# THE ROLE OF KERATINOCYTE DERIVED INTERLEUKIN-1 $\alpha$ AND TUMOR NECROSIS FACTOR $\alpha$ IN EPIDERMAL LANGERHANS CELL DEPLETION

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
DECEMBER, 1996

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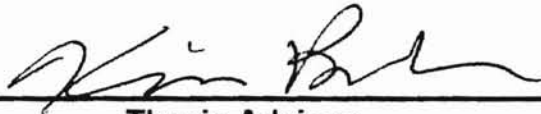

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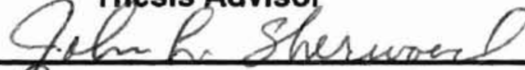

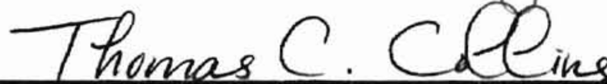

Dean of the Graduate College

## PREFACE

Advances in the area of skin immunology have been increasing at an explosive rate. With the discovery of skin associated lymphoid tissues and classification of the cells associated with this system, much has been learned about how the mammalian body protects itself from invasion by potentially harmful environmental factors. The purpose of this study was to determine, in part, the effect of staphylococcal enterotoxin A (SEA) on cells of the epidermis with regard Langerhans cell migration and cytokine production. This was achieved by Langerhans cell enumeration in murine epidermal sections following exposure to SEA, interleukin-1 $\alpha$ and tumor necrosis factor $\alpha$ through immunohistochemical staining.

Results suggested that keratinocyte derived interleukin-1 $\alpha$ and tumor necrosis factor $\alpha$ play a pivotal role in inducing Langerhans cell migration from the skin to the draining lymph node in response to antigenic challenge with SEA. Application of these two cytokines directly to skin sections resulted in depletion of Langerhans cells from the epidermis equivalent to that observed by treatment with SEA. Furthermore, the effects of interleukin-1 $\alpha$ and tumor necrosis factor $\alpha$ were blocked by pre-treatment with neutralizing antibodies against each cytokine.



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This work was funded through a grant from the Oklahoma Center for the Advancement of Science and Technology (OCAST).

I would like to express my deepest gratitude to Dr. Kim Burnham for giving me the opportunity to begin my research endeavors in his laboratory as an undergraduate and ultimately as a graduate student. His patience, understanding and guidance have made this experience very rewarding. I would also like to thank Dr. John Sherwood and Dr. David Demezas for serving on my committee and providing their thought provoking questions and insightful suggestions.

A very special recognition goes to my colleague Murali Bandla. I can never repay him for his advice, encouragement and friendship while working toward this degree. The same heartfelt thanks goes to my lab partners Gopi Shankar and Jeremy Johnson for always being there in good time and bad.

To Jason--I will forever be indebted to you for helping me to see the good in every situation and for sharing all the ups and downs that graduate school has to offer.

Finally, I dedicate this thesis to my parents, Rick and Beverly. None of this would have been possible without their unconditional outpouring of love, friendship and support. They have helped me to see this dream become a reality and I will be forever grateful. Mom and Dad, this is for you.

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# CHAPTER I

# LITERATURE REVIEW

## Integument

The integument, or skin, is the largest organ in the mammalian body and for many years, was the most misconstrued of all the organ systems. It has been widely accepted that the skin serves as a protective barrier, shielding the internal workings of the body from the potentially harmful effects of dehydration, toxic compounds and a vast array of infectious microorganisms and insects. However, the technologies of modern science have enabled many investigators to look beyond the surface of the skin and now associate the processes of temperature regulation and neuronal stimulatory senses of hot, cold and pain with capillaries and sensory nerves embedded within the deepest layers of the skin.

The physical barriers that the skin utilizes to effect protective functions are simple yet elegant in their design. The skin is covered with a fine layer of hairs which serve as a primary deterrent for many biting insects. The outermost layers of the skin are constantly sloughed off and bathed in salty sweat and acidic, oily sebum from glands located in the deep underlying layers (48). The acidic nature of the skins' surface and the constant loss of outermost cells plays a significant

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role in preventing large numbers of harmful microbes from penetrating the cutaneous surface.

Immunologically, the skin is more than just a physiochemical barrier protecting the body from the external forces of the environment. In very recent years, it has been widely established that a specialized relationship exists between the skin and the immune system that allows for immune protection for cutaneous surfaces without significant impairment of the skins' physiological responsibilities (7). This phenomenon is vitally important in understanding the roles individual cells of the skin may play in regulating the pathology of many infectious and potentially fatal diseases. Growing concern over the depletion of the ozone layer and over-exposure of organisms to the harmful effects of UVB radiation are of great importance to scientists worldwide. UVB has been shown to effectively cause the depletion of Langerhans' cells (LC), the major antigen presenting cell in the cutaneous layers, from mammalian skin (9, 39).The fate of LC after UVB exposure is unknown but it has been hypothesized that the absence of these protective cells and the continued exposure to the ionizing effects of the sun may significantly contribute the onset of skin cancer in susceptible groups of individuals (39).

Another main focus of skin immunology revolves around the more cosmopolitan subject of arthropod-borne disease. The World Health Organization reports that 2.5 million people die each year of malaria alone in Africa with roughly 1 million of those being children (34). Individuals living in the Caribbean and parts of southeast Asia are constantly faced with outbreaks of

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mosquito-borne dengue hemorrhagic fever. Malaria and other arthropod transmitted diseases, such as yellow fever, still remain a constant threat to those living in the jungle regions of Africa and South America. All of these pathologic conditions are the direct result of an insect bite through the protective defenses of the skin. More importantly, many researchers have determined that many arthropods have developed elaborate means by which to modulate the host immune system at the level of cutaneous immune responses in order to more effectively obtain a blood meal and thereby transmit pathogens (34).

In the past 20 years, much progress has been made in terms of understanding the importance of the skin as an immune organ. By examining the ultrastructure of the integument, further advances can be made in terms of effectively treating many trenchant pathologic conditions around the world.

## The Epidermis

The epidermis is composed of tightly packed layers of stratified squamous epithelium covered by an insoluble layer of keratin, a protein product of epidermal epithelial cell differentiation (48). Its primary function is to protect the collagenous dermal layer beneath it which is richly endowed with sensory nerves, blood vessels and vessels of the lymphatics. It can be divided into 5 distinct layers: the dermal epidermal junction, the stratum germanitivum or basal layer, stratum granulosum, stratum spinosum, and the stratum corneum or horny layer (63). Many different cell types contribute to the integrity of the epidermis,

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all with unique and highly specialized functions. However, one of the most important contributions the cells of the epidermis lend to an organism is the protection they offer with regards to initiating an immune response.

**SALT: Skin Associated Lymphoid Tissue**

The highly specialized nature of skin, which permits it to carry out its' various differentiated functions so superbly, requires that immunity expressed within its' confines be appropriate and consistent with maintenance of the tissue's physiologic functions (7). There are four functionally distinct cell types in the skin responsible for mediating immunity: dendritic epidermal T-cells (DETC) and circulating lymphocytes, endothelial cells, keratinocytes and Langerhans cells. Collectively, these cells have become known as skin associated lymphoid tissue (SALT). The concept of SALT is very recent with the most relevant findings being made only ten years ago. Keratinocytes contribute 90 to 95% of the actual cell mass of SALT and therefore create a very rich environment for the other cellular constituents of SALT because of their innate ability to produce large quantities of very potent regulatory cytokines (7, 22, 23). Subsets of circulating lymphocytes serve the skin by accurately identifying pathogenic antigens and effectively stimulating the appropriate humoral and cell mediated immune responses. Langerhans cells function as resident antigen presenting cells and have the unique ability to migrate out of the periphery into the dermal lymphatics and present antigen to T-helper cells in the draining lymph nodes. Lastly,

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endothelial cells lining the blood vessels will up-regulate and express a variety of cellular adhesion molecules which function to "traffic" circulating immune cells to sites of antigen challenge as shown in Figure 1 (7, 63).

## Dendritic Epidermal T-Cells

Before understanding the complex physiology associated with the DETC, it is first necessary to harbor reasonable knowledge concerning the structure and function of T-lymphocytes which are capable of circulating to the skin.

Skin seeking lymphocytes are CD4+ T-cells of the helper/inducer phenotype (7). These cells are responsible for inducing the cell mediated immune response through a variety of cytokine regulated pathways of cell activation and proliferation. This is achieved through the binding of the T-cell receptor (TCR) to a Major Histocompatability Complex Class II (MHC-II) molecule on the surface of an antigen presenting cell (12). The TCR is a heterodimer composed of an acidic alpha chain of 45 to 55 kDa and a basic $\beta$ chain of 40 to 50 kDa as illustrated in Figure 2. These chains are noncovalently associated with the gamma, delta and epsilon subunits of the CD3 molecule to form the functional TCR/CD3 complex as seen in Figure 3 (12). These cells circulate throughout the blood and home into specific sites of antigenic challenge.

In contrast, DETC do not express the CD4 cell surface antigens nor do they express the CD8 antigen associated with suppresser T-cells. However, they do express leukocyte common antigen (LCA) or Thy-1, which is present on both

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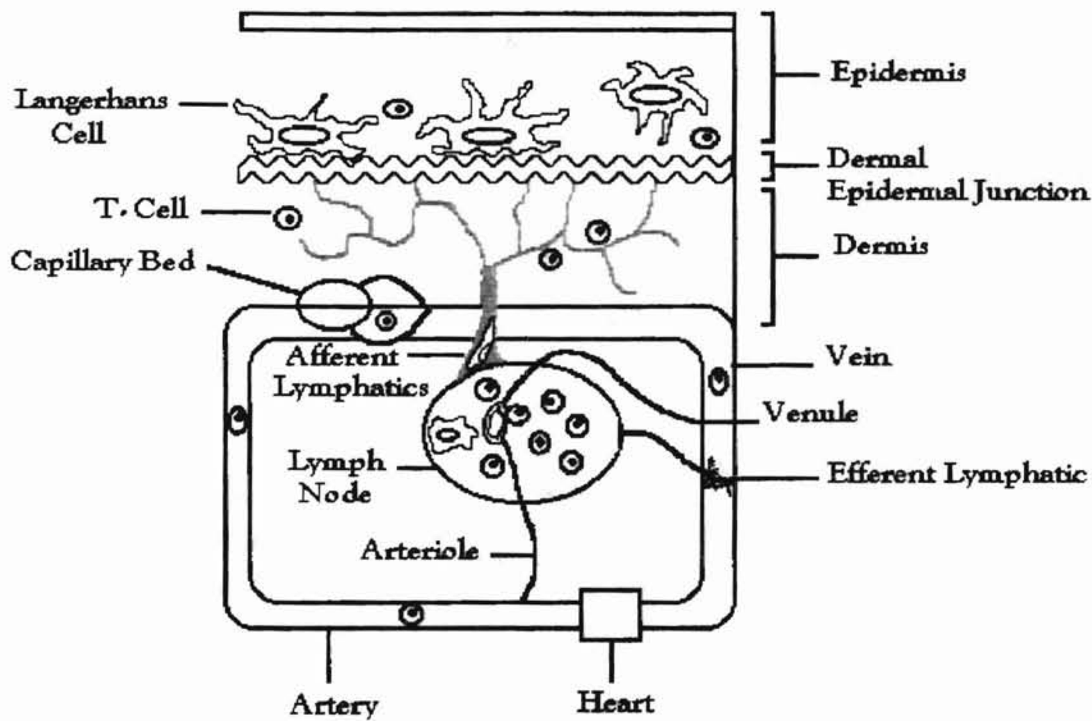


Figure 1. Skin Associated Lymphoid Tissue (SALT).

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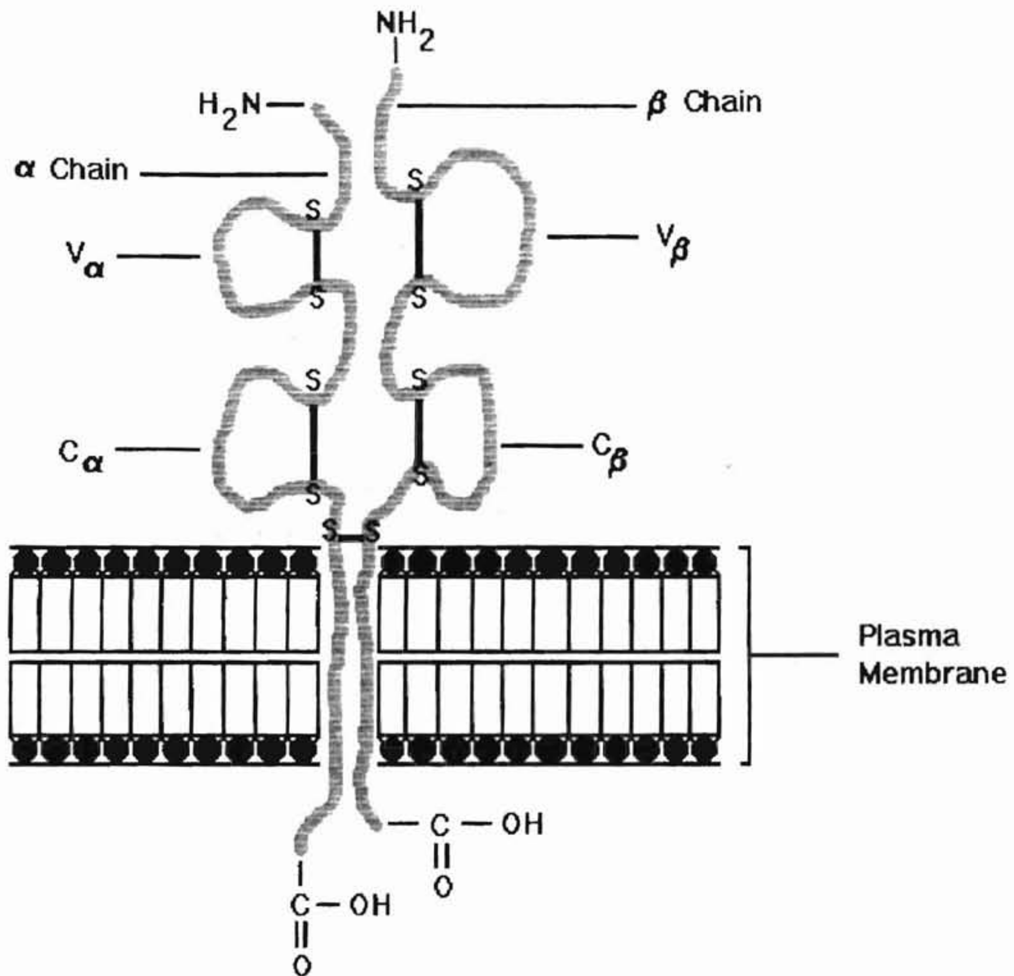


Figure 2. T-Cell Receptor Protein Structure.

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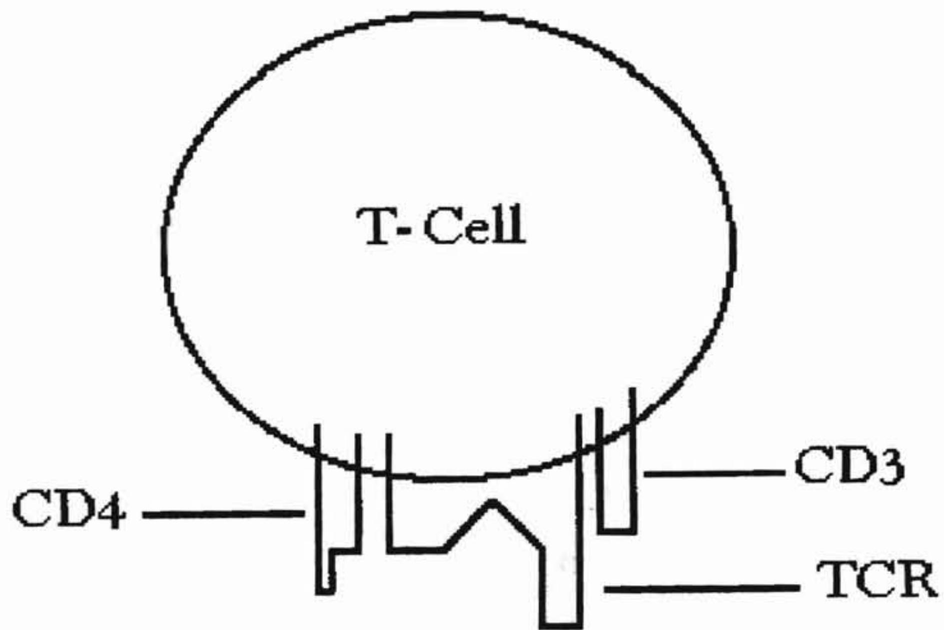


Figure 3. TCR/CD3 Complex

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CD4+ and CD8+ T-cell subsets. More importantly, these cells do not circulate in the blood periphery but are resident in the skin in the suprabasal region of the epidermis (7). The TCR is markedly distinct. It, like its CD4 relative, is associated with the CD3 molecule, but unlike the CD4 cell, the TCR is composed of gamma and delta chains (32).

The function of the DETC is still unclear and to date, DETC have only been observed in mice. It is thought that humans harbor a similar cell type but their identification has yet to be confirmed. Functionally, DETC are thought to play a significant role in immune surveillance and tolerance and may possibly be associated with MHC-I molecules, but no conclusive evidence for either theory has been officially accepted (5, 37, 51, 64, 65).

## The Keratinocyte

Keratinocytes are stratified squamous epithelial cells that differentiate to produce keratin-- thus the term epidermal keratinocyte (62). Growth occurs in the basal layers of the epidermis, and as the cells mature, they migrate upward in columns displacing the keratinized cells above (62). The primary function of the keratinocyte with respect to SALT is to create a favorable microenvironment in which the other cellular components of SALT can function. Keratinocytes are known to make a very complex array of cytokines, or proteins, which have regulatory control over other cells of the body. Some of the most important are Granulocyte/Macrophage- Colony Stimulating Factor (GM-CSF), IL-3, IL-6,

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Transforming Growth Factor β (TGF-β), TNF-α and IL-1 α, which is always constitutively expressed (7, 36) (see Appendix-I). However, when faced with epidermal antigenic challenge, these cells have the capability of dramatically up-regulating the expression of many or all of these cytokines.

In addition, keratinocytes possess two other important characteristics which make them a valuable component of SALT. First, keratinocytes are phagocytic and have the unique capability of taking up protein antigen and degrading it through proteolytic cleavage (7). This may be of critical importance with regard to antigen presentation. Currently, many researchers working in the area of skin immunology hypothesize that the conversion of large antigenic peptides into more particulate antigen can facilitate antigen uptake by the true antigen presenting cells of the skin, the Langerhans cells. However, further research is needed to support this theory.

Secondly, and perhaps more importantly, keratinocytes can be induced to up-regulate and express MHC-II on their cell surfaces in the presence of T-lymphocyte derived interferon gamma (IFN-γ) (7, 45). Morhenn et al. (1988) showed that keratinocytes incubated with both recombinant INF-γ and T-cell culture supernatants showed positive MHC-II expression in 90% of the cells after a 2 day incubation (45). It has also been suggested that IFN-γ can induce keratinocytes to up-regulate the expression of cytokines that intensify intra-epidermal immune responses (21, 50) and in 1988, Dustin et al. demonstrated that IFN-γ and TNF-α could increase the expression of intracellular adhesion molecule-1 (ICAM-1) on the surface of endothelial cells lining the walls of

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draining lymphatics (16). Up-regulated MHC-II can effectively aid LC in antigen presentation to circulating T-cells and cytokine and ICAM-1 expression may serve to activate T-cells and mediate lymphocyte trafficking thus effectively initiating a localized immune response.

## The Langerhans Cell

### Morphology

Langerhans cells are perhaps the most unique cellular component of SALT. Discovered nearly a century ago by Paul Langerhans, who is best known for his discovery of the islet of Langerhans within the pancreas, these cells were first thought to be associated with the nervous system (7). These assumptions were based on the fact that these cells could be positively stained with colloidal gold chloride. However, in 1960, Birbeck et al. disputed this finding and considered them to be derived from epidermal melanocytes (25). The theories as to the origin and function of LC at the time were based solely on the key morphological features of the cells first by light microscopy and later with electron microscopy.

Morphologically, LC are very distinct. They are distributed throughout the body in the stratified squamous epithelium, dermal connective tissues, lymphatics and associated skin appendages such as sebaceous gland ducts and outer hair root sheaths (25). In the epidermis, they are located in the suprabasal layer forming a continuous network and constitute 3 to 8% of the total cells (7, 17). The LC is highly dendritic in appearance and in the skin, these dendrites

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project upward towards the epithelial surface. The number of dendritic appendages varies and , as a result, Figueroa and Caorsi (1980) have classified these cells into five different categories based on the number of dendrites and the degree of branching (20). LC morphology is demonstrated in Figure 4.

The LC nucleus is extremely large and convoluted looking very much like the nucleus of granulocytic white blood cells. The cytoplasm contains a moderate to high number of mitochondria, a well developed endoplasmic reticulum and a very prominent Golgi apparatus (25, 67). However, there are surprisingly few lysosomes and those that are present are small and closely associated with the Golgi region (67). The cells are phagocytic but their potential for phagocytosis is significantly reduced in comparison to the phagocytic capability of macrophages.

The most distinctive cytoplasmic feature of the LC is the Birbeck granule. Discovered in 1961 by Birbeck, the "granule" is a rod or racquet shaped structure of linear, folded membranes enclosing a paracrystalline substance (25). They are randomly distributed throughout the cytoplasm and in the areas of the Golgi. They tend to be associated with a swollen, coated vesicle. This cellular organelle is specific for LC and is the only reliable morphologic marker for LC identification. Unfortunately, little is known about the origin, fate and function of the granule and it's study will undoubtedly be a very active venue of research in the future.

**Cell Surface Markers**

Like all cells in the mammalian body, LC express distinctive cell surface markers which serve as recognition sites for regulatory proteins and other

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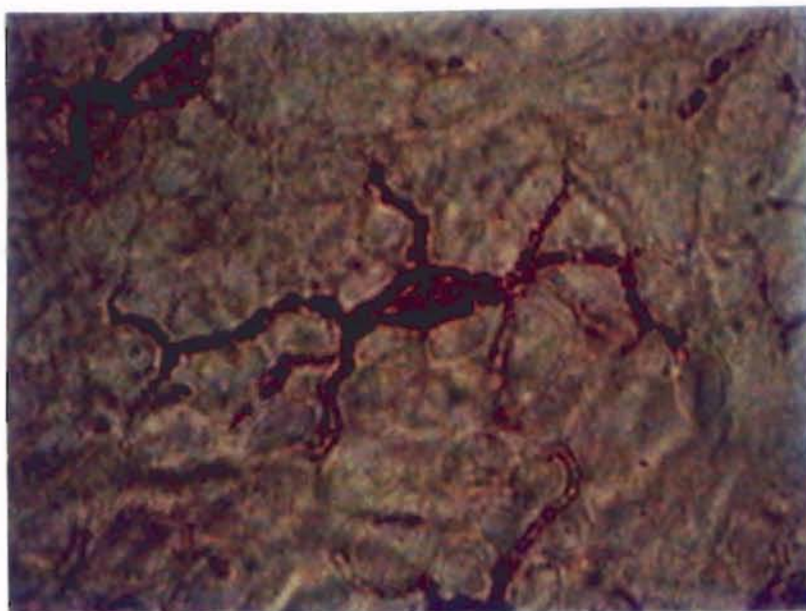

Figure 4. Langerhans Cell Morphology.

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biomolecules. These markers have also been utilized as a means of LC identification. During the 1960's, Wolff et al. discovered that LC possess relatively high amounts of cell surface nucleotide phosphatases, especially adenosine triphosphatase (ATPase) which could be exploited as a means by which to better visualize these cells within the skin (67). LC also exhibit surface marker characteristics reminiscent of those on cells of the monocyte-macrophage lineage in that they express Fc-IgG receptors, complement receptor C3 and most importantly MHC-II antigens (6, 53, 59, 67). The presence of Fc and C3 receptors hint towards the fact that LC may be involved in regulating humoral and complement mediated immune responsiveness while the presence of MHC-II implicates the LC in activating cell mediated immune responses.

## Ontogeny

As previously mentioned, LC were thought to be of neuronal or melanocytic origin although no direct evidence for either theory has been presented. However, in 1980, Katz and Tamaki provided the first evidence that LC are bone marrow derived through the use of bone marrow chimera studies (58). This was accomplished by observing that LC in the epidermis of an irradiated recipient mouse strain A reconstituted with an intravenous injection of donor strain B or A x B bone marrow cells were of B or A x B origin after 3 months. (57, 58). As a result, previous theories that LC were of monocyte-macrophage lineage have

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been widely accepted although the process of cell differentiation from monocyte progenation to LC remains poorly understood.

## Function

It has already been established that LC play a vitally important role in immune functions. Exactly what these cells do with respect to mediating the immune response has been an area of intense research for the past 10 years and, as a result, LC have been implicated in regulating allergy, delayed type hypersensitivity, graft rejection and most importantly, antigen presentation.

**LC and Allergy.** In 1992, Wang et al. determined that resident LC in human epidermis can bind monomeric IgE through a high affinity receptor recognized as FcεRI (62). It is of significant importance because cells that are normally responsible for mediating the allergic response, namely basophils and mast cells, also express Fc receptors to IgE immunoglobulin.

Under normal conditions, the FcεRI aggregation on the surface of basophils and mast cells generates a series of biochemical events that lead to the release of histamine, arachidonic acid derivatives and cytokines (4). This phenomenon is a type of antibody dependent cellular cytotoxicity and results in foreign cell death through cell mediated lysis. In contrast, LC aggregation of the FcεRI on the cell surface leads to the internalization of antigen by receptor mediated endocytosis via coated vesicles and endosomes (4). At this point, researchers

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can only speculate as to the true purpose of this function but it is believed that the Fc receptor mediated endocytosis of antigen plays a role in contact hypersensitivity and priming for antigen presentation.

**LC and Contact Hypersensitivity.** Delayed type contact hypersensitivity (DTH) results when an immunogen applied to the skin of a sensitized individual produces erythema and swelling as a result of accumulation of inflammatory cells such as lymphocytes, monocytes, macrophages and basophils around the capillary beds within the dermis (12). This process takes 24 to 72 hours to occur and the primary lymphocyte involved in this response is the CD4+ T-cell. In order for these cells to be active in mediating this type of immune response, antigen presenting cells must be present. Because macrophages do not normally enter the epidermis, the LC has been implicated as the primary APC involved in the DTH response at the cutaneous level.

In 1987, Macatonia et al. demonstrated that dendritic cells can be isolated from draining lymph nodes of mice whose skin was painted with contact sensitizers prior to harvest (41). These antigen presenting cells were positively identified as LC in 1980 by Silberberg-Sinakin and Thorbecke through the use of ferritin as a topical antigen and electron microscopy (55). These studies showed that the antigenic ferritin was readily taken up by LC and seen on their surfaces and within membrane bound inclusion bodies of the cytoplasm (55). It is now conclusive that LC are the primary cells in the epidermis capable of inciting a DTH reaction in response to repeated immune challenge with topical antigen

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and upon antigenic stimulation. During these responses, these cells migrate out of the epidermis to regions of the draining lymph nodes (30).

**LC and Graft Rejection.** Because LC are directly implicated in DTH through the uptake of antigen and subsequent migration to the draining lymph node, LC have also been implicated as being the primary inducers of allograft rejection. MHC-II or IA bearing dendritic cells arriving in the lymph node following contact sensitization at the site of a skin allograft were found to be of graft donor origin (14). Furthermore, the dendritic cells accumulating in the lymph nodes have been shown to contain Birbeck granules characteristic of LC. As a result, activated T-cells migrate to the graft site and destroy donor tissues and mediate graft rejection.

**LC and Classical Antigen Presentation.** Antigen presenting cells (APC) display a number of unique immune functions, such as phagocytosis and cytotoxicity (7, 12). Most importantly, APC have the uncanny ability to present foreign antigen in association with self MHC-II to antigen-reactive lymphocytes (12). This process is absolutely essential when an organism is faced with antigenic challenge because humoral and cell-mediated immunity cannot occur efficiently in the absence of APC.

Antigen presenting cells express self MHC-II surface antigens. Class II antigens are glycoproteins consisting of two noncovalently linked alpha and beta peptide chains. Each chain consists of extra cellular domains $\alpha1$ and $\alpha2$ and $\beta1$

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and β2, a connecting peptide, a transmembrane region and a cytoplasmic tail as shown in Figure 5 (12). In the classical concept of antigen presentation, APC encounter foreign antigen and internalize the particle via endocytosis to form a phagosome. It is here that the antigenic particle is proteolytically degraded into smaller particles. Elsewhere in the cytoplasm, MHC-II molecules are assembled and transported to the Golgi where they are coupled to the degraded antigenic particles, packed into Golgi vesicles and re-expressed on the cell surface. The classical model of antigen presentation is depicted in Figure 6.

It is important to note how critical MHC expression is with respect to inducing an effective immune response, for it is this particular peptide configuration which presents and effectively binds to the TCR. Neither MHC-II nor antigen alone can activate T-cells, the reason being that the TCR is highly specific. The MHC-II molecule binds antigen within a peptide binding groove located between the α and β chains. As previously mentioned, the TCR α and β chains are in close association with the CD3 molecule on the cell surface (12). When the APC presents antigen to the T-cell, the MHC-II α chain binds to the TCR α chain and the MHC-II β chain binds to the TCR β chain with the antigen sandwiched in the recognition groove formed by the joining of the two molecules as shown in Figure 7. It has not been determined exactly how the physical act of APC/TCR binding initiates T-cell activation through the TCR but it is believed to be a conformational change in the antigen bound MHC-II molecule which allows for the correct binding interactions(12).

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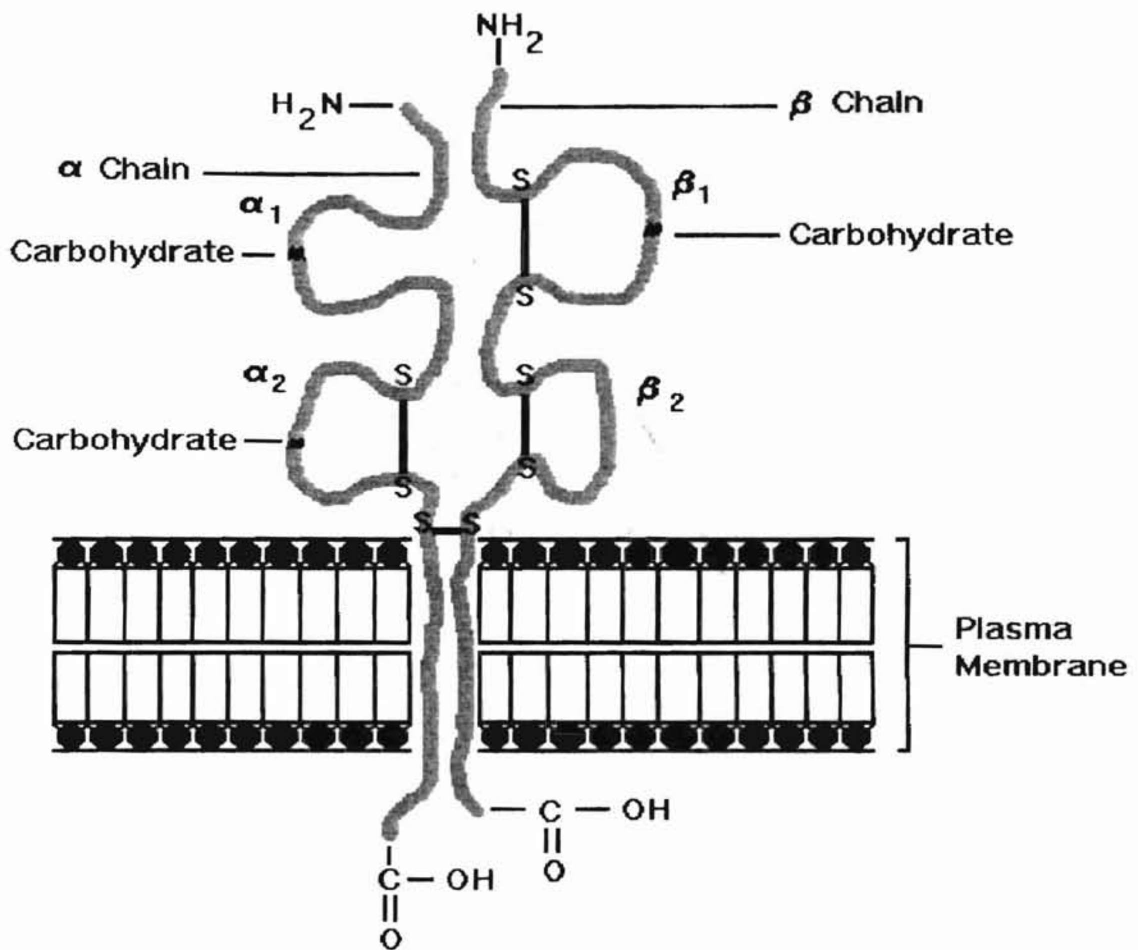


Figure 5. MHC II Molecule Protein Structure.

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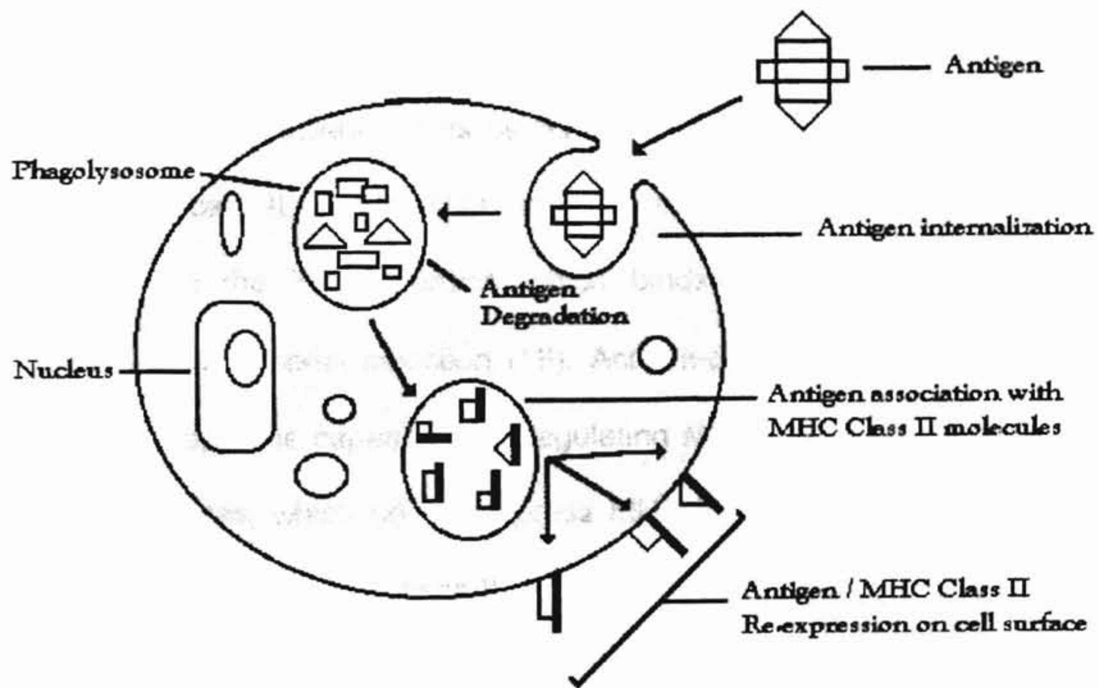


Figure 6. Classical Antigen Presentation.

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## T-Cell Activation

The binding of the T-cell to the LC is the first step in the initiation of the skin immune response (61). Subsequent steps involve a complex cascade of cytokines produced primarily by the activated T-cell. The first gene product expressed is interleukin-2. This cytokine is an autocrine growth factor for the activated T-cell. IL-2 production induces the increased expression of IL-2 receptors on the T-cell surface which binds more IL-2 leading to T-cell proliferation and clonal selection (18). Activated T-cells also secrete IFN-$\gamma$, a very potent cytokine capable of up-regulating MHC-II expression on cells, such as keratinocytes, which do not express MHC-II under normal conditions (23). IFN-$\gamma$ also functions to enhance IL-2 receptor expression on resting T-cells, up-regulate IgG Fc receptors on polymorphic nuclear cells and plays a pivotal role in aiding the body in clearing viral particles from circulation (23). Activated T-cells can also secrete TNF-$\alpha$ and $\beta$, IL-3, IL-4, IL-5, IL-6, IL-7 and GM-CSF (11). After T-cell proliferation and clonal selection, the activated T-cells leave the lymph node via the efferent lymphatics which empty directly into the circulatory system. Once in circulation, adhesion molecules expressed on endothelial cell surfaces function to "home" T-cell to the original site of antigenic challenge where they are manifested in a full scale cell mediated response (7, 61).

## Cytokines

In order to fully comprehend the magnitude of the skin immune response, it is important to understand which biomolecules regulate the response, effectively

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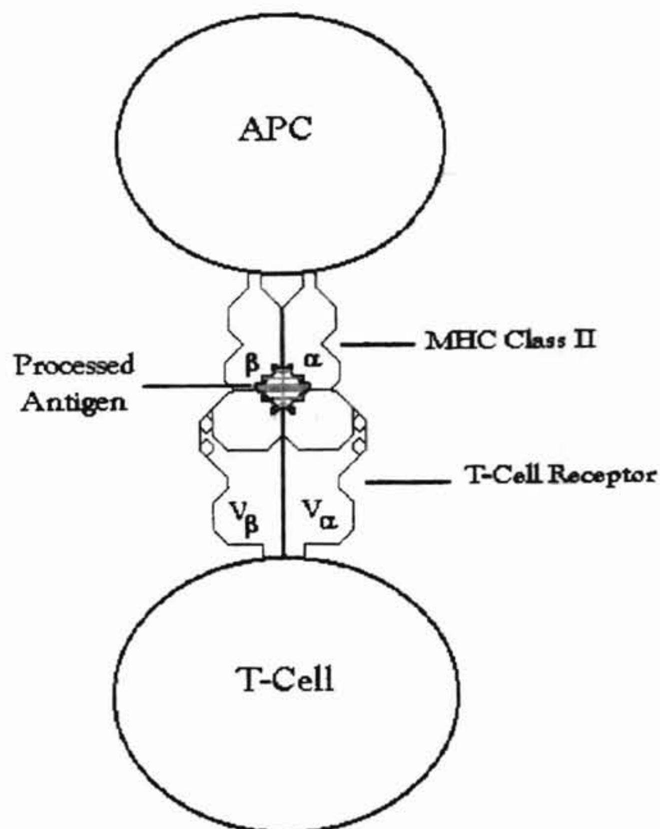


Figure 7. MHC II / TCR Interaction During Nominal Antigen Presentation. The location of antigen is situated in the antigenic peptide binding groove between the MHC and TCR molecules.

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turning it on and off with the advent of immune challenge and subsequent antigen clearing.

Cytokines are proteins which are secreted from producer cells that bind to specific receptors on the surfaces of target cells. Their mode of action is brought about inside the target cell by signal transduction across the plasma membrane (11) although many of these transduction pathways are complex and poorly understood. Functionally, cytokines have many physiological roles in multicellular organisms, most of which are dual or overlapping, such as regulating hematopoiesis, inflammatory responses and control of cytotoxic and phagocytic cells (11). Most importantly, cytokines are involved in regulating lymphocyte proliferation and the production of other cytokines.

In the epidermis, keratinocytes are the main cytokine producing cells (7, 26, 44). They secrete a broad spectrum of cytokines of various functions. For the purpose of this research, Il-1 $\alpha$ and TNF-$\alpha$ are the most relevant.

IL-1 $\alpha$ was originally called lymphocyte activating factor (LAF). It is a 17 kDa glycosylated peptide and is secreted in a precursor form which is later proteolytically cleaved to form the active molecule (22). Keratinocytes express IL-1 $\alpha$ constitutively and dramatically up-regulate it's synthesis under specific conditions (7). IL-1 $\alpha$ is absolutely essential for the proper activation of peripheral T-cells. After MHC/TCR binding and antigen recognition, the APC up-regulates the expression of IL-1 $\alpha$ which binds to numerous IL-1 $\alpha$ receptors on the surface of the T-cell. IL-1 $\alpha$ binding activates the transcription of FOS and other important proliferative cytokines (11).

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In addition to T-cell activation, IL-1 $\alpha$ has also been implicated in the activation of other immune cells, such as B-cells, monocytes, granulocytes and macrophages, and its expression can be induced by IFN-$\gamma$ (11, 22).

TNF-$\alpha$, like IL-1 $\alpha$, is also a 17 kDa polypeptide derived from an inactive precursor. It exists in multimers of two to three identical subunits containing many potential glycosylation sites (23). TNF-$\alpha$ is primarily secreted by activated monocytes or macrophages. In the epidermis, TNF-$\alpha$ is produced by the keratinocyte (26). TNF-$\alpha$ production can be induced by a wide range of stimuli. Primary inducers of TNF-$\alpha$ are bacterial endotoxins such as lipopolysaccharide (LPS). TNF-$\alpha$ can also be induced by other cytokines, most importantly IL-1 $\alpha$ and IL-6. Once secreted, TNF-$\alpha$ will bind to TNF receptors located on the surfaces of a variety of cell types and be internalized and degraded via a lysosomal pathway (23, 42). Results of TNF-$\alpha$ binding are manifested in very substantial cellular responses.

TNF-$\alpha$ functions primarily in the activation of polymorphonuclear cells, T-cells and B-cells, inducing them to proliferate and secrete immunomodulating cytokine. Upon antigenic challenge with bacterial endotoxin, TNF-$\alpha$ has been implicated as being a primary mediator of septic shock (11, 42) and in some cases has been shown to have a direct cytostatic or cytotoxic effect on some tumor cells (11). However, with regard to the experimental procedures presented here, it is most important to note that TNF-$\alpha$ has the ability to up-regulate the IL-1 $\alpha$ indicating that these two cytokines are synergistic in their activities.

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## Superantigens

The concept of the superantigen was brought to the forefront of immunolgical research in 1973 when Festenstein described unmapped gene loci encoding antigens that generated vigorous mixed leukocyte reactions (MLR) between major histocompatability complex non-identical strains of mice (19). Subsequent analysis determined that the responding cells of the MLR were T-cells and the antigens which stimulated their proliferation were encoded for in a loci in B-lymphocytes. As a result, these proteins were initially called minor lymphocyte stimulating antigens (Mls) or endogenous superantigens. With respect to their activity, Kappler et. al 1989 were able to show deletion of T-cell subsets from mice expressing the Mls antigens and that these subsets were defined by the variable β (Vβ) region on the TCR (29).

Further examination of endogenous superantigens revealed that these unique proteins were the result of viral DNA integration and protein expression within the host chromosome at a specific gene locus (1, 2). These findings fueled aggressive analyses of many viral infections in both mice and humans for the presence of viral superantigens. The most extensive research initially focused on mouse mammary tumor virus (MMTV).

MMTV was discovered at the end of the last century and was on of the first oncogenic retro-viruses described (2). To date, there are 53 known MMTV gene loci, 3 of which encode for superantigens (2, 56). In 1991, it was discovered that the nucleocapsid (N protein) of rabies virus also acts as a superantigen and

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currently, Epstien-Barr virus and human immuno-deficiency virus are under intense scrutiny in order to determine whether or not they also harbor potential superantigenic proteins (27).

With the discovery of endogenous viral superantigens, attention shifted towards a broad spectrum of bacterial proteins known to cause septic conditions in humans and lesser mammals. Janeway et al 1989 and Murray et al 1995 determined that many staphylococcal strains and group A streptococci produce exogenous superantigens (28, 47). The bacterial superantigens are 22 to 30 kDa pyrogenic proteins that are acid, heat and protease resistant (47). Most of the exogenous superantigens contribute to several acute illnesses such as food poisoning, toxic shock syndrome, septic shock and several autoimmune disorders (27, 47). Other bacteria known to produce disease causing superantigens include various strains of *Yersinia, Pseudomonas* and mycoplasma (43).

For the purposes of this research, discussions pertaining to superantigens will focus on properties of antigenic peptides produced by *Staphylococcus aureus.*

## Superantigen Functions

As previously mentioned, superantigens stimulate T-lymphocytes in a manner that depends upon the compositions of the variable region on the β chain of the TCR (27). In nominal antigen presentation, antigen/MHC-II binding induces T-

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cell specificity due to the interactions of several variable elements built into the TCR, notably V$\alpha$, V$\beta$, J$\alpha$, J$\beta$ and D$\beta$. (27, 44). It has been shown that the variable regions of the $\alpha$ and $\beta$ chains of the TCR interact to form a ligand binding site similar to that formed by the heavy and light chains of the immunoglobulin molecule (44). Once formed, the ligand binding site then contacts both the $\alpha$ and $\beta$ chains of the MHC-II molecule with the antigen sandwiched in the groove created between the two molecules. This binding then augments the production of cytokines and up-regulates clonal expansion of the activated T-cell (13).

In contrast, superantigens do not require antigen procession and internal MHC coupling by an APC in order to stimulate and immune response (10, 27, 47) but rather bind directly to the MHC-II molecule and the TCR outside the conventional antigen binding groove as seen in Figure 8 (27). Upon MHC-II binding, the superantigen/MHC-II complex interacts and binds only to the V$\beta$ region of the TCR (27, 47). All other variable regions of the TCR appear to play no detectable role in superantigen binding and, as a result, this allows for a very non-specific activation on any T-cell with the correct V$\beta$ element (27).

**Consequences of Superantigen Exposure**

Superantigenic challenge results in an immune response that is immediate and dramatic. The first event after exposure involves the binding of superantigen to exposed MHC-II on the surface of and APC (27, 43). This is followed by an

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explosive release of cytokines, such as TNF-$\alpha$ and IL-1 $\alpha$, from APC's (27, 43, 47). Superantigen is then presented to T-cells expressing the appropriate V$\beta$ TCR chain. It is important to note two fundamental elements of superantigen presentation.

First, IL-1 $\alpha$ is absolutely essential for the up-regulation and expression of the IL-2 receptor and corresponding cytokine in order to drive T-cell proliferation following activation (10, 11, 43). Second, in nominal antigen exposure, fewer than 1 in 10,000 T-cells are activated in order to produce a sufficient immune response (27, 47). However, because superantigens react with the V$\beta$ region of the TCR, as many as one-fifth of all T-cells may be stimulated by a particular superantigen (10, 27, 43, 47).

This massive, non-specific T-cell activation induces an even greater release of cytokines with TNF-$\alpha$ being the first one to two hours immediately following exposure and IL-1 $\alpha$ and IL-2 reaching maximum levels two to four hours after exposure (43).

Eventually, the superantigen activated T-cells undergo clonal deletion by apoptosis three to four days after activation. Subsequent exposure to the same superantigen results in T-cell anergy of a state of immune unresponsiveness in which the T-cell does not produce IL-2 or the IL-2 receptor and therefore, does not proliferate ( 11, 43, 47).

Physiological consequences of superantigen exposure can be extremely dangerous and often times fatal if left unchecked. With respect to staphylococcal enterotoxins, the first problem arises from the livers' inability to effectively clear

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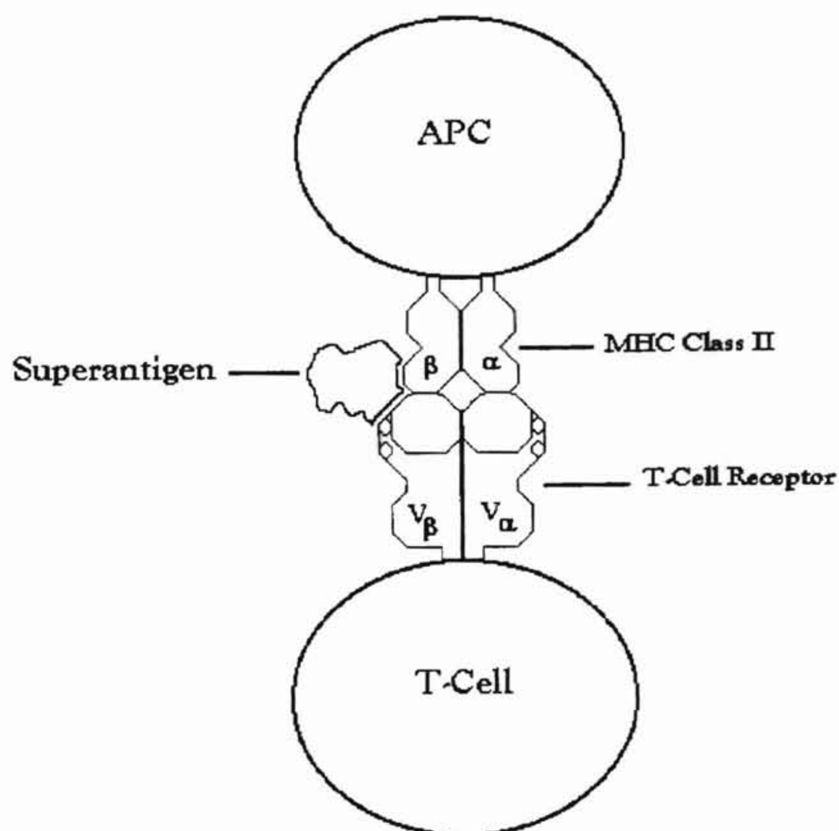


Figure 8. MHC II / TCR Interaction During Superantigen Presentation. The location of antigen is situated outside the antigenic peptide binding groove and is bound to the β regions of the MHC and TCR.

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the toxin from the body (27). This enhances an exogenous superantigens' lethal effects by $10^5$ fold thereby increasing the severity of symptoms in an infected organism (47).

Secondly, the massive release of cytokine, particularly TNF-$\alpha$, results in septic shock (10, 27, 47). Septic shock is known to be mediated by TNF-$\alpha$ and is characterized by capillary leakiness of fluid into intracellular spaces causing pain swelling (23).

Finally, superantigens have been implicated in autoimmune mediated disorders such as rheumatoid arthritis and multiple sclerosis. The release of cytokines, such as TNF-$\alpha$ and IL-1 $\alpha$, in response to superantigen exposure are believed to play a significant role in the activation of self-reacting T-cells that escaped clonal deletion within the thymus (47, 22, 23). These activated T-cells attack synovial tissues within the joints and augment tissue destruction, thus causing the painful and debilitating symptoms of arthritis. Likewise, multiple sclerosis occurs when self reacting T-cells cause demyelination of axons and chronic inflammation of CNS components (27, 47).

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### SEA Involvement in the Skin Immune Response

It has long been established that UVB radiation can induce LC migration from murine skin (3, 8, 39). However, the signals which initiate this migration have remained poorly understood. Burnham et al (1993) were able to show that UVB radiation induced LC depletion could be blocked by a panel of of inhibitors

known to block PKC, protein kinase associated G-proteins, GTP binding proteins, transcription and translation (9). These results suggested that LC depletion induced by UVB may involve a G-protein as well as *de novo* protein synthesis (9).

With the discovery of superantigens and the knowledge of their unique mode of immune cell activation, Pickard et al (1994) tested a selection of staphylococcal superantigens in order to examine the role LC MHC-II might play in immuno-modulating components of SALT (52). SEA was found to be the most potent enterotoxin resulting in almost complete LC depletion from the epidermis upon exposure.

In addition, experiments involving inhibitors of PKC, G-proteins, G-protein associated kinases, cAMP, transcription and translation all suggested that, like UVB induced depletion, SEA induced depletion involved a GTP binding protein and the synthesis of an unknown regulatory molecule or molecules (52). These molecules were hypothesized to be cytokines.

Shankar et al (1996) discovered that the addition of SEA directly to murine epidermal cell suspensions resulted in elevated levels of IL-1 $\alpha$ and to a lesser extent TNF-$\alpha$ when quantitated by Enzyme Linked Immunosorbent Assay (ELISA) (54). It was also observed that IL-1 $\alpha$ secretion by epidermal cells after treatment with SEA involved PKC (54).

Based on these findings, this research focuses on the possible roles IL-1 $\alpha$ and TNF-$\alpha$ play as primary inducers of LC migration when faced with antigenic challenge with SEA.

## Objectives and Hypothesis

The primary objective of this research was to determine whether or not the SEA induced production of IL-1 $\alpha$ and/or TNF-$\alpha$ could effectively stimulate LC depletion thus implication these two cytokines as initial mediators in the signaling pathway of LC migration.

Specific objectives addressed the following:

1. Can the depletion effects observed by SEA be blocked with antibodies against IL-1 $\alpha$ and TNF-$\alpha$?
2. Do IL-1 $\alpha$ and TNF-$\alpha$ induce LC migration from the skin?
3. Can the depletion effects observed by IL-1 $\alpha$ and/or TNF-$\alpha$ be blocked by neutralizing antibodies?
4. *Does a synergism exist between IL-1 $\alpha$ and TNF-$\alpha$ in their abilities to induce LC migration?*

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# CHAPTER II

# MATERIALS AND METHODS

## Animals

The animals used in this study were 6 to 8 week old female Balb/c strain mice obtained from Charles River (Wilmington, MA) and Jackson Laboratories (Bar Harbor, ME). All animals were maintained in Laboratory Animal Resources, a licensed facility at the School of Veterinary Medicine at Oklahoma State University.

## Buffers and Media

Phosphate buffered saline solution (PBS) containing 0.15 M sodium phosphate was used to hydrate skin sections during the preparative phase of each experiment in addition to preparing solutions of bovine serum albumin (BSA), non-fat dry milk and antibody dilutions. One liter of PBS contains 1.15 g anhydrous sodium phosphate ($Na_2HPO_4$, Sigma Chemical, St. Louis, MO), 0.2 g monobasic potassium phosphate ($KH_2PO_4$, Fisher Scientific, Fairlawn, NJ), 8.0 g sodium cholride (NaCl, Baker Chemical Corp., Phillipsburg, NJ) and 0.2 g

potassium chloride (KCl, Fisher) adjusted to 7.2 and then sterilzed by autoclaving for 20 minutes at 15 psi.

Solutions of bovine serum albumin (2, 5 and 10%) were prepared using crystalline BSA fraction V (Fisher) and filter sterilized by vacuum filtration through a 0.2$\mu$ Vacucap™ filter (Gelman Sciences, Ann Arbor, MI).

Tris buffered saline with 50mM Tris-HCl and 0.05% Tween-20 (TBS-Tween) was used to wash epidermal sections during immunohistochemical staining. One liter of TBS-Tween contains 138 mM NaCl, 2.7 mM KCl, 50 mM tris-HCl (Sigma), and 1.5 g Tween-20 (Sigma) in distilled deionized water.

In all experiments, skin sections were cultured in sterile Cellgro™ RPMI 1640 media (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (FBS, Sigma), 2.0 mM L-glutamine (Sigma), 100 U/mL penicillin (Sigma), 0.1 mg/mL streptomycin (Sigma), 0.1 mM MEM non-essential amino acids (Sigma), 0.1 mM sodium pyruvate (Sigma) and 0.1 mg/mL gentamycin sulfate (Sigma). All media was filtered through a 0.2 $\mu$ Vacucap™ filter.

### Toxins and Cytokines

Staphylococcal enterotoxin A (SEA, Toxin Technologies, Sarasota, FL), was applied to epidermal sections in concentrations of 50mg/mL and 100mg/mL dimethyl sulfoxide (DMSO, Sigma) in 25$\mu$L volumes in order to induce LC migration. Controls were pure DMSO with no cytokine or toxin.

Likewise, recombinant murine interleukin-1 α (Sigma) and tumor necrosis factor α (Sigma) were also used to study LC migration through topical application in a DMSO vehicle.

## Antibodies

Purified mouse-anti- mouse $Ia^d$ monoclonal antibody (Mab, Pharmingen, San Diego, CA) was used to positively stain MHC-II or Ia surface antigen on LC following experimental treatment. This antibody is an IgG isotype and is specific for the Ia molecules of the $H\text{-}2^d$ haplotype.

IgG fractions of polyclonal rabbit-anti-mouse IL-1 α and TNF α (Genzyme, Cambridge, MA) were administered in intraperitoneal injections for neutralization *in* a concentration of 100μg/mL PBS.

Monoclonal hamster-anti-mouse IL-1 α and TNF α (Genzyme) and monoclonal hamster-anti-mouse sheep red blood cells (SRBC) harvested from ascites (Cedar Lane, Westbury, NJ) were used in *in vivo* studies. All polyclonal and monoclonal antibodies were of the IgG isotype.

Whole rabbit serum (Sigma) was used as a control serum for *in vitro* studies.

## Preparation of Tissue Sections

All mice in the study had the dorsal hair removed by shaving followed by addtional removal with an over-the -counter depilatory (Neat™) and then euthanized in an ether chamber. The dorsal skin was then rinsed thoroughly with

sterile distilled water and 70% ethanol to maintain sterility and surgically removed in a sterile laminar flow hood.The skin was then placed in a sterile plastic petri dish and sectioned into the appropriate number of 1cm$^2$ pieces with a surgical scalpel.

Falcon™ 24 well tissue culture plates (Fisher) were prepared for skin culture by placing 1.5 cm sterile antibiotic filter discs (Fisher) 3 per well, which were then saturated with 600 μL supplemented RPMI 1640 media. Individual skin sections were then aseptically transferred to each well and placed epidermal side up on the center if each disc. Following experimental treatments, all sections were incubated at 37° C with 5% carbon dioxide for 48 hours.

## Harvesting Epidermal Sections

In order to enumerate epidermal LC through immunohistochemical staining, the epidermis must be sperated from the dermis while maintaining its integrity. This was achieved by floating each skin section after culture epidermal side up on 2M sodium bromide (NaBr, Sigma) in a 24 well plate for 1 hour at 37° C with 5% carbon dioxide. After incubation, the epidermis was gently scraped off the dermis as a thin sheet using a scalpel blade in a petri dish of sterile PBS. The epidermal sections were then stored in PBS prior to staining.

## Immunohistochemical Staining

Tissues were fixed for staining using HistoChoice™ tissue fixative (Fisher) for

20 minutes at 4°C. The samples were then incubated with 3% hydrogen peroxide ($H_2O_2$, Sigma) to quench endogenous peroxidase activity. Using a solution of 2% Carnation™ non-fat dry milk the sections were blocked at room temperature with gentle shaking for 2 hours. Blocking with a benign protein solution prevents non-specific binding of antibody to the well plate walls and random exposed sites on the skin sections. The primary antibody, anti-mouse $Ia^{d}$, was diluted 1:100, applied to the skin sections and allowed to incubate over night at 4°C with gentle shaking. The remaining reagents were components of the Vecta Stain Elite ABC Kit (Vector, Burlingame, CA). A biotinylated secondary antibody was diluted 1:10,000 and incubated under the same conditions as the primary antibody. Following secondary antibody binding, an aviden/peroxidase enzyme complex was allowed to couple to the antibody bound sections at room temperature for 1 hour. AEC peroxide substrate was then added to visualize antibody bound LC.

## Experimental Treatments

Cytokines and SEA were delivered topically to each skin section for culture *in vitro* and *in vivo* via a DMSO vehicle in 25µL quantities. Blocking antibodies were diluted in sterile PBS to a final concentration of 100 µg/mL PBS and delivered intraperitoneally in 100 µL volumes. These antibodies were then allowed to circulate sytemically for 2 hours prior to skin harvest.

# CHAPTER III

# RESULTS

The main objective of this study was to develop a better understanding of how superantigens and the cytokines they induce modulate components of the skin associated lymphoid tissues. Specifically, this project focused on SEA induction of IL-1 $\alpha$ and TNF-$\alpha$ expression and the possible roles these cytokines play in stimulating LC migration from the epidermis to the draining lymph nodes.

## *In vitro* Studies

### Effects of IL-1$\alpha$ and TNF-$\alpha$ on *In Vitro* LC Depletion

In order to ascertain how IL-1 $\alpha$ and TNF-$\alpha$ affect epidermal LC, cytokine levels observed by Shankar et al (1996) upon epidermal cell exposure to SEA were sequentially doubled in the amount expressed above the constitutive levels in order to produce dose response curves. IL-1 $\alpha$ was increased in increments of 34.0 pg/mL while TNF-$\alpha$ was increased in increments of 11.7 pg/mL. All cytokine dilutions were made in DMSO, applied to prepared skin sections and incubated for 48 h at 37$^{0}$ C in the presence of 5% carbon dioxide. After culture, epidermal sheets were removed and IA+ cells stained via immunohistochemical

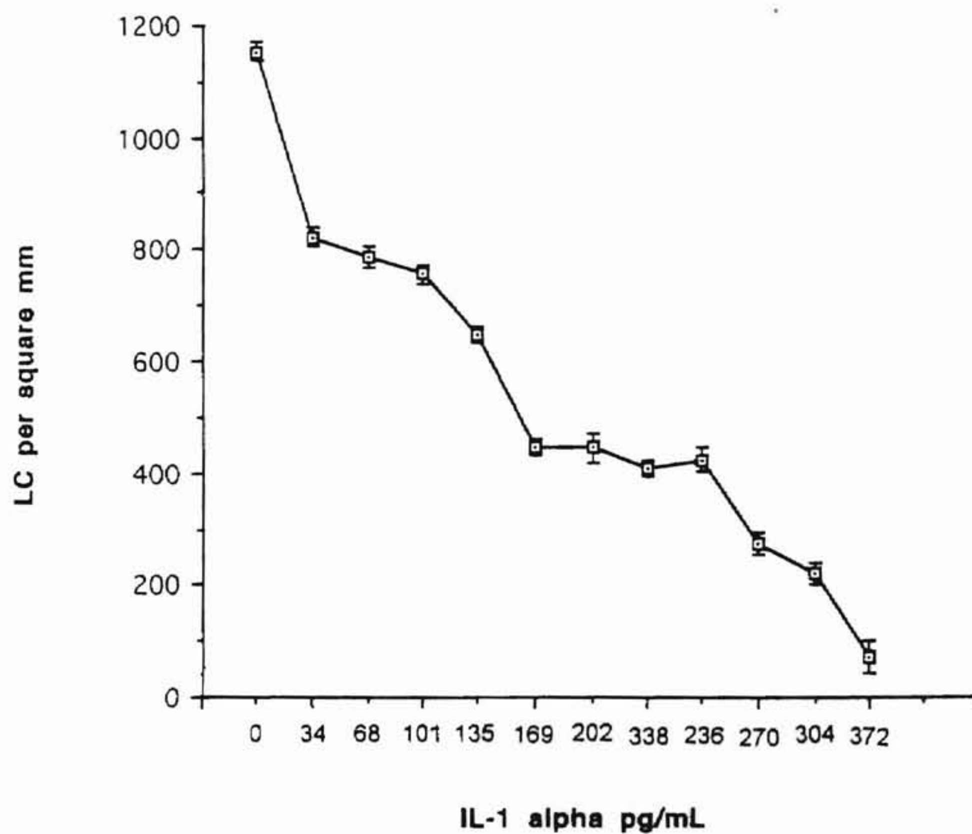


Figure 9. *In vitro* LC Depletion by IL-1 α. The values expressed are the mean generated by pooling data from 3 separate experiments.

techniques. Figures 9 and 10 depict the results of these experiments. As shown in Figure 9, increased levels of IL-1 $\alpha$ effectively stimulated the depletion of LC form the epidermis. TNF-$\alpha$ did not cause a dramatic depletion in LC but rather an initial depletion that tended to level out despite an increase in cytokine concentration (Figure 10).

**Effects of Blocking LC Depletion by SEA with**

**Polyclonal Anti IL-1 $\alpha$ and Anti TNF-$\alpha$**

**Antibodies *In vitro***

As previously noted, Pickard et al (1993) showed that SEA can effectively cause LC depletion from the epidermis (52). Prior to harvesting the epidermis for tissue culture, each mouse was intraperitoneally injected with 100 $\mu$L of PBS as a control, or polyclonal anti IL-1 $\alpha$ or antiTNF-$\alpha$ diluted to a concentration of 100 $\mu$g/mL. The mice were rested for two hours to allow for systemic circulation. Skin sections were harvested, treated with SEA and incubated for 48 h at $37^0$ C in the presence of 5% carbon dioxide. This was done to investigate whether or not antibodies against IL-1 $\alpha$ and TNF-$\alpha$ could effectively block LC depletion induced by SEA. As seen in Figure 11, SEA in both 50 $\mu$g/mL and 100 $\mu$g/mL amounts caused LC depletion. Treatment with anti IL-1 $\alpha$ blocked LC depletion by both SEA concentrations while treatment with anti TNF-$\alpha$ blocked depletion but to a slightly lesser extent. These data implicate both IL-1 $\alpha$ and TNF-$\alpha$ as primary mediators of SEA induced LC depletion from the epidermis.

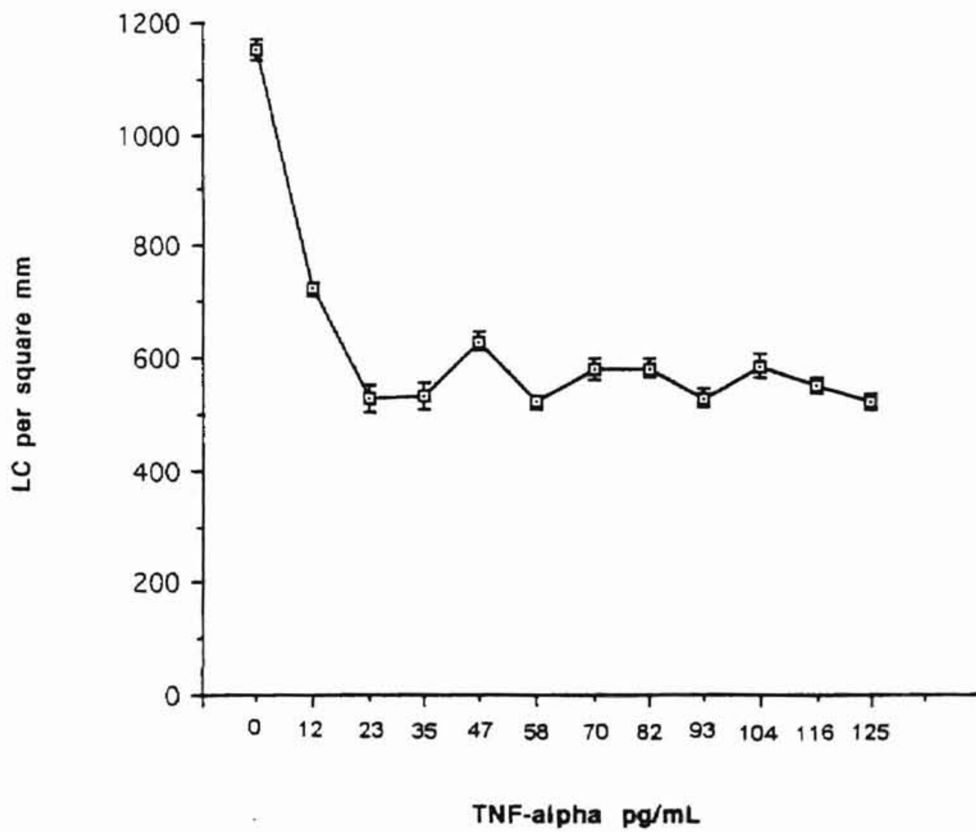


Figure 10. *In vitro* LC Depletion by TNF-$\alpha$. The values expressed are the mean generated by pooling data from 3 separate experiments.

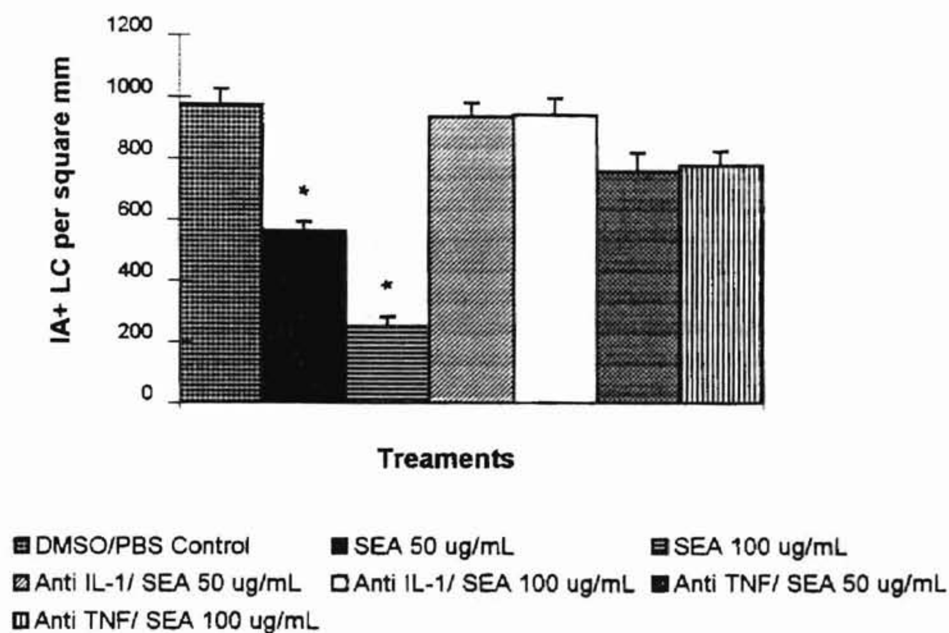


Figure 11. *In vitro* Blocking of LC Depletion By SEA with Polyclonal Anti IL-1 $\alpha$ and Anti TNF–$\alpha$ Antibodies. The values expressed are the mean +/- SEM generated by pooling data from 3 separated experiments. Asterisk denotes $p < 0.05$ as compared to the positive control by the Student t-test.

## .Effects of Blocking LC Depletion by IL-1 α and TNF-α with Polyclonal Anti IL-1 α and Anti TNF-α Antibodies *In Vitro*

To determine whether of not IL-1 α and TNF-α are involved in the induction signals of LC migration, recombinant IL-1 α and TNF-α were diluted to concentrations known to cause maximum LC depletion. This information was obtained from the dose response curves. Prior to cytokine treatment, the appropriate PBS control and antibody dilutions were administered to sample mice which were then rested for two hours as previously described. This was followed by skin removal, cytokine application and 48 h incubation at $37^0$ C in the presence of 5% carbon dioxide. Figures 12 and 13 clearly demonstrate that both cytokines are capable of inducing LC depletion based on the fact that pre-treatment with antibodies to both cytokines effectively inhibited the migration of LC from the skin.

## An *In Vitro* Investigation of Possible Cytokine Synergism

This study was conducted in order to test whether or not IL-1 α and TNF-α have synergistic effects in their abilities to induce LC migration. Sample mice were again pre-treated with the appropriate PBS control, anti SRBC control and polyclonal antibodies prior to tissue culture. This was followed by topical treatment with the designated cytokine and 48 h incubation at $37^0$ C in the

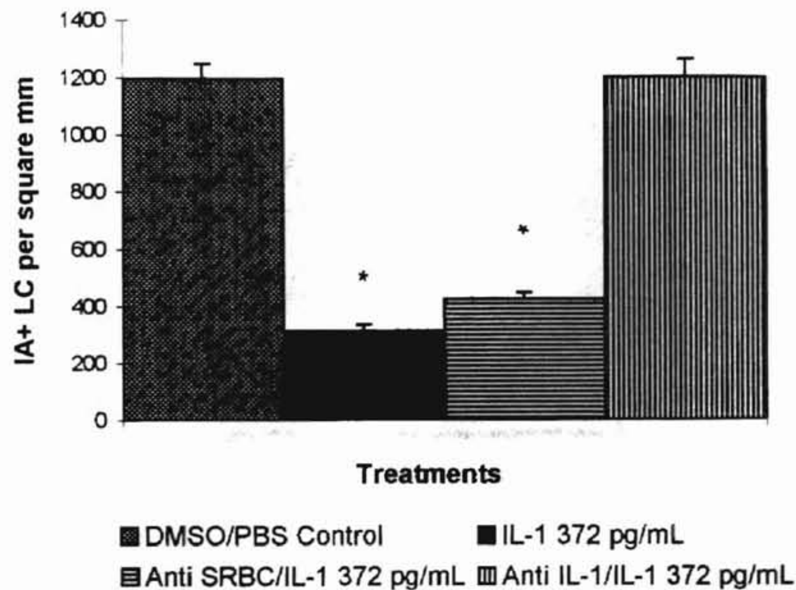


Figure 12. *In vitro* Blocking of Lc Depletion by IL-1 α with Polyclonal Anti IL-1 α Antibody. The values expressed are the mean +/- SEM gernerated by pooling data form 3 separate experiments. Asterisk denotes $p < 0.05$ as compared to the positive control by the Student t-test.

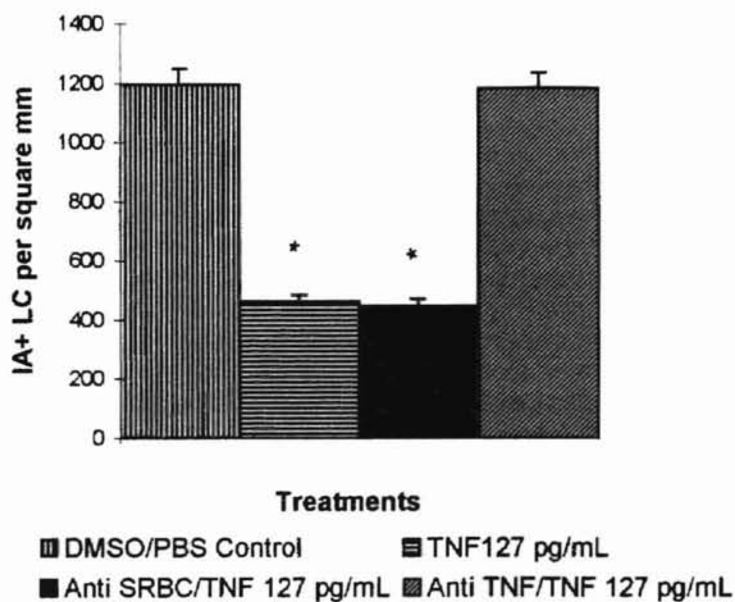


Figure 13. *In vitro* Blocking of LC Depletion by TNF-α with Polyclonal Anti TNF-α Antibody. The values expressed are the mean +/- SEM generated by pooling data from 3 separate experiments. Asterisk denotes $p < 0.05$ as compared to the positive control by the Student t-test.

presence of 5% carbon dioxide. Figure 14 indicates that there is no statistical difference between the anti SRBC/IL-1 α and anti SRBC/TNF-α groups. However, a difference was observerd between the anti SRBC treated groups and the DMSO/PBS control. Again, anti IL-1 α and anti TNF-α effectively blocked LC depletion by IL-1 α and TNF-α. Anti IL-1 α failed to block LC depletion by TNF-α thereby suggesting that TNF-α alone can induce LC migration while anti TNF-α blocked LC depletion by IL-1 α.

## *In vivo* Studies

### SEA Mediated LC Depletion *In Vivo*

All *in vivo* experiments were conducted to substantiate *in vitro* findings. Following removal of dorsal hair and surface sterilization of skin with 70% EtOH, sample mice were treated with 100μg SEA/mL DMSO vehicle at the base of the neck. Mice were kept in isolation for 48 h with the treated skin sections being harvested at the end of the incubation. Epidermal sections were removed by the same method employed in the *in vitro* studies and IA+ cells enumerated via immunohistochemical staining. Figure 15 shows significant LC depletion by SEA comparable to that observed in the *in vitro* studies.

### LC Depletion with IL-1 α and TNF-α *In Vivo*

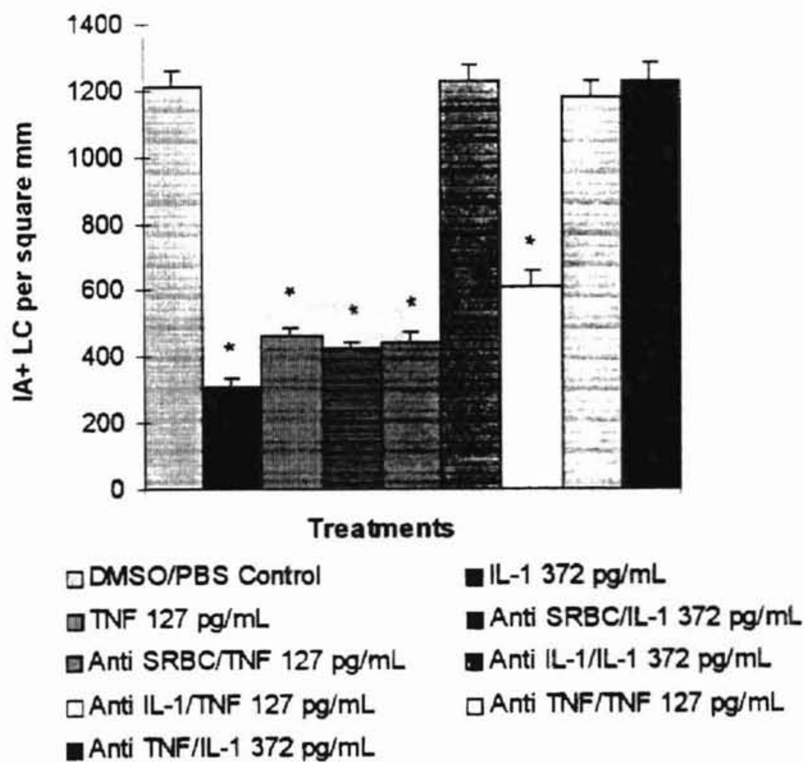


Figure 14. *In vitro* Cytokine Synergism Study. The values expressed are the mean +/- SEM generated by pooling data from 3 separate experiments. Asterisk denotes $p < 0.05$ as compared to the positive control by the Student t-test.

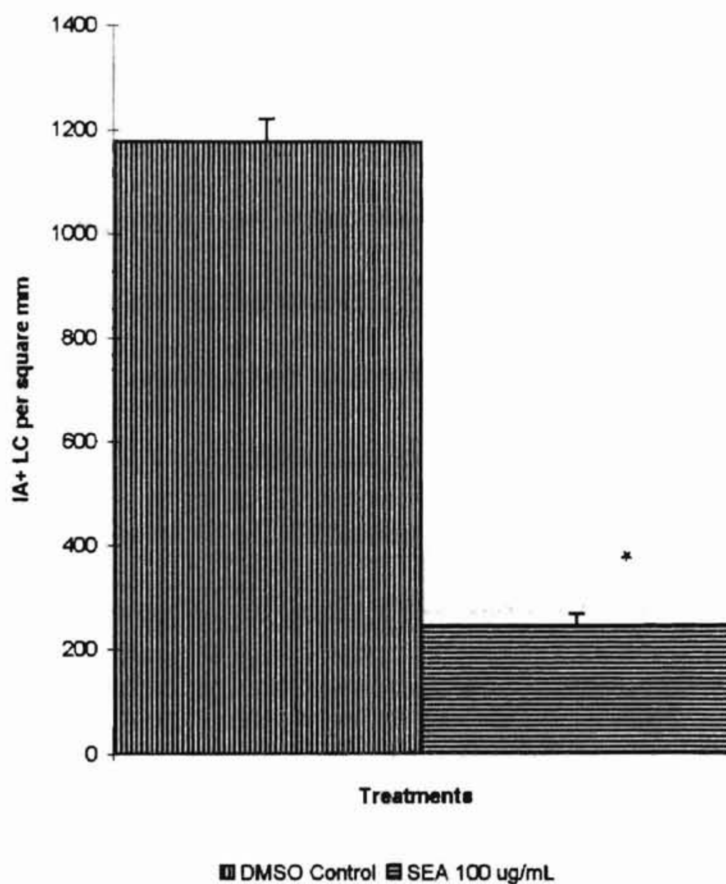


Figure 15. *In vivo* LC Depletion by SEA. The values expressed are the mean +/- SEM generated by pooling data from 3 separate experiments. Asterisk denotes $p < 0.05$ as compared to the positive control by the Student t-test.

This set of experiments focused on the effects of IL-1 α and TNF-α and their ability to induce LC migration *in vivo*. The experimental parameters were identical to the SEA *in vivo* studies with LC enumeration via immunohisotchemical staining. IL-1 α applied to the skin surface at a concentration of 372 pg/mL to induced LC migration comparable to that exhibited by treatment with SEA (Figure 16). These findings parallel those observed in the *in vitro* experiments.

When TNF-α was applied to the skin in a concentration of 127 pg/mL, LC depletion was observed comparable to that seen by SEA. However, there was a marginally higher level of LC depletion *in vivo* with TNF-α than was observed *in vitro*. Although the depletion levels differ slightly between the two studies, they do not exhibit a significant statistical difference and therefore, the higher level of depletion observed in the *in vivo* study might possibly be attributed to the affects of other immune components in the cytokine network.

## Effects of Blocking LC Depletion by SEA with Monoclonal Anti IL-1 α and Anti TNF-α Antibodies *In Vivo*

Sample mice were pretreated with 100 μL volume intraperitoneal injections of PBS, anti SRBC, anti IL-1 α or anti TNF-α dilutions. Following a two hour rest period, mice were administered 100 μL volumes of SEA in DMSO on the dorsal skin and kept isolated for 48 h. Skin sections were removed, epidermal sheets isolated and immunohistochemically stained for IA+ cells. Figure 17 shows that

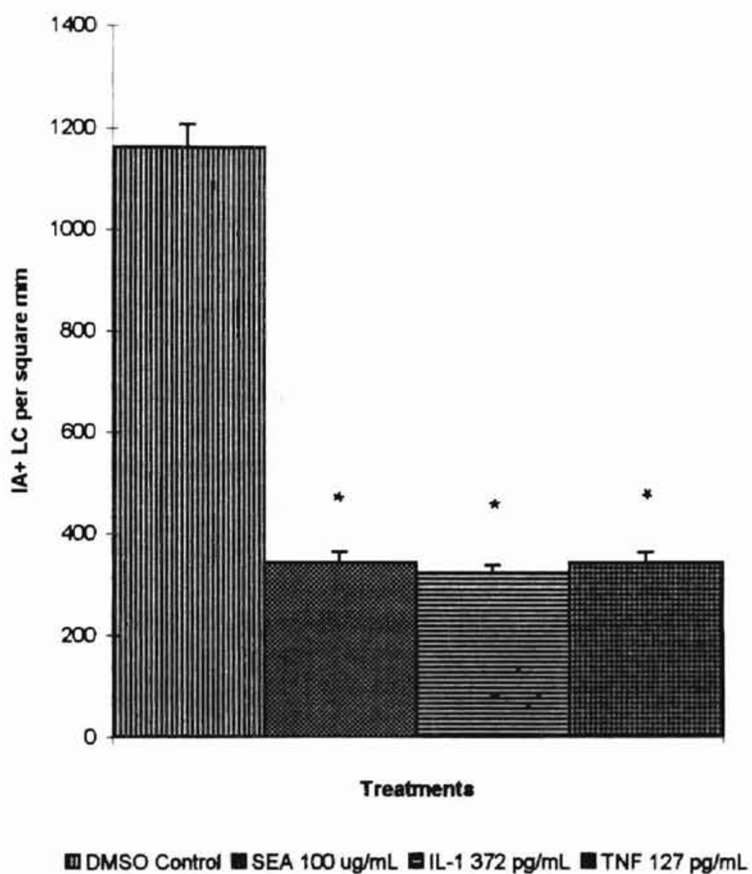


Figure 16. *In vivo* LC Depletion by IL-1$\alpha$ and TNF-$\alpha$. The values expressed are the mean +/- SEM generated by pooling data from 3 experiments. Asterisk denotes $p < 0.05$ as compared to the positive control by the Student t-test.

both antibodies blocked LC depletion keeping levels within the same statistical measurements as those observed in the DMSO/PBS control. These findings further implicate IL-1 $\alpha$ and TNF-$\alpha$ as primary mediators of SEA induced LC depletion *in vitro* and *in vivo.*

## Effects of Blocking LC Depletion by IL-1 $\alpha$ and TNF-$\alpha$ with Monoclonal Anti IL-1 $\alpha$ and Anti TNF-$\alpha$ Antibodies *In Vivo*

In order to determine if IL-1 $\alpha$ and TNF-$\alpha$ are primary inducers of LC migration from the epidermis *in vivo,* sample mice were again pre-treated the appropriate controls and antibody dilutions and rested two hours. Cytokines were applied to the dorsal skin and mice were kept isolated for 48 h. Skin sections were harvested as before and stained accordingly. Results in Figure 18 indicate that LC depletion does occur with IL-1 $\alpha$ and TNF-$\alpha$. Anti SRBC antibodies failed to block cytokine induced LC depletion and the data were used as a means by which to measure the ability of anti IL-1 $\alpha$ and anti TNF-$\alpha$ to block LC depletion. Both antibodies successfully blocked LC depletion by their respective cytokines.

## An *In Vivo* Investigation of Possible Cytokine Synergism

The final experiment in this study was conducted in conjunction with a similar

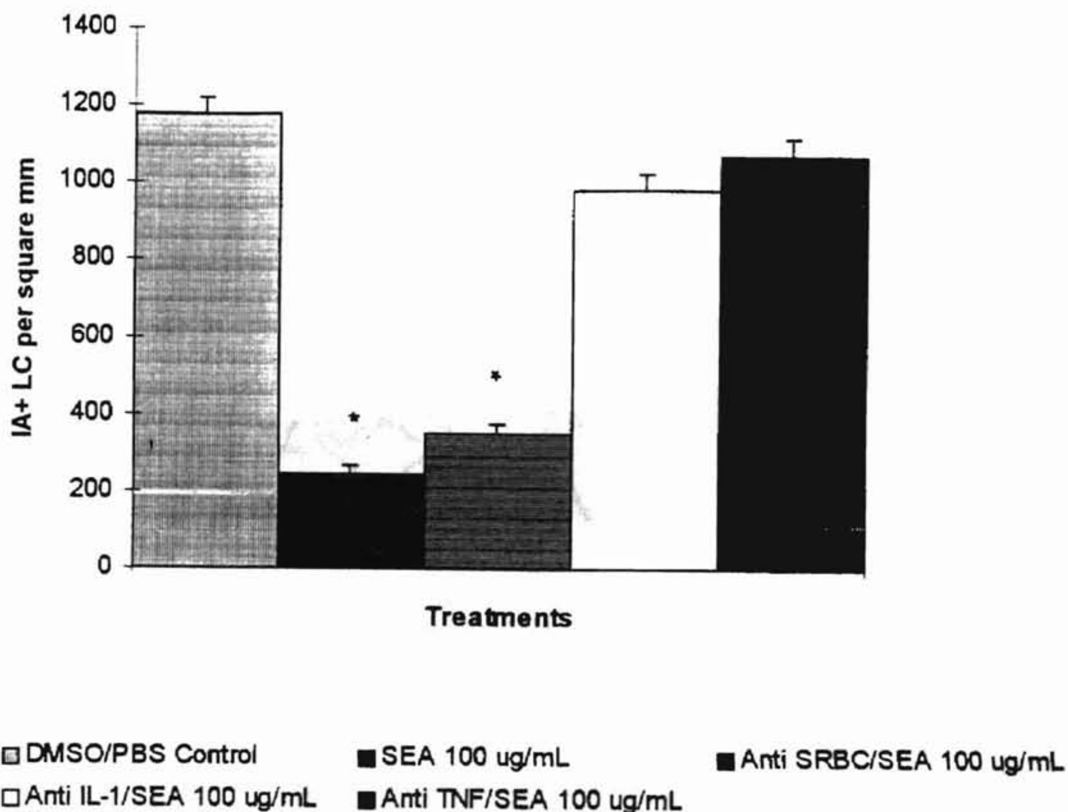


Figure 17. *In vivo* Blocking of LC Depletion by SEA with Monoclonal Anti IL-1α and Anti TNF-α Antibody. The values expressed are the mean +/- SEM generated by pooling data from 3 experiments. Asterisk denotes $p < 0.05$ as compared to the positive control by the Student t-test.

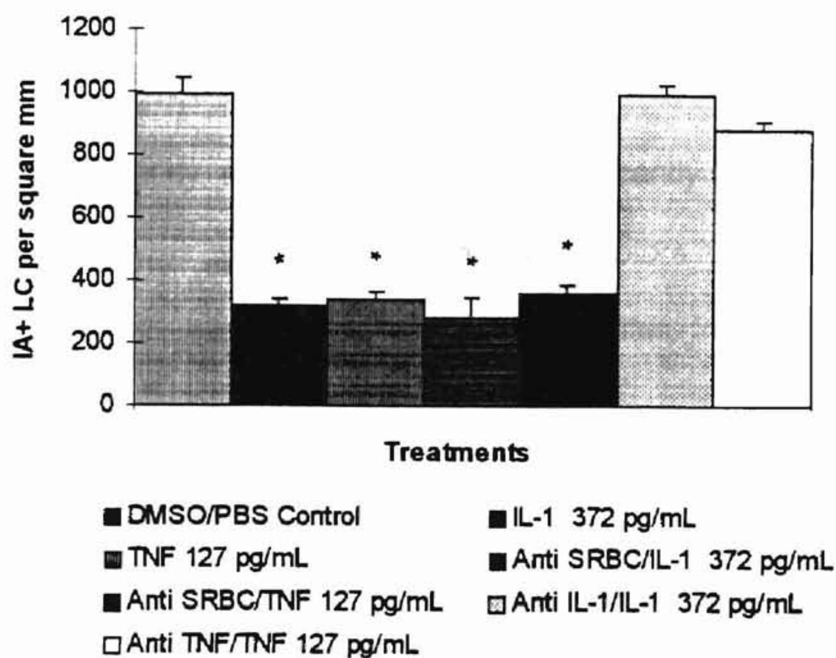


Figure 18. *In vivo* Blocking of LC Depletion by IL-1 α and TNF-α with Monoclonal Anti IL-1 α and TNF-α Antibody. The values expressed are the mean +/- SEM generated by pooling data from 3 separate experiments. Asterisk denotes $p < 0.05$ as compared to the positive control by the Student t-test.

*in vitro* study in order to determine if a possible synergism existed between IL-1 $\alpha$ and TNF-$\alpha$ in their ability to induce LC depletion *in vivo*. All experimental groups in Figure 19 are identical to those in Figure 18 with the addition of two experimental groups. The data shows that the sample mouse treated with anti IL-1 $\alpha$ and TNF-$\alpha$ cytokine exhibited no blocking of LC depletion by the anti IL-1 antibody. This is consistent with the data observed in the *in vitro* study. However, unlike the *in vitro* study, anti TNF-$\alpha$ failed to significantly block LC depletion by IL-1 $\alpha$. The differences may be due to a loss of receptors for TNF or IL-1 upon *in vitro* culture thereby resulting in fewer binding sites for both antibody and cytokine.

## Summary

Experimental observations involving the modulation of epidermal LC with SEA and keratinocyte derived cytokines IL-1 $\alpha$ and TNF-$\alpha$ have revealed significant information about their effects both *in vitro* and *in vivo*. SEA can cause dramatic depletion of LC from the epidermis. Recent findings demonstrating that SEA induced keratinocyte derived IL-1 $\alpha$ and TNF-$\alpha$ play a role in LC depletion have been substantiated by experiments in this study which revealed significant LC depletion with recombinant IL-1 $\alpha$ and TNF-$\alpha$ both *in vitro* and *in vivo*.

These findings are further supported by the ability of both polyclonal and monoclonal antibodies against IL-1$\alpha$ and TNF-$\alpha$ to block LC depletion induced by SEA , IL-1 $\alpha$ and TNF-$\alpha$.

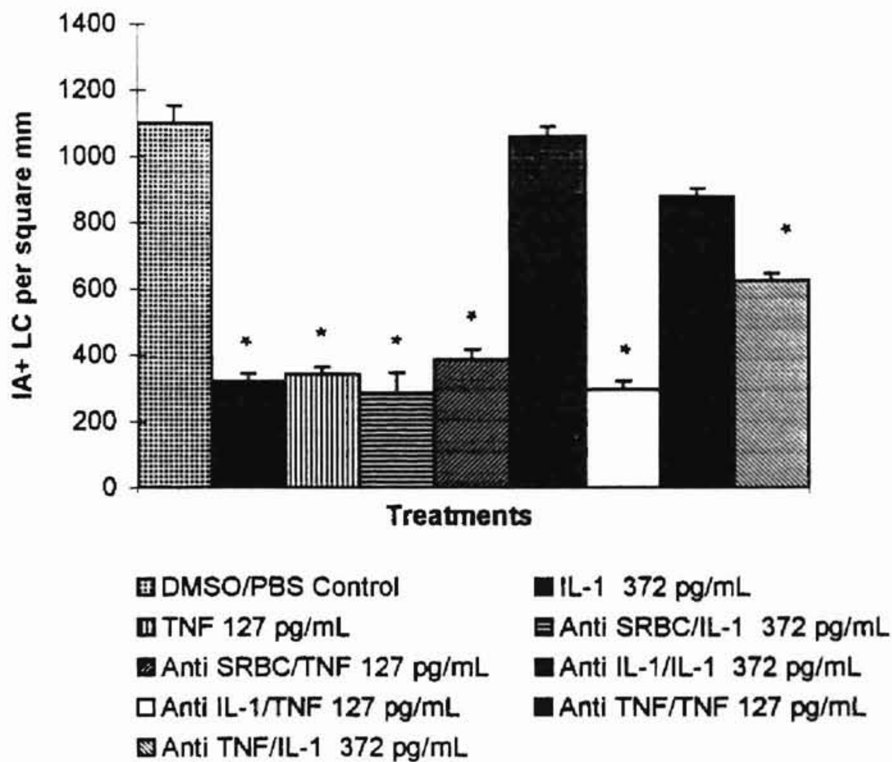


Figure 19. *In vivo* Cytokine Synergism Study. The values expressed are the mean +/- SEM generated by pooling data from 3 separate experiments. Asterisk denotes $p < 0.05$ as compared to the positive control by the Student t-test.

Finally, preliminary results indicate that a possible synergism exists between IL-1 α and TNF-α and their receptors. Anti TNF-α antibodies have the ability to block LC depletion by IL-1 α *in vitro*, and to a lesser extent *in vivo*. These data implicate IL-1 α as the primary cytokine mediator involved in initiating LC depletion in the presence of SEA.

Overall, these findings represent a possible first step in deciphering the cytokine pathway involved in LC depletion upon antigenic challenge and may offer valuable information in designing a model system for signaling mechanisms associated with the skin immune response.

# CHAPTER IV

# DISCUSSION

Much progress has been made in recent years with respect to the elucidation of a possible stimulus for LC migration from the epidermis to the draining lymph node. Early studies by Kripke *et al.* (1990) successfully showed that epicutaneous sensitization with FITC following a skin graft from a C3H mouse onto the dorsal region of a Balb/c mouse resulted in a dramatically elevated level of FITC labeled C3H dendritic cells in the Balb/c lymph nodes (33). These cells were positively identified as LC by the presence of Birbeck granules (33).

More compelling evidence came from studies involving UVB as a migratory stimulus. Exposure of both murine and human skin to various levels of UVB radiation resulted in LC depletion from the skin (3, 9, 39). In addition, experiments conducted in 1994 by Pickard *et al.* demonstrated through the use of several biological inhibitors that UVB induced migration could be blocked by inhibitors of transcription, translation and GTP binding proteins (52). Conclusions drawn from these data suggested that the stimulus for LC migration in response to UVB exposure involved regulatory signals perhaps involving G-proteins and protein synthesis (52).

The idea that *de novo* protein synthesis occurred in murine skin following UVB exposure was confirmed as a solid possibility through studies performed

by Ansel et al. (1988) in which they showed that murine keratinocytes were capable produced IL-1 $\alpha$ upon exposure to UVB and the bacterial toxin lipopolysaccharide (LPS) (3). Data obtained from various studies involving LPS instigated intensive research concerning the effects of staphylococcal superantigens on the skin immune system. In 1993, Pickard *et al.* successfully demonstrated that SEA can induce LC depletion comparable to that induced by UVB (52). In addition, data form these experiments indicated that, like UVB inhibition studies, LC depletion could be blocked by specific inhibitors of transcription, translation, G-proteins and associated kinases required for signal transduction (52). Again, this evidence suggests the possibility of *de novo* protein synthesis requiring the necessary kinase activity for proper cell signaling.

Based on earlier findings involving LPS and the production of IL-1 $\alpha$ by murine keratinocytes and the fact that TNF-$\alpha$ is also known to be produced by these cells (7, 22, 31), we hypothesized that one or both of these cytokines are up-regulated upon UVB or antigenic stimulation and, working through a signaling mechanism involving PTK and /or PKC, provide the necessary stimulus required for epidermal LC migration from the skin.

This hypothesis is supported by the observation that recombinant IL-1 $\alpha$ and TNF-$\alpha$ both caused a significant reduction in the numbers of IA+ LC from murine epidermis both *in vitro* and *in vivo.* IL-1 $\alpha$ appeared to be more effective at causing this depletion, taking the level of LC present in the epidermis down to that observed by SEA. This would initially suggest that IL-1 $\alpha$ is one of the primary biomolecules responsible for inducing migration. TNF-$\alpha$ appeared to

cause LC depletion similar to that observed by IL-1 α. However, once TNF-a concentration reached 23 pg/mL, LC levels tended to remain relatively unchanged.

This poses a unique situation with respect as to which cytokine is responsible for induction if at all. One possibility is that TNF-α is required by epidermal cells to provide the necessary signals for the up-regulation of other immunomodulating cytokines. Indeed, it has been shown that when Balb/c mice are treated with injections of staphylococcal enterotoxin B (SEB), serum levels of TNF-α peak at two h while IL-1 α levels are highest between four and six h (43).

Based on this information and the fact that TNF-α can stimulate the release of a host of cytokines form many different cell types, it seems likely that SEA induces TNF-α first and once optimum levels are reached for IL-1 α up-regulation, then IL-1 α is synthesized and thereby binds to its receptors.

To support this idea, experiments were performed in this study, both *in vitro* and *in vivo,* utilizing neutralizing polyclonal and monoclonal antibodies against both IL-1 α and TNF-α in the presence of SEA. Both antibodies were capable of blocking SEA induced LC depletion from murine skin. This clearly implicates both cytokines as contributing factors in superantigen mediated LC depletion. However, it should be kept in mind that it is the physical event of superantigen binding to exposed MHC II molecules on the surface of LC that is responsible for the resulting cytokine up-regulation and secretion.

The next logical step in the progression of this research focused on the actual cytokines and the effects they exude over immune components within the

skin. Cytokines are responsible for many cellular functions. Therefore, it is not surprising that they are also responsible for regulating immune functions. Initial studies by Lundquist and Back (1990) involving inducible cytokine secretion demonstrated that intracutaneous injections of IL-1 β in C3H/HeJ mice resulted in the decreased density of IA+ cells over a 2 day time period (40). They also observed that IL-1 β release occurred in purified LC upon exposure to UVB (40). This is of unique significance because IL-1 β is known to be constitutively expressed by murine LC and is thought to be involved in LC maintenance within the skin (7, 25, 40). In addition, both IL-1 α and IL-1 β bind to a common receptor (22) suggesting that in times of extreme injury, IL-1 β secretion and binding can augment LC depletion. However, no conclusive studies have been conducted to support this theory.

More compelling evidence upholding the idea that superantigen induced cytokine secretion plays a role in immune mediation was discovered by Matsuyama et al. in 1993. They concluded that binding interactions between SEB and MHC II induced the expression of genes encoding for the production of IL-1 and TNF in human keratinocytes (42). Antibodies generated against MHC II efficiently abolished SEB induced gene expression suggesting that MHC II is responsible for mediating gene expression (42). In addition, the use of three protein tyrosine kinase inhibitors successfully inhibited the expression of SEB induced IL-1 β mRNA. From this, they concluded that IL-1 β activity was regulated by protein kinase C (42). These findings are consistent with the findings of Shankar et al in which they observed that pre-treatment of murine

epidermal cell suspensions with SEA induced the expression and secretion of both IL-1 α and TNF-α (54) and that the expression of IL-1 α could be reversed with PKC inhibitors. Collectively, this data suggests that the binding of IL-1 α to target cells is partly responsible for inducing LC depletion upon antigenic stimulation and that the mechanism by which this occurs is regulated by PKC.

In order to ascertain how SEA induced production of IL-1 α and TNF-α affect the skin immune system with respect to antigen presentation and T-cell activation, maximum concentrations of both IL-1 α and TNF-α were obtained from dose response curves. Our data for both *in vitro* and *in vivo* studies indicate that both IL-1 α and TNF-α, when applied epicutaneously, are capable of inducing LC depletion equivalent to that observed by SEA. More importantly, pretreatment of sample mice with neutralizing polyclonal and monoclonal antibodies resulted in efficient blocking of LC depletion from the epidermis. This evidence serves to solidify the notion that both cytokines are directly involved in initiating LC migration.

With the discovery of this information, it is necessary to understand why IL-1 α and TNF-α are ideal candidates as inducers of LC migration. First, the biological activities of both cytokines provide compelling evidence. In addition to the functions already mentioned (See Chapter I) both IL-1 α and TNF-α stimulate the release of intercellular adhesion molecules (ICAM) from endothelial cells which line blood vessels and the draining lymphatics (31). The expression of ICAMs result in the adherence of neutrophils, monocytes lymphocytes and APC to vessel cell walls and may be involved in trafficking and directing these

cells to the appropriate regions of the body (3, 23, 31). Furthermore, TNF-α has been shown to play a role in up-regulating the skin immune response by increasing the number of MHC II required receptors for binding antigen (3, 31).

Finally, research conducted by Cumberbatch and Kimber involving TNF as a primary inducer of LC migration have shown that intradermal injections of recombinant TNF-α have effected significant LC migration while treatment with heat treated TNF failed to induce migration (15).

Therefore, it appears extremely likely that both cytokines are directly involved in LC depletion when murine skin is compromised directly by exposure to UVB, super antigens and cytokines.

The final experiments in this study focused on a possible functional synergism between IL-1 α and TNF-α both *in vitro* and *in vivo*. Very little research has been conducted on cytokine synergy with respect to superantigenic challenge so the data presented here is preliminary at best. However, it poses interesting possibilities with regard to cytokine function in skin immune responses.

*In vitro* results indicate that anti IL-1 α polyclonal antibody cannot completely block LC depletion by TNF. One possible explanation for this might be that TNF-α, when applied to the epicutaneous surface, stimulates the production of IL-1 a which in turn would bind to any IL-1 receptors that were left unblocked due to insufficient amounts of neutralizing antibody. Because most cytokines, IL-1 and TNF included, function at picomolar concentrations (22, 23, 43) the amount of IL-1 that might be produced may be enough to induce the partial depletion

observed.

Evidence further supporting these results were observed in the corresponding *in vivo* studies where anti IL-1 α again failed to block LC depletion by TNF-α. Based on previously documented evidence that TNF-α is produced before IL-1 α, the theory that unoccupied IL-1 α binding sites could be bound by TNF early on is a distinct possibility.

Additional support for this theory can be attributed to the second part of the study involving blocking experiments with anti TNF-α and treatment with IL-1 α. *In vitro,* anti TNF-α completely blocked LC depletion by IL-1 α. This data tends to indicate that antibody binding to TNF receptors blocks signals required to make IL-1 α thus blocking depletion.

However, duplication of this experiment *in vivo,* failed to exhibit the same level of blocking capability as that observed *in vitro.* The most likely explanation for this phenomenon might be that the binding of neutralizing antibody to TNF receptors, while inhibiting the production of TNF, still allows for the binding of IL-1 α to IL-1 receptors. This evidence would then strongly favor IL-1 as the primary biomolecule responsible for transmitting the necessary signals required by LC for migration upon antigenic challenge, superantigen or otherwise.

Continued experiments involving the use of superantigens as a means to modulate components of skin associated lymphoid tissues is currently one of the most intense areas of research in skin immunology. The implications of data discovered with respect as to how the skin immune system functions to defend

organisms from percutaneous antigenic challenge will have dramatic and far reaching effects in regions of the world where skin associated disease transmission remains a constant problem.

This study demonstrates that SEA induced IL-1 $\alpha$ and TNF-$\alpha$ are primarily responsible for inducing LC migration form the skin to the draining lymph nodes upon antigenic challenge. As a result, this data has provided a possible first step in a complex transduction pathway that remains to be elucidated.

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# APPENDIX - I

## EPIDERMAL CYTOKINES

| CYTOKINES | SOURCE | ACTIVITY |
|---|---|---|
| IL-1<br>alpha<br>beta | Several cell types | T-cell activation<br>NK cell activation<br>ICAM-1 expression<br>chemoattractant for neutrophils and macrophages |
| IL-3 | $T_H$ cells, NK cells, mast cells | Growth and differentiation |
| IL-6 | monocytes, $T_H$ cells, macrophages, bone marrow stem cells | Induces antibody secretion |
| IL-8 | macrophages, endothelial cells | Chemoattractant for neutrophils |
| TNF<br>alpha<br>beta | macrophages and like cells | Induces cytokine secretion<br>Cytotoxic and cytostatic to some tumor cell lines |
| IFN | leukocytes | Inhibits viral replication |
| G-CSF<br>M-CSF<br>GM-CSF | T-cells | Inflammatory response |
| TGF | platelets, | Wound healing |

| | | |
|---|---|---|
| alpha<br>beta | macrophages,<br>lymphocytes | IL-1 production<br>Chemoattractant for monocytes and macrophages |

ABBREVIATIONS: IL- interleukin; **TNF**- tumor necrosis factor; IFN- interferon; G-CSF- granulocyte colony stimulating factor; M-CSF- macrophage colony stimulating factor; GM-CSF- granulocyte/macrophage colony stimulating factor; TGF- transforming growth factor.

# VITA

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Master of Science

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