however, this inhibition was much less. Additionally, CD25 expression on the Ag-specific CD4 V β 8 T cells was greatly enhanced by LPS treatment in C57BL/6 mice, reaching over 80% at the 36-hour time point (Fig. 5.7B). In the absence of either TNFR this increase was at best around 50%. Thus, TNFR signaling appears to be inhibiting T cell accumulation yet enhancing CD25 expression.

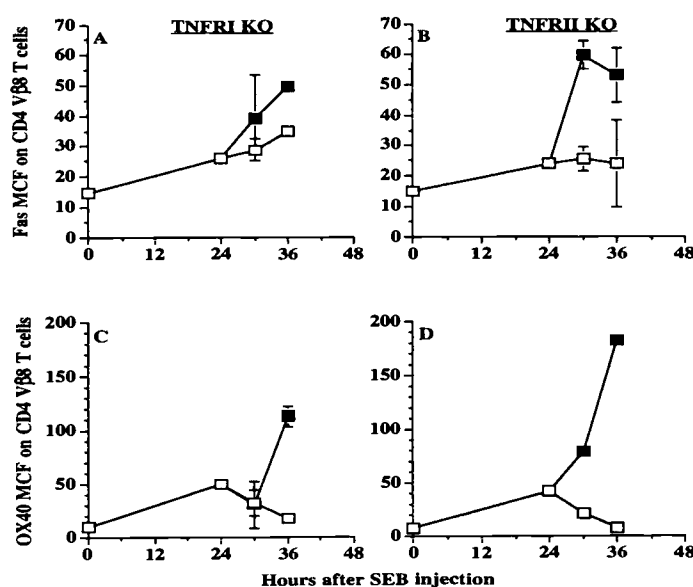


Figure 5.8: Fas and OX40 expression on TNFR-deficient T cells are enhanced by LPS injection. TNFR1 KO (A, C) and TNFR2 KO (B, D) mice were injected with 0.20 mg of anti-CD40 mAb, 60 μ g of SEB and 10 μ g of LPS. At hours 24, 30 and 36 after SEB injection, Ag-specific CD4 V β 8 T cells were analyzed for Fas (A, B) and OX40 (C, D) mean channel fluorescence (MCF) by flow cytometry. The OX40 MCF in the graph is the MCF on CD4 V β 8 T cells that are positive for OX40 expression, while the Fas MCF is the MCF on all CD4 V β 8 T cells. Data are from one representative experiment out of two performed.

Finally, to further investigate the effects of TNFR deficiency on surface molecule expression, Fas and OX40 expression was studied over 36 hours after

two- or three-signal stimulation. In both cases, LPS injection enhanced Fas and OX40 expression on the Ag-specific T cells (Fig 5.8). Thus, in the absence of TNFR signaling, these cells not only become more available to Fas-mediated death, but also can be stimulated very potently via OX40.

DISCUSSION

The initial goal of these studies was to examine early time points after antigen administration for clues to the causes of long-term T cell survival. A very striking observation from this work was that T cells exposed to stimuli that promote optimal memory development take much longer to begin their clonal expansion phase than T cells exposed to weaker stimuli.

Internalization of the TCR is a common occurrence observed after antigenic stimulation, but it is not an easy process to detect. Initial observations of this process were completed using identical T cell clones in vitro (21). The in vivo studies presented here monitored a small population of Ag-specific T cells (6% of all CD4 T cells, or 2% of all total cells, express V β 3 in the LN or spleen). If a fraction of this population internalizes its TCR, overall a small change will occur and be very difficult to monitor. Thus, although we could not directly observe internalization of the TCR on the SAg-reactive T cells, the BrdU data suggests that it may be occurring to some degree. The role of this phenomenon is somewhat undefined, but may be important in attenuating hyperresponsiveness.

It is perhaps expected that nearly all of the Ag-specific T cells express the high-affinity IL-2R after SAg exposure since IL-2 is secreted within two hours

after treatment (29). It is interesting, however, that these T cells re-express and maintain CD25 at increased levels on their surface after LPS signals are delivered. This would appear to be important for responses to IL-2. Since IL-2 is a cytokine that induces T cell proliferation, the fact that the three-signal stimulated T cells do not accumulate could mean that they are not responding to IL-2. However, the in vitro data in figure 5.4 shows that the receptor is functional and that these cells can respond to IL-2. No effect of IL-2 injection could be detected in vivo (Fig. 5.5). The T cells themselves are not making any IL-2, but there was some other source that was observed. Maybe this source of IL-2 is producing saturating amounts of IL-2 in vivo so that when IL-2 is injected in vivo it has no detectable effect.

To fully understand the role of IL-2 in the responses studied here, its signals must be blocked. An interesting experiment would be to block IL-2 with a mAb or use IL-2 knockout mice to observe whether early Ag-specific T cell responses are affected. If IL-2 is killing the T cells, neutralization of IL-2 might enhance their early proliferation and long-term survival. If it was required for survival, responding T cells would die in its absence.

Such IL-2 blocking experiments have been done in the Jenkins laboratory (30). Experiments in mice injected with antigen and LPS showed that blockade of IL-2 enhanced both proliferation and survival of the responding T cells. IL-2 can promote T cell death (27). Thus, the inhibition of growth observed in Fig. 5.1 may be due to T cell death resulting from IL-2 signaling. Unfortunately, no Ag-specific T cell death could be reliably detected in the responding T cell populations by flow

cytometry. Fig. 5.4 showed that these cells proliferate in vitro in response to IL-2, but they may be dying soon afterward. An interesting experiment would be to purify the responding T cells from the mice at these early time points and give them IL-2 in vitro, but instead of measuring proliferation, monitor them for death. As mentioned before, such an experiment might explain whether IL-2 signaling is an important cause of this early deletional phase.

Some studies have suggested that DNA laddering, one of the characteristics of apoptosis, is the cause of this early deletion phase (10) although whole LN populations were examined in these studies, and not just responding T cells. Other more recent work found very little apoptosis in the antigen-specific T cell population until after the peak of clonal expansion (31). The majority of the dying cells were found in the proliferating population and not in the non-responders. Thus, the role of apoptosis in the growth inhibition observed here is unclear.

Like IL-2, TNF- α is secreted within a couple of hours after SAg administration (29, 32). Blockade of TNF- α both by mAb treatment and by the use of receptor knockout mice prevented the delayed accumulation of T cells as well as the increase in CD25 expression observed under normal conditions. Unfortunately, in mice injected with TNF- α in the place of LPS, no consistent or significant effect was observed in the early or long-term T cell response (data not shown). Thus, TNF- α may be detrimental to early survival, and although earlier studies pointed to a long-term survival-inducing role for TNF- α in T cell responses (4), its absence or

inhibition seems to actually enhance T cell activation and survival (Fig. 5.6 and 5.7).

What could be occurring during early T cell responses in this three-signal model is that the SAg is inducing IL-2 and TNF- α secretion which is causing the T cells to die or internalize their TCR. Once these cytokines and the SAg are cleared from the system, the T cells recover and begin their clonal expansion. Injection of LPS is likely inducing TNF- α that is acting on the responding T cells to increase expression of CD25. The IL-2 that is secreted binds to CD25 and again either kills off the cells or signals them to internalize their TCRs. The former option would be detrimental to the response, while the latter choice might allow for preparations to be made for a switch to a memory T cell.

The prolonged delay observed in this model seems to be important for memory T cell development, but not required. It is very clear that long-term survival is often preceded by a slow initial response. What could be the significance of this? Maybe this delayed response represents a switch to a memory phase. Three-signal stimulation enhances Fas and OX40 expression even in the absence of TNFR signaling (Fig. 5.8). OX40 is a potent costimulatory signal that may be crucial for memory responses (33). Furthermore, in T cells that survive in response to SEA/LPS stimulation, Fas death pathways are uncoupled (4). Thus, this delayed accumulation could be due to the responding T cells taking some time to increase the expression of anti-apoptotic molecules (c-FLIP or Bcl-2 family

members) and enhance the expression of other molecules that stimulate memory induction.

The cellular processes occurring during the early phases of T cell activation in response to SAg and three-signal stimulation are still unclear. Although migration can account for the disappearance of the responding cells, apoptosis and TCR internalization are likely causes. The antigen-specific T cell population may also be responding in a heterogeneous fashion. There is some evidence that V α chains can influence SAg binding to the TCR (34, 35). If the responding T cell population uses different V α chains, they may not all respond equally to stimulation. Some may respond quickly and die off, while others may take longer to initiate activation and as a result, survive.

The significance of this delay seems to suggest that memory T cells take longer to develop. Whether this is due to prolonged TCR internalization and memory preparation or to a selection process that kills off weak responders in favor of robust responders is unclear. To date, the phenomena observed here have not been the object of much study. They are very intriguing, however, and may not only help to provide clues to how long-lived T cells are generated in this model, but how SAg and long-term immune responses initiate.

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CONCLUDING THOUGHTS

The use of a combination of SAg, costimulation and LPS *in vivo* provides a very effective model for studying the mechanisms behind memory T cell induction. The numbers of surviving T cells generated after three-signal stimulation are consistently and dramatically larger than those observed with one or two signals (Figs. 3.2, 3.3, 4.1 and Table 3.2). Thus, knowing exactly how this survival signal is being induced is critical, not only to understanding this model, but also for improving current disease treatments based on manipulating a T cell response.

The work presented in this dissertation has provided some interesting new information regarding T cell survival and the role that adjuvants play in that process. In the context of three-signal stimulation, many proinflammatory cytokines, as well as NF- κ B activity are not required for long-term survival (Figs. 4.7, 4.8, 4.9). This is quite surprising given that all of these molecules have been reported as having anti-apoptotic functions (1-5). However, both cytokines and NF- κ B activity can contribute to cell death (6-9). It may be that cytokine and NF- κ B signals are more apoptotic in this model. TNF- α does appear to be at least inhibitory, if not pro-apoptotic, during the early phases of a T cell response in our model system (Fig. 5.7). Or, it could be that cytokine signals and NF- κ B transcription may promote T cell survival, yet in their absence other survival pathways may substitute. Such is the difficulty of *in vivo* experiments.

What is evident from this work is that the costimulatory signal does not seem to matter. CD40 (CD28-mediated) and OX40 costimulatory signals can both

synergize with LPS to optimize T cell survival. Thus, we ultimately favor a three-signal hypothesis that extends the two-signal hypothesis, which explains T cell activation, to include T cell survival (Fig. 6.1). In this model, both antigen and costimulation are essential for fully activating T cells. But, to optimally keep those responding T cells alive, an inflammatory or adjuvant signal must be delivered. The more T cells that result from the activation phase, the more that can be induced to survive by LPS.

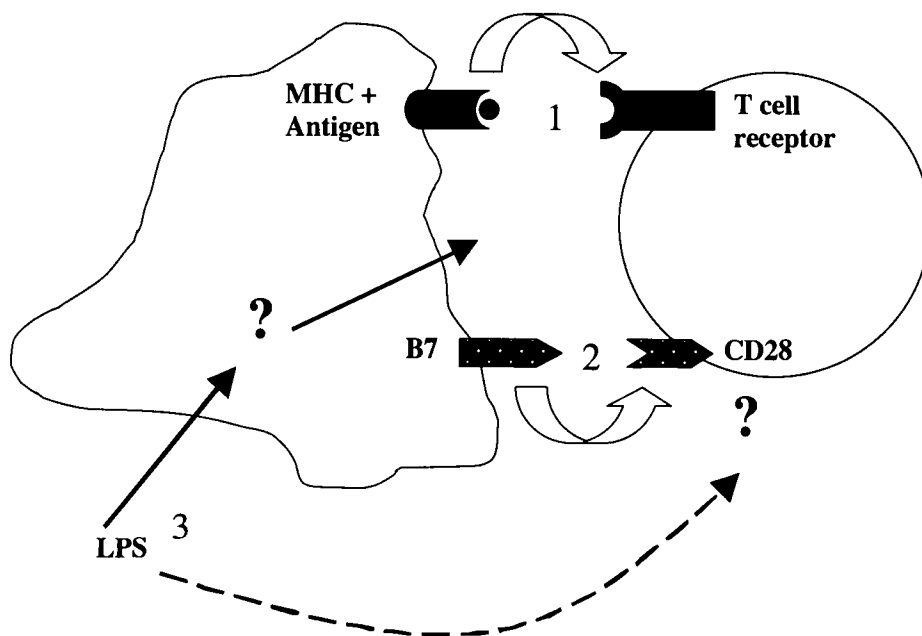


Figure 6.1: The Three-Signal Hypothesis. Signals 1 (Ag) and 2 (costimulation) promote excellent T cell activation, but poor long-term survival. The presence of signal 3 (LPS, or any adjuvant) during this activation phase is required to produce optimal T cell survival and memory induction. How LPS specifically enhances survival is still unknown.

WHAT DOES CYCLOSPORIN TELL US ABOUT T CELL SURVIVAL?

To elucidate the mechanism of T cell survival induction, it was crucial that we find something that could inhibit it. In the search for this survival inhibitor, a very common and quite heavily studied immunosuppressant, cyclosporin A (CsA), surprisingly did the job. T cell survival was completely inhibited in its presence (Fig. 4.12). The primary mode of action of CsA is to inhibit calcineurin (10, 11). CsA does this by binding to cellular cyclophilins and forming a complex that can bind to and inhibit calcineurin, a dephosphatase necessary for the activation of many transcription factors, the primary one being NFAT (See Fig. 1.7). Thus, transcription of T cell survival genes by NFAT or calcineurin-regulated transcription factors could be the means to keeping these cells alive.

What genes are induced by NFAT? Many of the known targets of NFAT activation are cytokine genes (12). Most interleukins, as well as TNF- α and IFN- γ , are transcribed by NFAT and are blocked by CsA administration. The same goes for many cell surface molecules like CD40L, FasL and the IL-2R α -chain (12). Little is known, however, about NFAT-mediated transcription of survival genes such as those in the Bcl-2 family. Some studies have suggested a role for NFAT proteins in Bcl-2 expression, but the significance of that may not be fully appreciated until now (13).

SAg-stimulated T cell death is enhanced by CsA treatment (14). Additionally, CD40 stimulation and subsequent NFAT activation in B cells is sensitive to CsA (15). However, CD28 costimulation in T cells is supposedly

unaffected by CsA (16, 17). Additionally, CsA can inhibit B cell proliferation, but when stimulated with LPS, those B cells are resistant to the inhibition (18). In the three-signal model, the SEA and CD40 signals we are delivering are sensitive to CsA, but the CD28 and LPS signals are not. This assumes, however, that B7 is still upregulated after CD40 stimulation. If CsA blocks B7 induction, then we would observe very poor T cell activation, essentially similar activation as observed with SEA alone. Such a poor response in the presence of CsA would lead to enhanced death, possibly death that cannot be overcome by LPS.

Thus, the inhibition observed here might be due to an irreversible death stimulus that affects the SAg-reactive T cells in the 24 hours between SAg and LPS administration. If the T cells do not proliferate, then there will be much fewer around for LPS to rescue, and thus low numbers of long-lived Ag-specific T cells will result. An experiment testing the timing of CsA treatment is absolutely critical for understanding the role of calcineurin-regulated pathways in this survival process. CsA treatment is often ineffective if given too long after an antigenic stimulus (11, 19, 20). Thus delivery of CsA 12 hours after SAg, but still prior to LPS treatment might elucidate whether the survival effect really is mediated by NFAT or related pathways. As an interesting side note, both LPS and cytokine signaling do not utilize calcium mobilization, something that is required for NFAT activation (12). To find a role for calcineurin and calcium release in response to LPS signaling would be truly novel.

Another complicating factor in the interpretation of the CsA data is that NFAT activation may not be the only affected pathway. Other transcription factors are modulated by calcineurin (21). AP-1, NF- κ B, and octamer associated protein (OAP)-1 can all be affected to varying degrees by CsA (11, 22, 23). Thus any of these transcription factors, alone or in combination, may be the key to T cell survival.

To really test whether NFAT is involved, there are many options available. NFAT1 and NFAT4 knockout mice are available and could be injected with three-signals and the resulting T cell survival analyzed (13, 24, 25). These may not be the best models to use, however. There is the possibility of redundancy of function between NFAT family members, where in the absence of one member, others can substitute. Additionally, the phenotypes of these mice might affect the results. T cells in NFAT1 knockout mice hyperproliferate in response to antigens, while NFAT4 knockout mice have reduced numbers of peripheral T cells as a result of defective selection in the thymus.

Since there may be redundant functions for NFAT proteins, other mouse models could inhibit all of them, but would still globally affect T cell responses, and as such, may also not be suitable. Mice deficient in the tyrosine kinases lck or ZAP-70 would inhibit NFAT activation due to blockade of calcium and TCR signaling pathways. These mice, however, have very few peripheral T cells due to lack of survival in the thymus, thus they may not be of much use (26, 27). Additionally, p21ras is absolutely required for NFAT activation, and T cells

transgenic for a dominant negative version of it can shut down NFAT, primarily through inhibition of the MAP kinase pathway (28, 29). Such experiments were only done in vitro. Mice deficient in p21 do exist, and are viable, but surprisingly they have increased numbers of memory CD4 T cells and defective mechanisms of tolerance induction to some antigens (30). These observations alone seem to argue against a role for NFAT in T cell survival. Overall, mice deficient in any of these components would inhibit NFAT, but unfortunately, might have other unwanted side effects that would make interpretation of experimental data difficult.

Interestingly, there could be a cooperative function for NFAT and NF- κ B in the survival response. Although there has not been demonstrated any direct interaction between NFAT and NF- κ B, they both can bind to similar DNA sequences and initiate transcription. NFAT can bind to κ B-like sequences found in the TNF- α , IL-8 and E-selectin promoters, as well as in the HIV-1 LTR (31-34). In the case of the human IL-4 P sequence, NFAT and NF- κ B even compete for binding (35). NF- κ B can be inhibited by CsA, but this inhibition is weaker, and is thought to occur later than that observed for NFAT (11, 22, 23). An NF- κ B activation pathway dependent on calcium mobilization and new protein synthesis is what is sensitive to CsA; other activation pathways not requiring new protein synthesis, such as through cytokine or LPS receptors, are supposedly unaffected (10, 36). Thus, CsA could be interfering with a combined effect of NFAT and NF- κ B transcription, or disrupting interaction or competition between them. Maybe in the absence of NFAT (which is more potently and rapidly affected by CsA), NF- κ B

promotes apoptosis (Fig. 4.12), but in the absence of NF- κ B, NFAT can promote survival (Fig. 4.9). Thus, long-term T cell survival in his model might depend on a balance between the activity of these important transcription factors (Fig. 6.2).

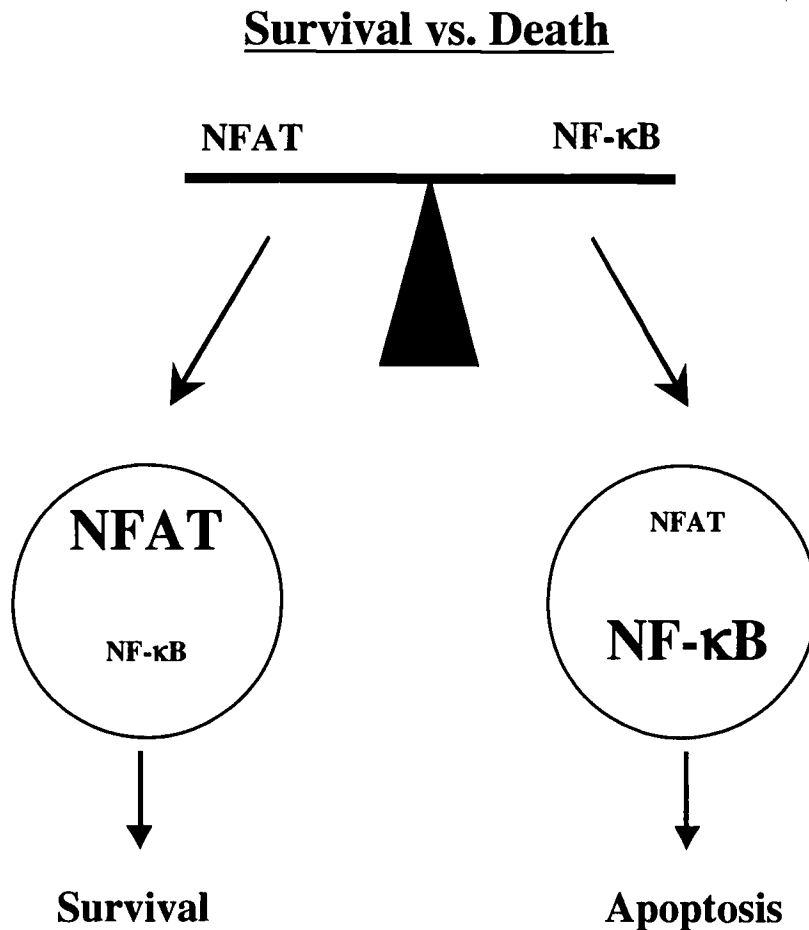


Fig. 6.2: T cell survival may be a balancing act between transcription factors like NFAT and NF- κ B. T cell survival may occur when NFAT transcriptional activity is greater than NF- κ B activity. When NF- κ B predominates, apoptosis may occur. Different ratios of these transcription factors might also affect whether a survival factor is made, or cell death is initiated.

WHAT SPECIFIC MOLECULES COULD BE PROMOTING SURVIVAL?

Previous work done with surviving SEA/LPS-treated T cells found that they were resistant to Fas-mediated death (1). This could be due to the expression of a molecule called cellular FLICE-inhibitory protein (c-FLIP) (37). Normally, when Fas is ligated, its death domain binds to and activates the caspase FLICE that begins the destruction of the cell. When c-FLIP is present, it binds to the Fas death domain, sequestering it away from FLICE. FLICE cannot bind to the Fas complex and thus will not trigger death. Thus, T cell survival may be due to uncoupling death pathways via inducing expression of protective molecules such as c-FLIP. The protein c-FLIP only protects against receptor-mediated death. It does not play a role in other forms of apoptosis such as growth factor withdrawal, which also likely plays a role in activated T cell death. Thus, even if c-FLIP expression were to be enhanced and maintained, this would not completely account for long-term T cell survival.

As mentioned earlier, proteins of the Bcl-2 family may be induced during these *in vivo* treatments (38, 39). The primary candidate is Bcl-x_L. This protein is upregulated upon T cell activation and is found in memory cells (40-42). Whether this is expressed in the T cells generated by three-signal stimulation is not known, but to truly know if Bcl-x_L expression is required for long-term T cell survival, a knockout mouse is needed. Such mice have been made, but they die before birth and thus cannot be used (43). What could be done is to inject Bcl-x_L-deficient embryonic stem cells into a RAG knockout mouse that lacks B and T cells. The

recipient mice would survive and develop Bcl-x_L-deficient T cells, and upon three-signal stimulation could be examined for long-term T cell survival. Another possibility is to use transgenic mice that have the Bcl-x_L protein under the control of the lck promoter (44). Such mice constitutively express Bcl-x_L only in their T cell populations. Injection of SAg and costimulatory mAb could be performed on these mice to see if T cell survival can be obtained in the absence of LPS when Bcl-x_L is constantly expressed. Or, another variation would be using mice that have a dominant negative version of Bcl-x_L under the control of the lck promoter. Here, the mice would develop normally, and only their T cells would be deficient in Bcl-x_L. The ability of these deficient T cells to survive long-term after three-signal stimulation would be an easy experiment. To date, such mice have not been created.

Both c-FLIP and Bcl-x_L proteins could be the key players in T cell survival, alone, or even more likely, in combination. Production of these molecules could be what is delaying the early accumulation of T cells in the peripheral lymphoid tissues that was observed in Fig. 5.1. Possibly, the T cells hold back their response until complete preparations for memory generation have been made. Neutralization of TNF- α enhances early T cell responses, but still generates effective long-term survival (Fig. 5.6). Along this line of reasoning, TNF- α might somehow slow down the production of survival molecules, or the switch to a memory cell. When blocked, these processes may be accelerated. This being said, even if survival proteins such as c-FLIP and Bcl-x_L are the cause of survival, uncovering exactly

how they are produced and maintained would still require study. These proteins are not under random control processes. Something must stimulate their production and activity.

WHAT SOLUBLE SIGNALS COULD FAVOR SURVIVAL?

Aside from NFAT, there are other possible sources for the survival signal. We tested some cytokines that may have been responsible (Fig. 4.5), but not all of them. Other strong cytokine candidates for inducing the survival signal are IL-15 and IFN- α/β . These cytokines have not been investigated in the three-signal model, and may be important. IFN- α/β has been shown in vitro to directly act on activated T cells and prevent their death (45). In vivo, poly I:C, a synthetic RNA molecule that induces IFN- α/β also stimulates memory CD8 T cell proliferation (46). This effect, however, was attributed to downstream IL-15 induction (47). Additionally, in those studies, IL-15 stimulated the proliferation of preexisting CD8 memory cells; it did not necessarily generate those memory T cells. Furthermore, IL-15 only affected CD8 memory cells, but not CD4.

Our treatments generate very effective CD4 and CD8 T cell survival. Furthermore, our rt-PCR data (Fig. 4.5) show that some enhancement of IL-15 mRNA is induced after LPS stimulation. This change is rather small, but could be significant. IL-15R α -chain knockout mice do have reduced numbers of memory phenotype CD8 T cells, but whether this cytokine is necessary for their generation, or is more important for their maintenance remains to be determined (48). However, recent findings in the Lefrancois laboratory suggest that T cell memory

can be induced in the absence of IL-15 (49). Thus, overall IL-15 does not seem to be the likely cytokine candidate causing T cell survival. IFN- α/β seems to be the best remaining candidate for survival. This is a strong possibility because recent work in the Tough laboratory found that IFN- α/β is essential for the adjuvant effect observed with CFA (50). Thus, the effects of three-signal stimulation need to be examined in mice deficient for these cytokines to fully determine their role in T cell survival.

THE ROLE OF T CELL RECOGNITION OF LPS

In order for LPS to cause such an enhancement in long-lived T cell populations, some extracellular signal must be initiating the T cell survival response. Most of the literature suggests that the APC is the crucial mediator of the effects on T cells that are observed with LPS. And while this indirect interaction may be very important during an immune response, it may not be the only method of T cell stimulation. There are actually quite a few poorly studied mechanisms by which T cells could be directly responding to LPS itself.

If the T cell survival signal is delivered by a secreted soluble factor binding to T cells, the source of the factor could be NK1.1 T cells. These T cells recognize glycolipid antigens presented by an MHC class I-like molecule named CD1 (51, 52). Thus, it may be that LPS is being presented to these NK1.1 T cells and activating them. NK1.1 T cells are not well understood, but much research done within the last year suggests they can induce rapid and early production of cytokines necessary for T cell development (53). Some researchers believe these

cells are a crucial link between the innate and adaptive immune responses and that they may provide clues to the mechanism by which adjuvants work (54).

Currently, only presentation of synthetic and mycobacterial lipids by CD1 has been studied. Mycobacterial antigens are an essential constituent of Complete Freund's Adjuvant (CFA), and thus could be initiating enhanced immune responses via CD1 presentation. Nothing is currently known about the ability of LPS to be presented by CD1. It may not be possible, but if it is, LPS presentation to these NK1.1 T cells may initiate their secretion of a T cell survival factor, or at least a cytokine that can induce one. Mice deficient in CD1 have been created (55, 56) and could easily be used to examine whether three signals can induce long-term T cell survival in the absence of these NK1.1 T cells.

LPS presentation to T cells may also occur by another route. Recent work found that the LPS from *Brucella abortus* can bind to MHC class II molecules and be presented to T cells (57). Such presentation induced T cell activation that was inhibited by anti-*Brucella* LPS and anti-class II mAbs. This response was limited only to *Brucella* LPS and did not work with LPS from *Escherichia* and *Shigella* species. The work in this thesis was done with *Salmonella* LPS, which was not studied by this group. If this presentation pathway is real, and can function with *Salmonella* LPS, it would be a truly novel way of stimulating T cell responses.

Finally, T cells might be able to respond directly to LPS by binding to it via toll-like receptors (TLRs), that until very recently were only thought to play a role in innate cell responses (58). TLRs can actually be found on T cells in small

amounts (59). Signaling via TLRs is mainly known to activate NF- κ B transcription, but can activate AP-1 and MAPK pathways as well (60, 61). Demonstrating T cell activation by direct binding of LPS via TLRs would also be novel.

WHAT CAN THIS KNOWLEDGE DO FOR HUMAN HEALTH?

A better understanding of how memory T cells are generated could assist in the treatment of many diseases. First, vaccine technology could benefit. T cells are central to humoral and cellular adaptive immune responses and can enhance an already effective innate response. If the means of inducing this T cell survival signal could be incorporated into a vaccine, many immune responses could be improved and many diseases that are currently difficult to treat might be cured.

Additionally, the observation that CD8 T cells respond to this survival signal could allow for better tumor therapies. CD8 T cells are major players in anti-tumor responses, both by direct cytolytic activity and attraction of phagocytic cells. A better CD8 T cell response as well as a better CD4 response could conceivably improve the treatment of many forms of cancer.

Finally, an understanding of how T cells are maintained in a long-lived state could be turned against them to treat autoimmune diseases. A chronic inflammatory environment characterizes many autoimmune diseases. If the response could be eliminated, the painful symptoms of such conditions could be alleviated.

Ultimately, at a basic cellular level, this work strives to contribute to the growing knowledge of the mechanisms of memory T cell induction. On a farther reaching level, hopefully, many chronic or fatal conditions can be treated by the application of these studies into more applied work.

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