The Role of Cholera Toxin and Tape Stripping in Epicutaneous Immunization

by

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Abstract

In this study I have characterized the role of cholera toxin (CT) and tape stripping (TS) in eliciting and enhancing cellular immune responses generated by the epicutaneous application of peptide. In addition, the role of TLR4 signaling in the induction of skin elicited cellular and humoral immune responses was investigated. The CD4 T cell response to antigen was studied using a TCR transgenic mouse that has rearranged TCRα and TCRβ genes allowing it to express a T cell receptor specific for Ova323-339 in complex with I-A\textsuperscript{d} MHC class II molecules. To characterize the CD8 T cell response, naïve C57BL/6 mice were immunized with an MHC class I restricted epitope of ovalbumin, namely SIINFEKL (Ova254-267), and the resultant peptide specific CTLs were enumerated using multimeric Class I MHC-peptide complexes. The function of the CTLs was characterized using cytotoxicity assays. The effect of CT and TS on DC migration was also determined. When used together, TS and CT enhanced peptide specific CD4 T cell proliferation above levels achieved with either treatment alone. Similarly, CD8 T cell proliferation was optimal in mice treated with both modalities. The generation of functional CTL through the skin did not require IL-12p40, a key Th1 promoting molecule, suggesting a role for other Th1 promoting factors. Both CT and TS enhanced the immigration of DCs to the draining lymph nodes in Balb/c mice revealing a potential mechanism for their adjuvant effects. Finally, TLR4 signaling was not required for the generation of epicutaneous T cell or antibody mediated immune responses in these model systems. Epicutaneous immunization is a promising approach for the generation of epitope-specific T cell responses. As such, it may provide a practical, cost effective alternative to conventional needle immunization.
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1. Introduction

Skin is the single largest organ in the mammalian body. It provides a physical barrier between internal organs and the external environment. This barrier is water impermeable, retains moisture, protects the host from the damaging effects of ultraviolet radiation and retains structural integrity in the face of mechanical trauma. Being in close proximity to the external environment also makes the skin an important port of entry for invading pathogens. As such, one of its most vital functions is providing the first line of defence against invading entities (Junqueira et al. 1998). Accordingly, the surface layers of the skin contain the main components of the immune system. In this study, I harnessed the skin immune system to initiate systemic immune responses.

1.1 Structural layers of the skin

Epidermis

The skin can be divided into two functionally distinct layers, the epidermis and the dermis. The epidermis is the most superficial layer and in humans is composed of five sub-layers which move outwards from the dermis in the following order: stratum basale, stratum spinosum, stratum granulosum, stratum lucidium, and stratum corneum. The stratum corneum is the most superficial layer of the epidermis and can be 15-20 cell layers deep in humans and 2-3 cell layers deep in mice. The lipids contained within the stratum corneum layer function to provide a physical barrier to penetration by environmental agents and minimize water loss (Junqueira et al. 1998).

Cell types found within the epidermis

The important cell types found within the epidermis include keratinocytes (KCs), Langerhans cells (LCs), and leukocytes. Keratinocytes are the largest cell population of the epidermis and the other cells are found interspersed among them. Their primary role is to form the structural scaffold of the epidermis. They are also known to produce soluble proteins, known as cytokines, that can modulate immune responses. The cell type that truly distinguishes the skin as a functional immune organ is the Langerhans cell (LC). Initially characterized as being of neuronal origin due to their long dendrite by Paul Langerhans, LCs have been reassigned to the dendritic cell lineage over the past two decades. Langerhans cells can be found in the basal and suprabasal layers of the mammalian and murine epidermis. Although LCs and KCs are present in human
and murine epidermis, there are differences in the cellular composition of the epidermis in these two organisms. For instance, the murine epidermis contains epidermal dendritic TCRγδ-bearing cells that are not found in human skin while human skin contains epidermal dendritic TCRαβ-bearing cells, which are, absent from murine skin. In addition, both the human and murine epidermis contain other cells with a recognized role in immune responses, namely macrophages and mast cells (Bos 1997).

Dermis

The dermis, unlike the epidermis, consists of two layers composed of different tissue types. The outermost papillary layer is composed of loose connective tissue, while the deeper reticular layer contains dense connective tissue with type I collagen. The dermis serves to join the epidermis to the subcutaneous tissue (hypodermis) through a basale lamina that is found between the stratum basale of the epidermis and the papillary layer of the dermis. The main cell types found within the dermis include fibroblasts, macrophages, mast cells and dermal dendritic cells (DCs). It also includes cells of the vascular and lymphatic endothelium (Junqueira et al. 1998).

1.2 Elements of an immune response

The mammalian immune response consists of two integrated and interdependent processes: innate and adaptive immunity. Under homeostatic conditions, both arms are turned off. Activation of the immune system as a whole, however, requires activation of innate immunity only as events downstream of an activated innate immune response serve to initiate an adaptive immune response.

Innate immunity

Danger Signals and initiating innate immunity

The primary function of innate immunity is to recognize the presence of danger, perform some immediate damage control, and initiate the antigen specific adaptive arm of the immune response. The activation of the adaptive arm is achieved through the activation of professional antigen presenting cells, the most potent of which are DCs. Dendritic cells are designed to recognize signals that communicate the presence of danger in the surrounding tissue, signals that are expressed by tissues undergoing damage, necrotic death, destruction and/or are under distress (Gallucci and Matzinger 2001). During an infection, these signals can be produced either by the host (endogenous), or by the invading organism (exogenous). Examples of putative endogenous
danger signals include heat shock proteins (hsp) and certain pro-inflammatory cytokines, such as interleukin-1 (IL-1). Exogenous signals consist of pathogen associated molecular patterns (PAMPs). These are molecules and substances that are restricted to pathogens and not found within the host (Gallucci and Matzinger 2001). One of the most potent bacterial PAMPs is the gram-negative bacterial cell wall constituent lipopolysaccharide (LPS). Other examples include viral double stranded RNA and bacterial unmethylated GC rich DNA sequences (Medzhitov 2001)

**How DCs respond to danger signals**

Dendritic cells perceive danger signals, whether endogenous or exogenous, by expressing cell surface receptors that recognize these signals as ligands. One of the best-known receptors in this regard are the pattern recognition receptors (PRRs) that recognize PAMPs. They are the mammalian homologue of the family of Toll-like receptors (TLR) originally identified in *drosophila*. Although initially thought to recognize exogenous PAMPs exclusively, recently many endogenous danger signals have been identified that also signal through these receptors. Regardless of the receptor used, these danger signals are able to promote the activation of resting DCs (Medzhitov and Janeway 2000).

Resting DCs within tissues actively internalize proteins and antigens from the surrounding tissue (exogenous antigens) and present them on their cell surface along with proteins synthesized within the DC (endogenous antigens). Exogenous and endogenous antigens are generally processed via two distinct pathways, the MHC class II and MHC class I pathways respectively. This separation becomes relevant at the later stages of an immune response because the T cell receptor (TCR) is restricted to either one or the other MHC molecule. Therefore, CD4 T cells can only recognize an antigen presented in complex with MHC class II molecules and CD8 T cells can only recognize an antigen in complex with MHC class I molecules (Banchereau et al. 2000). The simple act of presenting antigen on its cell surface, however, does not allow resting DCs to initiate an adaptive immune response. Dendritic cells presenting antigen must be stimulated in order to provide the necessary co-stimulatory signals to prime naïve T and B cells. Key features of activated DCs are the following: 1) Reduced ability to internalize and process antigens for presentation. 2) Increased expression of antigen/MHC complexes on cell surfaces. 3) Up-regulation of co-stimulatory molecules that are essential for stimulation of naïve T cells, and 4) expression of certain chemokine receptors that direct DC movement along a chemokine gradient.
leading from the tissue to secondary lymphoid organs (Banchereau et al. 2000). DCs undergo this activation process upon receiving danger signals.

**Adaptive Immunity**

**Activation of adaptive immunity**

As part of the activation process, DCs receive signals that stimulate their migration to the secondary lymphoid organs where cells of the adaptive immune response reside. The secondary lymphoid organs include lymph nodes (LN), the spleen, and Peyer’s patches in the small intestine. Secondary lymphoid organs are the site of initiation of adaptive immunity since they house the main effector cells of adaptive immunity (Zinkernagel 2003). In the secondary lymphoid organs mature (activated) DCs displaying antigen acquired in the periphery deliver two signals that activate naïve CD4 T cells. Signal one is provided by the engagement of the T cell receptor (TCR) with the antigen/MHC complex on DC surfaces and signal two is provided by the interaction of the co-stimulatory molecules expressed on DC surface with their respective receptors on T cells. Both signals are required to achieve full activation. Immature DCs that are capable of delivering signal 1 but not signal 2 induce T cell apoptosis or a state of in-activation referred to as anergy (Baxter and Hodgkin 2002).

**Deciding on the class of response**

The main effector mechanisms of adaptive immunity can be categorised into two classes, Th1-driven (CD8 T cell mediated; cellular) and Th2-driven (antibody mediated; humoral). The class of response initiated is dictated by cytokines secreted by activated DCs and CD4 T cells. The main cytokine responsible for initiating Th1 immunity is interleukin-12 (IL-12). Th2 immunity is stimulated by IL-4. In addition to promoting their respective classes of immune responses, each cytokine also performs a counter-regulatory function by turning off the production of the other cytokine, thereby strengthening its own signal (Jenkins et al. 2001). Mature DCs migrate to the T cell rich zones of secondary organs, deliver signal 1 and 2, and induce clonal proliferation of T cells. The cytokines secreted by the mature DC will determine which class of effector functions are activated.

**Effector mechanisms of adaptive immunity**

Activation of CD4, CD8 T cells or B cells results in the acquisition of effector function by these cells and the ability to home to the tissue in which the DCs originally perceived the danger signal. Activation of CD4 T cells results in differentiation of cells into either Th1 or Th2 helper
T cells (depending on the cytokine milieu). Effector CD4 T cells function to further direct the class of immune response through the secretion of specific cytokines. Th1 CD4 T cells can facilitate the activation of naïve CD8 T cells through the production of interferon gamma (IFNγ), while Th2 CD4 T cells facilitate the activation of B cells by producing the cytokine IL-4 (Jenkins et al. 2001). Upon activation, CD8 T cells differentiate into cytotoxic T lymphocytes (CTL) capable of killing cells expressing antigen recognized by their TCR. All nucleated cells are capable of displaying endogenous proteins in the context of MHC class I molecules on their cell surface. CTL are therefore able to recognize virally infected cells as those cells expressing a viral antigen/MHC class I complex on their cell surface. Similarly, tumor cells display antigens from proteins that they are actively synthesizing (Barry and Bleackley 2002). The main effector mechanism acquired by B cells upon activation is the secretion of antibodies specific for the antigen responsible for engaging the B cell receptor (BCR). Antibodies function to bind and neutralize soluble infectious particles preventing them from infecting cells and targeting them for destruction by the complement system. However, they are ineffective at clearing cells that have already been infected with a virus (Janeway et al. 1999).

Initiation of immunity in the skin

Given the processes described above the skin becomes an immunologically relevant organ because the tissues that comprise it are capable of sending out danger signals and the resident tissue APC are capable of acting in response to them. Accordingly, the immunologically relevant tissues of the skin are those that house professional APC, the epidermis and the dermis. The professional APC that can be found within these tissues are the Langerhans cells and dermal DCs respectively (Nickoloff 2002). Despite the existence of two distinct DC subsets, the majority of immune responses elicited within the skin have been attributed to activation of Langerhans cells and not dermal DCs. One reason for this may be that since the epidermis lies above the dermis any physical or environmental trauma directed at the skin as a whole would be absorbed by the epidermis first before continuing on to the deeper layers. Secondly, dermal DCs have been difficult to identify due to lack of knowledge regarding their distinguishing features (Bos 1997). Thus, most investigations into immune responses originating in the skin have assumed that LCs are the professional APC involved.

Immune responses in the skin are generated, as in any other tissue, after the detection of danger. The main cells of the epidermis that participate in sending out endogenous danger signals in
response to injury or stress are KCs. One of the most well known danger signals to be released within the epidermis by KCs is interleukin-1 (IL-1) (Murphy et al. 2000). Other major cytokines released by KCs under stress include IL-6, IL-8, and tumor necrosis factor alpha (TNFα). All of these cytokines can act as potent danger signals since they serve to activate the resident tissue APC, the LC (Bos 1997). Langerhans cells act as sentinels within the epidermis, internalizing various endogenous and exogenous antigens found within their vicinity and displaying them on their cell surface along with proteins they synthesize (Nickoloff 2002). Therefore, any foreign particle introduced into the skin would be engulfed by LCs, processed and displayed. Given the segregation of the processing pathways into exogenous versus endogenous, most of the antigens acquired from the surrounding environment by LCs are displayed in complex with MHC class II molecules. Once LCs receive a danger signal they undergo maturation and begin to migrate through the various layers of the skin into the tissue draining lymphatic vessels that carry them into the nearest tissue draining LN (Jakob et al. 2001). There they will present their load to the resident CD4 T cells and set in motion the steps of the adaptive immune response.

1.3 Principles of Immunization

*Immunological basis of vaccines*

**Immunological Memory**

An essential feature of a successful immune response is the generation of antigen specific immunological memory. Once the danger that stimulated the response is contained, APC no longer receive danger signals and return to their resting state. The cells of adaptive immunity that have been activated require periodic re-stimulation to continue exerting their effector functions. In the absence of activating signals these cells undergo activation induced cell death (AICD), whereby approximately 90% of the T and or B cells undergo apoptosis. The remaining 10% of cells become memory cells with a majority re-circulating to populate the tissue in which they first encountered their antigen (Badovinac et al. 2002, Murali-Krishna et al. 1998). The establishment of immunological memory allows the immune response to mount a stronger, faster immune response upon a secondary challenge with the same antigen (Zinkernagel 2003). Immunological memory forms the rationale for the field of vaccinology. By vaccinating an individual with a weaker form of an infectious agent, vaccines allow for the activation of immunity and the generation of immunological memory while minimizing morbidity. Consequently, the goal of a successful vaccine is to establish a large memory population. Since

**Limitations of vaccines**

The current limitation of most vaccines is that they stimulate only the humoral immune response allowing only humoral and not cellular memory to be generated (Liu 1997). Vaccines that make use of live attenuated viruses however, are able to stimulate cellular immunity. An example of such a vaccine is the measles/mumps/rubella vaccine (Gans et al. 2001). Cellular immune responses are critical for effectively controlling infections that reside within cells, where antibodies cannot reach (Seder and Hill 2000). Thus, one of the biggest challenges facing the field of vaccinology in the 21st century is to construct vaccines that can stimulate both arms of adaptive immunity and thereby provide protection that is more complete. In addition to redesigning vaccines to allow more complete protection, there is a great deal of interest in moving away from needle administration to a less invasive and needle free mode of vaccination (Levine 2003). The need to find alternatives to the needle stems from the inherent problems associated with needle use. First, the use of needles requires proper personnel training to ensure used needles are disposed of properly and not reused. Even with personnel training one cannot completely eliminate needle reuse, and thus there is the risk of spreading needle borne diseases such as Hepatitis B and C and HIV (Aylward et al. 1995, Kane et al. 1999). Second, needles are perceived to be invasive and pain causing. These factors can influence compliance with vaccination regimens especially those requiring boosters. Third, the equipment and personnel training required increase the price of a needle administered vaccine making it less feasible for mass immunizations in developing and third world countries (Levine 2003). A good example that illustrates the benefits of needle free vaccines is the oral polio vaccine which proved quite effective in eradicating polio (Hull et al. 1997).

**Epicutaneous Immunisation**

**Definition**

A promising alternative to delivering vaccines via needles is to harness the power of the skin immune system by delivering immunizing solutions into the surface layers of the skin (epicutaneous immunization). Since the structural components of the cutaneous immune system
can be found in the most superficial layers of the skin, i.e. the epidermis, solutions applied to the skin surface do not have to penetrate deep in order to elicit an immune response. In addition, measures such as limited tape-stripping (TS) of the skin have been shown to remove the surface layers of the stratum corneum facilitating penetration of solutions applied topically (Dreher et al. 1998, Marttin et al. 1996). Thus, delivery of vaccines via the skin could potentially be accomplished without the need for invasive delivery procedures.

Recent progress in epicutaneous immunisation

Over the past few years several groups have illustrated that epicutaneous immunization (ECI) can be used successfully to elicit protective immune responses (Glenn et al. 1998a, Kaiserlian and Etchart 1999, Misra et al. 1999, Tang et al. 1997). The current focus in the field is to characterize the types of immune responses that can be elicited through ECI. It has been used successfully in animal models to elicit immune responses to non-infectious proteins and/or antigens (Beignon et al. 2001), viruses (El-Ghorr et al. 2000, Mueller et al. 2002) and tumor antigens (Seo et al. 2000). In addition to administering the agent against which an immune response is desired, antigen or protein based vaccines must also provide the danger signal(s) required to initiate immunity. Exogenous danger signals included in immunization solutions are referred to as adjuvants. Common examples include LPS, bacterial enterotoxins, and bacterial DNA sequences.

Despite the large number of studies that have investigated the feasibility of ECI most have focused on the humoral immune response as a measure of the effectiveness of the technique. A humoral response to a number of different antigens has been generated using a variety of different adjuvants, such as cholera toxin (CT) (Glenn et al. 1999), lymphotoxin (Beignon et al. 2001), and bacterial CG rich DNA sequences (Beignon et al. 2002) through the skin. ECI has been found to generate a wider variety of antibodies as compared to the oral route (Glenn et al. 1998b) with prominent antigen-specific IgA responses (John et al. 2002). What is less well characterized is the ability of ECI to generate cellular immunity. Although the generation of antibody responses provides indirect evidence for the activation of CD4 T cells through ECI, little is known about the in vivo activation of antigen specific CD4 and CD8 T cells in response to ECI (Hammond et al. 2001, Seo et al. 2000).
1.4 Thesis objectives

We hypothesised that CT and TS are efficient mechanisms for stimulating cellular immune responses through the skin. Based on this hypothesis our objectives were two fold. First, to explore the possibility of stimulating optimal CD4 and CD8 T cell responses through ECI with an antigen plus adjuvant (CT). Second, to study the immune mechanisms that may be stimulated in response to danger signals elicited by the immunising procedure. To accomplish this, ECI immunisation in this thesis was carried out by i) TS to expose deeper epidermal layers and induce mild tissue injury that may stimulate release of endogenous danger signals and ii) application of CT, a bacterial enterotoxin commonly used as an adjuvant that may provide exogenous danger signals.
2. Materials and Methods

2.1 Animals Used

Inbred Balb/c and C57BL/10ScSn (B10) mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). Inbred C57BL/6 (B6) mice were obtained from Charles River Laboratories (Wilmington, MA, USA). BALB/c-TgN (DO11.10) mice, expressing a TCR recognizing the class II epitope of ovalbumin (Murphy et al. 1990) and C57BL/6-Tg (OT-1) mice expressing a TCR recognizing the class I epitope of ovalbumin (Hogquist et al. 1994) were both obtained from Jackson laboratories and subsequently bred within the animal unit facility of the BC Research Institute for Children’s and Women’s Health, Vancouver, Canada. B6.129S1-Ii12btm1Jm (IL-12p40ko) (Magram et al. 1996), C57BL/10ScNcr (TLR4ips-del) (Poltorak et al. 1998), and C.C3H-Lpsd (Vogel et al. 1994) were also obtained from Jackson laboratories. All experiments were carried out on 6-8 week old mice and approved by the Animal Care Committee of the University of British Columbia.

2.2 Cell Lines

EL4 and EG7-Ova (EL-4 transfected to express OVA) (Moore et al. 1988) mouse lymphoma cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured upright in 75cm$^2$ tissue culture flasks (Sarstedt). EL4 cells were cultured in cRPMI. EG7-Ova cells were cultured in cRPMI supplemented with 0.05 mM 2-mercaptoethanol and 0.4 mg/ml G418.

2.3 Peptides used

Full Ova protein (OVA V) was purchased from Sigma. The peptides used are the class I restricted epitope of ovalbumin protein, (SIINFEKL) (Ova8) and the class II restricted epitope, (ISQAVHAAHAEINEAGR) (Ova323). The 2C peptide (SIYRYYGL) was used as a control for the specificity of the K$^b$-Ova-tetramer. All three were synthesized using FMOC chemistry, purified by reverse phase high-performance liquid chromatography to > 80% purity at the Nucleic Acid and Peptide Synthesis Facility of the University of British Columbia.

2.4 Lymphoid organs collected

The CD4 and CD8 T cell response was characterised in the auricular, cervical, brachial, axillary, and inguinal lymphnodes (LN). The CD8 T cell response was also characterised in the spleen. DC migration studies were conducted using only the ear draining auricular LN. The author
acknowledges the help extended by the Ardavin lab in locating the auricular LN (Anjuere et al. 1999). Please refer to appendix A.1 for illustration. All lymphoid organs were collected in RPMI 1640 supplemented with 100u/ml Penicillin (GibcoBRL), 100 μg/ml Streptomycin (GibcoBRL), 2g/L sodium bicarbonate (Fisher) and 10% fetal bovine serum (GibcoBRL) (cRPMI).

2.5 Adoptive transfer of Transgenic T cells

The DO11.10 adoptive transfer model used was first described by Mark K Jenkins (Kearney et al. 1994, Pape et al. 1997). Transgenic CD8 T cells were isolated from OT-1 mice while transgenic CD4 T cells were isolated from DO11.10 mice. Single cell suspensions of LN and spleen collected from 6-8 week old mice were prepared. Cells were washed twice with phosphate buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS), referred to as 2% FPBS. Cells were spun at 450g for 5 min at 4C. Before washing, splenocytes were depleted of red blood cells using RBC lysis buffer (8.29 g NH₄Cl (0.15 M), 1 g KHCO₃ (1.0 mM), 37.2 mg Na₂EDTA (0.1mM) dissolved in 1 L distilled H₂O, pH adjusted to 7.2-7.4). After the last wash cells were re-suspended in 10 ml of 2% FPBS, passed through a 70 um nylon cell strainer (BD Biosciences), and labelled with CD4 (DO11.10 cells) or CD8 (OT-1 cells) microbeads (Miltenyi Biotech) as per the manufacturers instructions. After labelling cells were washed, re-suspended in 2% FPBS and passed through MS positive selection columns (Miltenyi Biotech). The eluant was discarded and the purified CD4 or CD8 T cells collected in a separate 15-ml Falcon tube. Cells were counted, re-suspended in PBS to a concentration of 5-8 million/ml and labelled with 5 uM carboxyfluorescein diacetate succinimidyl ester (CFSE Molecular Probes, Eugene OR) at 37C for 12 min. CFSE uptake was stopped by adding FBS and cells pelted and re-suspended to 50 million/ml in PBS. A small aliquot of the purified cells was stained with anti-CD4 (DO11.10 cells) or anti-CD8 (OT-1 cells) monoclonal Ab and analysed by FACS to confirm the purity of the population. Subsequently, 1ml insulin syringes (Becton Dickenson) were loaded with 100 μl of the cell solution and injected intravenously (tail vein) into naïve Balb/c (DO11.10 cells) or C57BL/6 (OT-1 cells) mice. Mice were restrained in a 50 ml conical Falcon tube with breathing holes and the tail vein dilated for 1-2 minutes using a heat lamp prior to the injection.

2.6 Epicutaneous Ear Immunisation

Immunisation of adoptively transferred mice

Mice were sedated with an i.p. injection of a ketamine/xylazine cocktail containing 80-120 mg/kg ketamine and 8-10 mg/kg xylazine. Immunization was carried out one day following the
adoptive transfer. Balb/c mice were immunized with 200 μg of Ova323 ± 25 μg of whole CT (Sigma) with or without barrier disruption (by tape-stripping). C57BL/6 mice were immunized with 25 μg of Ova8 ± 25 μg of CT with or without barrier disruption. In groups where mice were tape stripped first, the dorsal and ventral sides of the ear were both tape stripped 8 times using cellophane tape (Scotch Brand 3710 adhesive tape). The ear was then cleansed with an acetone soaked cotton tip prior to immunization to facilitate removal of the lipid barrier. Peptide and CT were diluted in PBS and 12.5 μl (25 μl/ear) painted onto the dorsal and ventral side. Mice were immunized on one ear only. Mice were immobilized for approximately 40 minutes, to allow the solution to dry thus preventing loss of peptide through grooming. The CD4 T cell response in the skin draining LN was assessed 4 days after the immunization. The CD8 T cell response was analyzed 3 days post immunization.

**Immunisation of naïve mice**

To generate a CD8 T cell response naïve C57BL/6 or IL12p40ko mice were anesthetized as stated above and immunized with 25 μg of Ova8 ± 25 μg of CT with or without TS. Mice were primed and boosted seven days later (on the contra-lateral ear) (thus both ears were used for the immunization). The CD8 T cell response in the skin draining LN and the spleen was assessed 14 days post priming.

To generate an antibody response naïve Balb/c mice were anaesthetised as stated above and immunised with 200 μg of full OVA protein and 25 μg of CT after TS. Solution was applied to the dorsal and ventral sides. Mice were boosted on the contra-lateral ear 7 days later. Antibody titres in the blood were determined 14 days post priming.

**2.7 DC immigration assay**

A previously described method was employed with minor adjustments (Ruedl et al. 2000). A 5% solution (in ethanol) (Fisher) of a green fluorescent dye, CellTracker (5-chloromethylfluorescenc diacetate, Molecular Probes, Eugene OR) was applied to the dorsal sides of the ears of naïve C57BL/6 or naïve Balb/c mice. Mice were tape stripped just before application of the solution. 25 μg of CT was dissolved in ethanol and applied to the same site 40 minutes after CellTracker. Twenty-four hours later the auricular LN were collected and teased apart with scissors. LN were re-suspended in RPMI 1640 supplemented with 100U/ml Penicillin, 100 μg/ml Streptomycin, 2g/L sodium bicarbonate, 5% fetal bovine serum, 1mg/ml Collagenase D (Roche, Basel
Switzerland), and 40 μg/ml DNAse (Boehringer Mannheim, Mannheim, Germany) for two 30-minute incubations at 37°C. Cells were collected in 0.1 M Di Sodium EDTA buffer (Sigma) (pH 7.2) supplemented with 5% fetal bovine serum and 5 μg/ml DNAse and passed through a 70 um nylon cell strainer (BD Biosciences). After washing 2x with EDTA buffer, cells were re-suspended in 2ml of EDTA buffer and layered onto 1 ml of NycoPrep 1.077A (Accurate). After centrifuging at 800g for 15 minutes at 4°C cells at the interface were collected and immuno-stained in EDTA buffer. A chemical irritant, dibutylthalate (DBT) (Sigma) stimulates optimal DC immigration and 5% CellTracker dissolved in a 1:1 mixture of dibutylthalate and ethanol was used as a positive control.

2.8 Antibody staining and FACS analysis

At the endpoint of each immunization, mice were sacrificed using carbon dioxide asphyxiation and lymphoid organs collected in cRPMI. Single cell suspensions were made as described above for adoptive transfer and cell surface antibody staining carried out in 96 well round bottom plates (Becton Dickenson) using 1.5 million cells per well. Immuno-staining of isolated DCs was carried out in microtitre tubes (Bio Rad) and antibodies diluted in EDTA buffer instead of 2% FPBS. Antibodies used to identify peptide specific CD4 T cells included KJ1-26 PE (Cederlane), a monoclonal Ab specific for the transgenic TCR expressed by DO11.10 CD4 T cells (Haskins et al. 1983), and anti-CD4 Cyc (BD Pharmingen). Dendritic cells were identified using biotinylated anti-CD40 (Cederlane) and anti-CD11c PE (BD Pharmingen). Peptide specific CD8 T cells were identified using a PE conjugated K^b^-Ova tetramer (graciously provided by Jacqueline D Trudeau and Dr. Rusung Tan and synthesized according to Altman 1996) and anti-CD8a FITC (Cederlane). Non-specific binding of the K^b^-Ova tetramer to B cells was excluded from the analysis by gating out cells staining positive for a B cell marker, CD45R using anti-CD45R/B220 PerCP (BD Pharmingen). Streptavidin APC (BD Pharmingen) was used as the secondary antibody for all biotinylated primary antibodies. All antibodies were diluted 1/100, except for the K^b^-Ova tetramer, which was diluted 1/250 or 1/500. Titration of the K^b^-Ova tetramer revealed these to be the optimal concentrations as they yielded good fluorescence intensity while minimizing non-specific staining. Antibody dilutions were carried out in 2% FPBS for lymphocytes or EDTA buffer for DCs. Staining was carried out in a volume of 50 μl. Cells were incubated with the antibodies for 30 minutes on ice in the dark. Cells which were stained with the K^b^-Ova tetramer were incubated with the antibodies + the tetramer for 1hr. Cells were washed 3x with 2% FPBS (or EDTA buffer for DCs) and spun at 800g for 1 minute.
between stains. 200,000 antibody stained cells were analyzed using four-channel fluorescence activated cell sorting (FACS) performed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) and Cell Quest software (Becton Dickinson).

2.9 Cr Release Assay

A previously described method was used (Sheikh et al. 1999). 30 million spleen cells (depleted of red blood cells) from C57BL/6 or IL12p40ko immunized mice were cultured with 1.5 million irradiated (25,000 Rads) EG7-Ova cells in 10ml of cRPMI in a 25cm² tissue culture flask (Sarstedt) standing upright. Cells were cultured for 5 days at 37C. On day 5 non-adherent cells (effector cells) were collected, washed, and counted. EG7-Ova and EL4 cells were used as target cells. Target cells were labeled with 0.1mCi Cr⁵¹/million cells for 90 minutes at 37C (Cr⁵¹ Amersham Pharmacia). Excess Cr⁵¹ was removed by washing cells 3x with PBS. Labeled target cells were cultured with effector cells in 96 well round bottom plates at three ratios. Plates were spun at 150g for 2 minutes prior to incubating for 4 hours at 37C. Control groups included target cells cultured in cRPMI (minimum) and 5% Triton X detergent (maximum) (Sheikh et al. 1999). After 4 hours, cells were spun at 800g for 2 minutes and 150 μl of the supernatant collected in microtitre tubes (Bio Rad). Samples were read using a gamma counter and % lysis calculated as follows:

\[
\text{sample cpm} - \text{minimum cpm} \times 100
\]
\[
\text{maximum cpm} - \text{minimum cpm}
\]

2.10 Antibody ELISA

7 days after the last boost, mice were anesthetized with Avretin (Sigma) containing 250 mg/kg of tribromoethanol (approximately 600 μl for a 25gm mouse) and bled from the jugular vein. Blood was collected in 1ml-ependorf tubes and allowed to clot at room temperature for 1 hour. The contents of the tube were spun down and the serum removed from the top layer. A previously described protocol for detecting Ab titres was used (Saloga et al. 1994). 96 well flat bottom ELISA plates (VWR) were coated with 5-μg/well ovalbumin protein in a volume of 50 μl. The protein was diluted in coating buffer (8.4 g NaHCO₃ and 3.56 g Na₂CO₃ dissolved in 1L of distilled water, pH 9.5). Plates were sealed and incubated overnight at 4C. Wells were blocked with 150 μl of blocking buffer (1% bovine serum albumin (Roche) in PBS) for 1 hour at RT. For Ova specific IgG and IgG1, serum samples were started at a 1/100 dilution and two fold serial dilutions carried out. The standards for IgG and IgG1 (Sigma) were started at 810 ng/ml and two
fold serial dilutions carried out. For Ova specific IgE and IgG2a, samples were started at 1/10 and two fold serial dilutions carried out. The standard for IgE (laboratory-generated) was started at 40 arbitrary units (AU) and for IgG2a (laboratory-generated) at 32 AU and two fold serial dilutions for each carried out. Plates were sealed and incubated at RT for 2hrs. For IgG, IgG1, and IgG2a detection antibodies (all from Zymed laboratories, San Francisco CA) were diluted 1/2000, 1/2000, and 1/500 respectively in blocking buffer and added to wells in a volume of 100 µl. Plates were then washed 7x with washing buffer before adding the enzyme substrate solution. For IgE plates were first incubated for 1hr at RT with 100 µl/well biotin-conjugated anti-mouse IgE (BD Pharmingen) diluted 1/200 in blocking buffer. Plates were washed 7x with washing buffer and then incubated for 1hr at RT with a 100 µl/well streptavidin-HRP (BD Pharmingen) diluted 1/1000 in blocking buffer. After washing plates 5x a 100 µl of peroxidase substrate solution was added to each well (Kirkegaard & Perry Laboratories, Gaithersburg MD). Plates were incubated for 45 minutes to an hour at RT in the dark. The reaction was terminated using 50 µl/well of 5% sodium dodecyl sulfate (SDS Sigma) and the optical density read at 405 nm. Antibody titres were extrapolated from a standard curve generated using linear regression analysis.

2.11 Statistical analysis

Statistically significant differences between groups were calculated using two-tailed Student’s t tests or non-parametric analysis of variances (ANOVA). A p < 0.05 denotes statistically significant differences. All analysis were done using Prism 3 (GraphPad software, San Diego CA).
3. Results

Antigen Specific Cellular Immune Responses

Epicutaneous immunization has become a viable alternative to the conventional mode of delivery of vaccines, namely intra-muscular injections (Babiuk 2000). Several groups have shown that epicutaneously administered protein or peptide is able to penetrate the skin surface and elicit potent antibody responses (Glenn et al. 1999). Whether optimal cellular responses can be elicited through this route is less well characterized. Cholera toxin, a well-known adjuvant, has been used successfully to enhance mucosal immune responses and shown to have stimulatory effects on DCs (Gagliardi et al. 2002). Tape stripping (TS) prior to immunization performs two functions. First, it removes some of the surface layers of the stratum corneum (Dreher et al. 1998, Marttin et al. 1996) facilitating penetration of the solute applied. Second, TS causes a limited amount of tissue damage and/or distress, causing the release of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα). Hence, one of the main objectives of this thesis was to determine whether a combination of CT and TS could be used to efficiently induce CD4 and CD8 T cell responses following epicutaneous immunization with peptide.

3.1 Epicutaneous immunisation with cholera toxin and tape stripping enhances CD4 T cell proliferation

To characterize the CD4 T cell response to epicutaneously applied peptide we used an adoptive transfer model developed by Mark Jenkins (Kearney et al. 1994, Pape et al. 1997). CD4 T cells were purified from a pool of TCR transgenic DO11.10 spleen and lymph node cells, labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and injected i.v. into naïve Balb/c mice. CFSE was used to track adoptively transferred cells and follow T cell proliferation in vivo. CFSE is a fluorescent marker, which binds to all proteins within the cytosol. The intensity of CFSE is serially diluted as the cellular contents are halved among the daughter cells with each successive cell division. As such it serves as a tool to follow, in vivo, the number of cell divisions undergone by the transferred cells in response to immunization. The CD4 T cell response was characterized on the Balb/c genetic background as these mice are known to mount predominantly Th2 immune responses. The Th2 bias is thought to result from the presence of a higher number of IL-4 producing NK1.1+ T (NKT) cells. In comparison, C57BL/6 mice produce Th1 skewed immune responses as a result of a higher number of IFNγ producing NK and NKT cells (Guler et al. 16)
One day following transfer, the Balb/c chimeric mice were immunized with the class II restricted epitope Ova323 with or without TS the ear skin and with or without the adjuvant CT. The extent of proliferation of the transferred cells in response to the epicutaneous immunization was determined 4 days later by FACS analysis of lymphocytes collected from skin draining LN. Simultaneous staining with the clonotypic TCR specific antibody, KJ1-26, and anti-CD4 monoclonal antibody (mAb) identified the ova-specific transferred TCR transgenic T cells.

The cells that stain with both the anti-TCR antibody, KJ1-26 and anti-CD4 mAb are found in the upper right hand quadrant of the density plots and are indicative of peptide specific clonotypic CD4 T cells (Fig. 1A). The greatest proliferation of peptide specific CD4 T cells was achieved with both TS and CT with a mean of 6.3 ± 1.0% peptide specific CD4 T cells. In comparison peptide immunization with CT alone resulted in 1.3 ± 0.2% (p<0.001) peptide specific CD4 T cells. Similarly, immunization with TS only resulted in 3.0 ± 0.7% (p<0.001) peptide specific CD4 T cells (Fig. 1C). While immunization with Ova323+TS resulted in a greater percentage of peptide specific CD4 T cells than immunization with Ova323+CT this difference was not statistically significant (p>0.05). Hence, optimal proliferation was seen in mice tape stripped and immunized with the adjuvant CT. Adoptively transferred but otherwise un-manipulated mice served as controls. This group showed on average 0.7 ± 0.2% peptide specific CD4 T cells. The CFSE profiles (Fig. 1B) illustrate the percent proliferation undergone by the transferred cells. Each peak to the left indicates a successive cell division, with the right most peak indicative of transferred naïve T cells. The CFSE profile (Fig. 1B) of the group immunized with Ova323+CT+TS shows that more than 90% of the transferred cells are recruited to proliferate in response to immunization with Ova323. In the group immunized with Ova323+TS while 80% of the cells underwent one or more divisions, 20% did not proliferate. Thus, the use of an adjuvant, CT, in epicutaneous immunization allows for a greater number of peptide specific T cells to become activated, enhancing the peptide specific CD4 T cell response by 5 fold relative to immunization with Ova323+TS. In comparison to the control group, Ova323+CT+TS resulted in a 9 fold increase in the number of peptide specific T cells, Ova323+TS resulted in a 4 fold increase and Ova323+CT immunization did not show a significant increase in proliferation above the control (Fig. 1C). Similar results were obtained if 5x10^6 million (Fig. 1D) transgenic T cells were transferred instead of 10x10^6 million cells (Fig. 1C).
Figure 1. Effect of CT and TS on CD4 T cell proliferation

Naive Balb/c mice were immunized with 200 ug of Ova323 ± 25 ug of CT with or without tape stripping one day following i.v. transfer of CFSE labelled DO11.10 transgenic CD4 T cells. Lymphocytes were isolated from skin draining LN 4 days later. A) Peptide specific CD4 T cells were identified by staining with the TCR clonotypic antibody KJ1-26 and anti-CD4 monoclonal antibody and analysed using FACS analysis. Numbers indicate percent of CD4 T cells that are peptide specific. B) Number of cell divisions undergone by Ova323 specific CD4 T cells. Histograms are gated for KJ1-26, CD4 positive T cells (i.e. cells in the upper right hand quadrant in A). Scatter plots show aggregate data from experiments carried out with C) 10x10^6 or D) 5x10^6 million transgenic T cells. Each dot is representative of a single mouse. Nonparametric students t test was used to calculate p values. p < 0.05 denotes statistical significance between groups. Data is representative of three or more independent experiments.
3.2 Cholera toxin and tape stripping enhance CD4 T cell proliferation in part by inducing Langerhans cell emigration

One factor that influences the magnitude of T cell proliferation is the amount of peptide presented in secondary LN organs (Jenkins et al. 2001). Since both CT and TS were found to be effective mechanisms for promoting optimal CD4 T cell responses, we investigated whether their effectiveness as adjuvants might stem in part from effecting LC migration from the epidermis to the LN thus increasing the number of peptide loaded DC in the LN.

The effect of CT and TS on LC migration was tracked using a fluorescent dye, CellTracker. The dye was painted onto the dorsal ear surface of Balb/c mice after TS and/or with CT. The ear draining auricular LN were collected 24 hours later since previous studies have shown peptide to be present in the draining LN by 24 hours after application (Ruedl et al. 2000). Dendritic cells were isolated from LN using Collagenase D digestion and enriched using density centrifugation. Cells in region 1 (R1 in Fig. 2A) were identified as DCs based on their size (FSC) and granularity (SSC). The degree of staining with anti-CD40 and anti-CD11c monoclonal antibodies, two classic markers of DCs, of cells contained in R1 allowed for the identification of three distinct DC subsets as defined by Reudl et al (Fig. 2B) (Ruedl et al. 2000). Only subset III expressed a significant number of CellTracker positive cells (Fig. 2C). The study by Reudl et al defined subset III to be of epidermal origin, specifically LCs, based on the presence of Birbeck granules and expression of E-cadherin, both markers of epidermal LCs. Both CT and TS increased the percentage of CellTracker positive DCs in the draining auricular LN compared to CellTracker applied alone (control) (Fig. 2B & 2C).

3.3 Epicutaneous immunisation with cholera toxin and tape stripping enhances CD8 T cell proliferation

In addition to studying the effect of TS and CT on promoting a CD4 T cell response we also wanted to determine whether these modalities had a similar effect on the CD8 T cell response. Previously, immunization through the skin has been shown to favor the induction of Th2 antibody responses (Wang et al. 1996b). However, both direct (Seo et al. 2000) and indirect (Celluzzi and Falo 1997) evidence suggests that CD8 T cell priming can be accomplished by skin derived antigen-presenting cells.
Figure 2. Effect of CT and TS on LC emigration to the draining lymph nodes in Balb/c A green fluorescent dye, CellTracker, was applied in ETOH onto the dorsal side of each ear with or without tape stripping. In groups that received cholera toxin, the toxin was applied 40 min after CellTracker, also in ETOH. 24 hours later ear draining auricular LN were collected and dendritic cells isolated and purified. A) The size (FSC) and granularity (SSC) of the population (R1) analyzed as DC B) Three different DC subsets (I, II, III) were identified by the degree of staining with anti-CD11c, and anti-CD40 mAb staining. C) Dot plots show percent cell tracker positive DC in subset III. Few cell tracker positive cells were found in the other two subsets (data not shown). D) Bar graph illustrates the mean cell tracker positive DC found in the individual treatment groups with error calculated as standard error of the mean. p < 0.05 denotes statistical significance between groups. Staining in a naive mouse was used to set gates. Data is representative of three or more independent experiments.
We wanted to determine whether 1) CD8 T cell responses could be generated through epicutaneous immunization and 2) CT and TS enhanced the CD8 T cell response. As mentioned earlier, C57BL/6 mice represent an IFNγ rich environment compared to Balb/c mice and therefore are an ideal strain to study CD8 T cell responses. Naïve C57BL/6 (B6) mice were immunized with 25 μg of Ova8 with or without TS and/or CT. Mice were boosted on the contra-lateral ear 7 days later and skin draining LN collected 14 days after the initial priming for enumeration of peptide specific CTL. Ova8 specific CD8 T cells were visualized using cell surface staining with K\(^b\)-Ova tetramer and anti-CD8 mAb. Tetramers consist of 4 peptide/MHC complexes bound to a fluorochrome through a biotin molecule attached to the MHC molecule at the carboxy terminus. In solution the peptide/MHC molecule binds to T cells expressing a TCR specific for the peptide contained within the binding groove of the MHC molecule. Conjugation to a fluorochrome thus allows tetramers to be used as a tool to enumerate peptide specific T cells within a given lymphocyte population (Klenerman et al. 2002). The K\(^b\)-Ova tetramer used herein consists of Ova8/MHC class I molecules and can be used to enumerate Ova8 specific CD8 T cells within the skin draining LN and spleen. Cells staining positive for both the tetramer and CD8 are present in the upper right hand quadrant of the density plot (Fig. 3A) and represent peptide specific CD8 T cells. Immunization with both CT and TS elicits an 11 fold greater response than TS alone and a 3 fold greater response than CT alone. Note also that CT alone resulted in a 4 fold greater response than TS alone (Fig. 3B). Consequently, similar to the effect seen with the CD4 T cell response optimal CD8 proliferation is achieved with both TS and CT. However, unlike the CD4\(^+\) T cell response in Balb/c mice, CT alone significantly enhanced T cell proliferation in C57BL/6.

3.4 IL-12 is not necessary for generation of functional cytotoxic T lymphocytes through epicutaneous immunisation

The fact that CT can be used as an adjuvant to enhance CD8 T cell activation and proliferation is novel given that CT inhibits the production by DCs of a key Th1 promoting cytokine, IL-12 (Braun et al. 1999). IL-12 is known to promote Th1 responses by stimulating naïve CD4 T cells to differentiate into effector Th1 cells capable of secreting IFNγ. The absence of IL-12 allows Th2 cytokines to be up-regulated (Trinchieri and Scott 1999) and thus CT has traditionally been associated with promoting Th2 responses. The results presented in Fig. 3 suggest that CT can also promote Th1 responses. We therefore wanted to determine the importance of IL-12 signalling in eliciting CD8 T cell responses.
Figure 3. Effect of CT and TS on CD8 T cell proliferation

Naïve C57BL/6 mice were primed and boosted 7 days later on the contra-lateral ear with 25 μg of Ova8 +/- 25 μg of cholera toxin with or without tape stripping. Lymphocytes from skin draining LN were collected 14 days after priming and peptide specific CD8 T cells enumerated by A) simultaneous staining with Kb-Ova tetramer and anti-CD8 monoclonal antibody and FACS analysis. Numbers in density plots indicate percent of CD8 T cells that are Ova8 specific. B) Scatter plot shows aggregate data from 3 independent experiments. Each dot is representative of a single mouse. Nonparametric students t test was used to calculate p values. p < 0.05 denotes statistical significance between groups. Data is representative of three independent experiments.
To do so naïve B6 and IL-12p40ko mice were immunised in a manner similar to that described in Fig. 3. Mice in each group received 25 μg Ova8 and 25 μg of CT during the prime and boost after TS. Staining with the multimeric MHC complex and anti-CD8 mAb revealed that IL-12p40ko mice achieve smaller numbers of activated peptide specific CD8 T cells compared to wild type B6 mice (Fig. 4A). However, data on the functional status of the cells, (Fig. 4C), illustrates that CD8 T cells from IL-12p40ko mice are just as efficient at killing target cells expressing Ova8 as CD8 T cells primed in wild type mice. Both groups are able to kill just over 50% of the target cells at the highest effector to target ratio. Thus, IL-12 is not required for functional CTL generation through the skin but is necessary for optimal CD8 T cell responses as it increases the number of activated CTL.

3.5 Cholera toxin and tape stripping do not affect dendritic cell emigration in C57BL/6

Since TS and CT are also able to optimize CD8 T cell proliferation in C57BL/6 we wanted to determine whether CT and TS affected DC immigration in a manner similar to that observed in Balb/c (Fig. 2). The study was carried out in the same way as described for Balb/c above only using C57BL/6 mice. Cells found in R1 (Fig. 5A) were analyzed for expression of CD40, CD11c and CellTracker. Similar to the findings in Balb/c mice, three distinct DC subsets were identified based on the degree of staining with anti-CD11c and anti-CD40 mAbs (Fig. 5B). When these subsets were analyzed for CellTracker expression both subset II (Fig. 5D) and subset III (Fig. 5C) expressed a significant percentage of CellTracker positive cells. This is in contrast to the results obtained in the Balb/c where only subset III (Fig. 2A) was found to have significant expression of CellTracker. These results indicate that, in B6, two distinct skin derived APC might be migrating to the draining LN as opposed to one APC type in the Balb/c. Also, in contrast to the results obtained in the Balb/c, a much higher percentage of CellTracker positive cells in subsets II and III were found in the control group of B6 mice suggests a higher basal rate of emigration. CT and TS did not enhance DC emigration above this basal rate (Figs. 5C and 5D). To ensure that DC migration above control levels could be achieved in B6 mice, CellTracker was applied along with a chemical irritant, DBT. DBT strongly induced CellTracker positive cells to migrate to the draining LN (Fig. 5C and 5D). Thus, the lack of effect of CT and TS on promoting DC migration above control levels in B6 mice is not due to an inherent lack of response of skin DCs in B6 mice to epicutaneous stimuli. Hence, the stimulatory effects of CT and TS on CD8 T cell proliferation in B6 mice stem from effects other than enhanced DC emigration.
Figure 4. Role of IL-12 in inducing functional CTL through epicutaneous immunization Naïve C57BL/6 or IL-12p40ko mice were primed and boosted 7 days later on the contra-lateral ear with 25 ug of Ova8 + 25 ug of cholera toxin after tape stripping. Lymphocytes from skin draining LN were collected 14 days after priming and peptide specific CD8 T cells enumerated by A) simultaneous staining with Kb-Ova tetramer and anti-CD8 monoclonal antibody and FACS analysis. Numbers in density plots indicate percent of CD8 T cells that are Ova8 specific. B) Scatter plot shows aggregate data from two or more independent experiments. Each dot is representative of a single mouse. Nonparametric students t test was used to calculate p values. p < 0.05 denotes statistical significance between groups. C) The ability of peptide specific CD8 T cells to lyse target cells expressing Ova8 was determined in a standard Cr release assay using the Ova8 expressing EG7 cell line as target cells. Cells were plated at three different effector to target ratio’s. Dots represent mean of triplicate values and error bars were calculated as standard error of the mean. Data is representative of two independent experiments.
Figure 5. Effect of CT and TS on LC emigration to the draining lymph nodes in C57BL/6 A green fluorescent dye, CellTracker, was applied in ETOH onto the dorsal side of each ear with or without tape stripping. In groups that received cholera toxin, the toxin was applied 40 min after CellTracker, also in ETOH. 24 hours later ear draining auricular LN were collected and dendritic cells isolated and purified. A) The size (FSC) and granularity (SSC) of the population (R1) analyzed as DC B) Three different DC subsets (I, II, III) were identified by the degree of staining with anti-CD11c, and anti-CD40 mAb staining. Bar graphs show the mean CellTracker positive cells found in C) subset III and D) subset II with error bars indicating standard error of the mean. Few CellTracker positive cells were found in subset I (data not shown). Staining in a naive mouse was used to set gates. Data is representative of three or more independent experiments.
Innate Immunity

Results presented thus far demonstrate that CT and TS work well as adjuvants to enhance the adaptive immune response to epicutaneously administered peptide. As adjuvants, CT and TS may function to release danger signals at the site of application resulting in activation of resident APC (Bendelac and Medzhitov 2002). In the above experiments we showed that CT and TS are both effective at inducing DC emigration to the tissue draining LN in Balb/c, an action restricted to activated DC. As such, we wanted to explore specific immune mechanisms that may be induced by TS to support DC activation.

Tape stripping is already known to activate epidermal Langerhans cells through the release of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα) (Ozawa et al. 1996) within the epidermis (Enk et al. 1993, Wood et al. 1997), that function as endogenous danger signals (Gallucci and Matzinger 2001). Another key mechanism involved in activating immune responses is signaling through TLRs (Medzhitov 2001, Medzhitov and Janeway 2000). Several endogenous ligands that signal through TLR4 have been identified including heparin sulfate (Johnson et al. 2002), hyaluronic acid (Termeer et al. 2002), fibronectin (Okamura et al. 2001) and heat shock proteins (Asea et al. 2002, Breloer et al. 2001, Bulut et al. 2002, Ohashi et al. 2000). Expression of many of these ligands is associated with tissue injury and or cell stress (Asea et al. 2002, Johnson et al. 2002, Okamura et al. 2001, Termeer et al. 2002). We hypothesized that TS may trigger the release of some or all of these TLR4 ligands by inducing injury to the epidermis. In addition to its potential importance in inflamed or distressed tissues, TLR4 signaling has also been found to be important for induction of Th2 immune responses to inhaled allergens in the lung mucosa. The expression of TLR4 on epidermis resident LCs and KCs (Curry et al. 2003, Song et al. 2002) suggests that TLR4 signaling may play a role in cutaneous immune responses. Thus, we wanted to determine whether cellular immune responses mounted to ECI with peptide plus adjuvant relied on TLR4 signaling.

3.6 Antibody responses to epicutaneously administered protein do not require TLR4 signalling

To study the role of TLR4 signaling in inducing humoral responses to ECI, the antibody response in wild type Balb/c mice was compared to that achieved in TLR4 deficient Balb/c mice (C.C3H/HeJ). This strain contains a proline to histidine mis-sense point mutation at position 712 in the cytoplasmic domain of the tlr4 gene. The mutation results in endotoxin
Figure 6. Role of TLR4 signaling in humoral responses to epicutaneous immunization Wild type and TLR4<sup>def</sup> mice were immunised with 200 ug ovalbumin protein plus 25 ug cholera toxin after tape stripping. Mice were primed and boosted 14 days later on the contralateral ear. Three weeks after priming serum was collected and assayed for Ova specific antibodies using ELISA. Each dot is representative of a single mouse. Scatter plots show aggregate data from 2 independent experiments.
hyporesponsiveness due to defect signaling transduction. The C.C3H/HeJ strain has been used successfully by several groups to study the effects resulting from a lack of TLR4 signaling in various experimental models (Dabbagh et al. 2002, Eisenbarth et al. 2002, Guillot et al. 2002, Haeberle et al. 2002, Rescigno et al. 2002). Mice were immunized with 200 µg of full Ova protein plus 25 µg of CT after TS. Mice were boosted 14 days later on the contra-lateral ear. Three weeks after priming serum was collected and assayed for Ova specific antibodies. Ova specific IgG1, IgE, IgG and IgG2a titres were assayed and found to be equivalently induced in both wild type and TLR4 deficient mice (Fig. 6). Thus, TLR4 signaling does not appear to be required for induction of humoral responses to epicutaneously administered protein.

3.7 Cellular responses to epicutaneously administered peptide do not require TLR4 signalling

Participation of TLR4 signaling in cellular responses was next characterized. The CD4 T cell response was studied using the adoptive transfer system described above. Antibody staining and FACS analysis revealed little difference in peptide specific CD4 T cell proliferation between wild type and TLR4 deficient Balb/c mice (Fig. 7A). The mean peptide specific CD4 T cell frequency in wild type and TLR4 deficient Balb/c mice was 3.1 ± 1.3% and 3.4 ± 1.4% respectively (Fig. 7B).

The CD8 T cell response was characterized using the wild type C57BL/10ScSn (B10) and TLR4 knockout C57BL/10ScNCr strains. The response was characterized using the immunization described above for B6 (Fig. 3) as well as an adoptive transfer system similar to the one described for the CD4 T cell response. In the adoptive transfer model, OT1.1 TCR transgenic mice recognizing Ova8 were adoptively transferred into naïve C57BL/6 mice that were subsequently immunized with 25 µg each of Ova8 and CT subsequent to TS. Responses in skin draining LN were characterized 3 days post immunization. Enumeration of peptide specific CD8 T cells in both systems was accomplished by simultaneous staining with Kb-Ova tetramer and anti-CD8 monoclonal antibody. In the adoptive transfer system (Fig. 8A and 8B) 22.2 ± 1.3% and 18.7 ± 2.6% peptide specific CD8 T cells were identified in wild type and TLR4 knockout mice respectively. In mice immunized without adoptive transfer (Fig. 8C) 0.41 ± 0.07% and 0.61 ± 0.23% peptide specific CD8 T cells were identified in wild type and TLR4 knockout mice respectively. Thus, in both systems similar responses were achieved in WT and TLR4 knockout mice. As such TLR4 signaling does not appear to be activated in response to tissue injury.
mediated through TS and does not play a role in enhancing humoral or cellular immune responses to ECI with peptide plus adjuvant.
Figure 7. Role of TLR4 signaling in CD4 T cell responses to epicutaneous immunization Naive wild type and TLR4^{def} mice were immunised with 200 μg of Ova323 plus 25 μg of CT after tape stripping one day following i.v. transfer of 5x10^6 CFSE labelled DO11.10 transgenic CD4 T cells. Lymphocytes were isolated from skin draining LN 4 days later. A) Peptide specific CD4 T cells were identified by staining with the TCR clonotypic antibody KJ1-26 and anti-CD4 monoclonal antibody and analysed using FACS. Numbers in density plots indicate percent of CD4 T cells that are peptide specific. B) Scatter plots show aggregate data from 6 independent experiments. Each dot is representative of a single mouse. Nonparametric students t test was used to calculate p values. p < 0.05 denotes statistical significance between groups.
Figure 8. Role of TLR4 signaling in CD8 T cell responses to epicutaneous immunization. A) Naive C57BL/10ScSn (wild type) and C57BL/10ScNCr (TLR4<sup>lps-del</sup>) mice were immunised with 25 µg of Ova8 plus 25 µg of CT after tape stripping one day following i.v. transfer of 5x10<sup>6</sup> CFSE labelled OT1.1 transgenic CD8 T cells. Lymphocytes were isolated from skin draining LN 3 days later. Peptide specific CD8 T cells were identified by staining with the Kb-Ova tetramer and anti-CD8 monoclonal antibody and analysed using fluorescence activated cell sorting (FACS). Numbers in density plots indicate percent of CD8 T cells that are peptide specific. B) Bar graph illustrates the mean peptide specific CD8 T cells in the two strains with error bars calculated as standard error of the mean. Nonparametric students t test was used to calculate p values. p < 0.05 denotes statistical significance between groups. Data is representative of two independent experiments. C) Naive Naive C57BL/10ScSn (wild type) and C57BL/10ScNCr (TLR4<sup>lps-del</sup>) mice were primed and boosted 7 days later on the contra-lateral ear with 25 µg of Ova8 plus 25 µg of cholera toxin after tape stripping. Lymphocytes from skin draining LN were collected 14 days after priming and peptide specific CD8 T cells enumerated by simultaneous staining with Kb-Ova tetramer and anti-CD8 monoclonal antibody and FACS analysis. Numbers in dot plots indicate percent of CD8 T cells that are Ova8 specific. Data is representative of two independent experiments.
4. Discussion

The objective of this study was to characterise the role of CT and TS in inducing optimal cellular immune responses to peptide applied epicutaneously. The findings of this thesis are as follows: 1) TS and CT differentially promote CD4 and CD8 T cell proliferation, and induce optimal proliferation in both T cell subsets when employed together, 2) CT and TS stimulate LC emigration in Balb/c mice, 3) IL-12 is not required for the generation of functional CTL through the skin, and 4) TLR4 signalling is not involved in adaptive immune responses elicited through epicutaneous immunisation (ECI) using TS and/or CT.

4.1 Epicutaneous route of administration is effective at promoting a cellular response

Type of responses characterised in the literature using ECI

Humoral Immune Response

Epicutaneous immunization has garnered attention and study over the past few years, as the immuno-stimulatory capacity of the skin becomes clear (Glenn et al. 1998a, Hogan and Burks 1995, Partidos et al. 2001, Williams and Kupper 1996). Most of the studies that have explored epicutaneous immunization have looked at the antibody response as a measure of activation of the immune system. It is now well established that antigens painted onto the skin surface can elicit protective antibody responses (Beignon et al. 2001, Glenn et al. 1998b, Scharton-Kersten et al. 1999). This has been shown mainly with the immunogen CT, which is able to elicit mucosal and systemic antibody responses when applied to the skin (Chen et al. 2000). Furthermore, the antibodies generated are functional as they afford protection against a lethal intranasal challenge of CT (Glenn et al. 1998b). The skin can also be used to elicit a humoral response against non-immunogenic protein peptides by co-administering the peptide with an adjuvant (Beignon et al. 2001). Humoral responses to a number of different antigens have been generated using a variety of different adjuvants, such as CT (Glenn et al. 1999), LT (Beignon et al. 2001), and CpG (Beignon et al. 2002) sequences through the skin. In addition to eliciting an antibody response epicutaneous immunization has been found to generate a wider variety of antibodies as compared to the oral route (Glenn et al. 1998b, John et al. 2002).

Cellular Immune Response

In contrast to activation of humoral immunity through epicutaneous immunization not many studies have determined whether cellular immunity can also be effectively activated through the
skin. In fact no study has looked directly at in vivo activation of CD4 T cells in response to peptide + adjuvant immunization through the skin. The few studies that do characterize a CD4 T cell response have done so by indirect methods, such as assaying for antigen specific proliferation of in vivo primed spleen cells upon in vitro re-stimulation. In addition, these studies look at peptide specific cytokine production, a feature characteristic of activated effector CD4 T cells (Hammond et al. 2001). The CD8 T cell response is even less well characterized than the CD4 T cell response with only one study showing CTL induction in response to percutaneous peptide application (Seo et al. 2000). Hence, one of the primary objectives of this study was to determine whether CT and TS could promote the induction of cellular immune responses to ECI.

**Results of this study**

As depicted in Figs. 1 and 3 epicutaneous immunization with TS and CT stimulated an increase in peptide specific CD8 and CD4 T cell numbers in the skin draining LN.

**The CD8 response achieved through ECI**

**Conventional versus non-conventional antigen presentation**

The finding that peptide plus adjuvant administered directly onto skin is effective at eliciting CD8 T cell immunity is important given the segregation of peptide processing into class I and class II pathways. Given this division in processing pathways it is surprising that peptide administered epicutaneously and thus destined for the exogenous processing pathway was able to stimulate CD8 T cell responses that are restricted by MHC class I molecules. A phenomenon by which exogenous peptides and/or proteins can gain access to the class I processing pathway and subsequently be expressed in complex with MHC class I molecules by APC is termed cross-presentation (Banchereau et al. 2000). Several studies have shown that proteins and large peptide fragments can be processed in this manner resulting in CD8 T cell cross priming (Belz et al. 2002, Nelson et al. 2000). As illustrated in Fig. 3, ECI is able to illicit CD8 T cell responses to peptide antigen suggesting that either cross-presentation (that is the internalization of the peptide antigen – with subsequent Class I MHC restricted display) is responsible for the presentation of Ova8 in the context of MHC class I molecules on DC surfaces or that other mechanisms are involved. *In vitro* studies suggest that DCs pulsed with peptide are able to present the peptide without internalization (Brossart and Bevan 1997, Celluzzi et al. 1996, Porgador and Gilboa 1995). It is suspected that soluble free floating extracellular peptide is able to directly bind either
empty MHC class I molecules displayed on DC surfaces or displace lower avidity peptides from the MHC molecule (Celis 2002). This phenomenon has been described for both bone marrow derived DCs (Porgador and Gilboa 1995) as well as epidermal DCs (i.e. LCs) (Celluzzi and Falo 1997). This process has been described not only for peptides that require no cleavage of extra amino acid residues but also for peptides that require removal of a few amino acid residues. These later peptides may be processed at the cell surface by cell surface associated proteases (Celis 2002). Although cell surface antigen loading has not been demonstrated in in vivo models it is possible that soluble peptides that are able to elicit CD8 T cell responses may be presented in just such a manner. Ova8 peptide utilized in Fig. 3 is an 8 a.a. long peptide that has a demonstrated avidity for the H-2K\textsuperscript{b} MHC class I molecule. Thus, it is formally possible that MHC class I molecules on epidermal resident LCs are loaded directly with Ova8 without prior peptide internalization and/or processing. Whether true internalization and cross-presentation occurs or exogenous peptide loading occurs remains to be determined.

Potential pitfall of peptide based immunisation

The fact that small peptides can be applied directly to the skin surface and successfully stimulate CD8 T cell immune responses offers a promising alternative to current vaccines which employ attenuated or killed whole micro-organisms to elicit immune responses. ECI with tumor derived or viral peptides could be employed to stimulate potent CTL that will recognize and kill appropriate target cells. However, if the main mechanism through which peptides contained in vaccine formulations are acquired by APC is through direct loading of MHC molecules on cell surfaces, then the peptide used must be chosen with care. Specifically, synthetically prepared peptides must resemble the epitopes presented by infected cells through the conventional intracellular class I processing pathway. In other words, peptide vaccines must stimulate CTL capable of recognizing the physiologically relevant target epitope of a given protein or peptide (Celis 2002). The Ova8 peptide used in Fig. 3 meets this requirement since the same epitope is generated by in vivo processing of the whole protein and expressed by the ovalbumin transfected tumor cell line, EG7.
4.2 Cholera toxin and tape stripping are potent adjuvants for the induction of cellular immunity to epicutaneous immunization

Use of CT as an adjuvant in mucosal immunity

Oral Route

The use of adjuvants in vaccine formulations is important because they provide danger signals that serve to activate DCs resulting in the activation of adaptive immunity. The gram-negative bacterial enterotoxin CT is among the most potent adjuvant so far characterized. The potency of CT was demonstrated most convincingly by its ability to abrogate immune tolerance to ingested antigens. It is believed that antigens, including orally ingested food antigens, which make contact with the gut mucosal immune system elicit tolerance so as to prevent elicitation of an immune response against foreign but non-harmful food antigens (Mayer et al. 2001, Strobel and Mowat 1998). In 1984 Elson et al demonstrated that keyhole limpet hemocyanin (KLH) antigen when co-administered orally with CT was able to elicit anti-KLH IgA antibodies (Elson and Ealding 1984). In fact, nanogram amounts of the toxin can be used to induce activation of gut immunity. This is important because clinical systems associated with this toxin, including diarrhea are initiated upon ingestion of microgram amounts thus making it possible to use CT as an adjuvant while avoiding the harmful effects of the toxin (Lycke and Holmgren 1986). In addition to activating antigen specific gut immunity, CT has also been used to enhance anti-viral immune responses. It has been used to enhance antibody responses to the influenza virus as well as to increase protective anti-viral responses to Sendai virus in the respiratory tract through oral co-administration (Chen and Strober 1990, Liang et al. 1988). Another feature that demonstrates the potency of CT as an adjuvant is its ability to promote long-term (22 months) memory antibody responses to orally administered antigens (Vajdy and Lycke 1992).

Interperitoneal and rectal route

Cholera toxin has also been shown to be an effective adjuvant when given intraperitoneally or intrarectally. It’s potency as an adjuvant has spurred an interest to use it to elicit protective immune responses to the human immuno-deficiency virus (HIV), one of the greatest medical challenges of the 21st century. Intrarectal immunization with an HIV peptide plus CT has been shown to induce CTL in secondary organs such as Peyers patches, lamina propria, and the spleen. More importantly, mice immunized as such were protected against an intrarectal challenge with a recombinant vaccinia virus expressing the HIV-III B gp160 glycoprotein (Belyakov et al. 1998). Thus, for the most part, the adjuvant like properties of CT have been
established primarily in relation to mucosal immunity and antibody responses.

Transcutaneous Route
More recently, it has been shown that transcutaneous administration of CT elicits protective antitoxin IgG, IgA, IgG1, IgG2a, IgG2b, and IgG3a antibodies (Glenn et al. 1998b). Whether it can also augment cellular immunity to epicutaneously administered antigens remains to be characterized.

Class of immune response associated with CT
In addition to eliciting a humoral immune response, CT also affects the polarization of that response. Adaptive immune responses can be polarized as either Th1 or Th2. Both types of responses are characterized by signature cytokines and effector mechanisms. With respect to CT, the humoral responses induced either in the mucosa or systemically have found to be Th2 dominated, as illustrated by the induction of Th2 antibodies such as slgA, IgG1, IgE, IgG2b (Pape et al. 1997, Xu-Amano et al. 1993). Th1 antibodies such as IgG2a are not as strongly induced in response to CT immunization. Immunization with CT is also reported to enhance IL-4, and IL-5 production by CD4 T helper cells, a feature which further supports the characterization of CT in the literature as a Th2 promoting adjuvant (Gagliardi et al. 2000, Hammond et al. 2001).

Tape-stripping as a method to enhance immune responses
In addition to using a traditional adjuvant such as CT, another mechanism employed in this thesis to enhance adaptive immune responses was to tape strip the ear skin prior to immunization. Although not an adjuvant in the traditional sense of the word, TS has been shown to enhance certain types of responses. Nishyima et al studied the effect of TS on a model cutaneous immune reaction, skin contact sensitization. Contact sensitization of the ear skin with chemical sensitizers such as DNFB resulted in significant ear swelling upon re-challenge. The effector mechanisms considered responsible for this immune response are secretion of pro-inflammatory cytokines and infiltration by activated CD8 T cells. Tape stripping of the skin prior to exposure to DNFB resulted in a greater degree of ear swelling, suggesting that TS augments the effector mechanisms elicited by contact sensitization (Nishijima et al. 1997). Also, in vitro studies with Langerhans cells isolated from tape stripped epidermis show these cells to be able to stimulate syngeneic and allogeneic T cell proliferative responses, suggesting the acquisition of an antigen presentation function by tape stripped LCs (Katoh et al. 1997, Nishijima et al. 1997).
Results of this study

Given the well-defined adjuvant role of CT and to a lesser extent TS, we employed both mechanisms in our immunization protocol. While TS has been shown to affect skin related immune responses, no studies have looked at whether CT could be used to enhance CD8 T cell responses to epicutaneously co-administered peptide. The results of this thesis show that CT and TS when used together can enhance both the CD4 and the CD8 T cell response after ECI with the appropriate peptides (Figs. 1 and 3).

Cholera toxin and CD8\(^+\) T cell responses

As discussed above CT has traditionally been found to promote Th2 immunity. Given this, it was novel to find that it could also promote CD8 T cell responses, a prototypic Th1 response (Szabo et al. 2003) when administered epicutaneously. As illustrated in Fig. 3, while the greatest CD8 response was seen in the group treated with CT and TS, CT alone was also able to elicit a response, albeit two fold smaller. The ability of CT to promote Th1 immunity calls into question the classification of CT as a solely Th2 promoting adjuvant. The primary mechanism by which CT is thought to selectively promote Th2 immune responses is through its ability to inhibit IL-12 production by DCs (Braun et al. 1999). IL-12 is considered to be the key cytokine responsible for switching on Th1 immunity, while at the same time actively suppressing the induction of Th2 immunity by shutting off Th2 promoting cytokine production (Gately et al. 1998).

However, research over the last few years has questioned the absolute necessity of IL-12 for stimulating CD8 T cell responses (Piccotti et al. 1998, Wan et al. 2001). Studies have also investigated the effect of IL-12 deficiency on the hosts’ ability to fight off viral infections. IL-12p40 knockout mice infected with mouse hepatitis virus show minimal liver damage and produce polarized Th1 cytokine responses (Schijns et al. 1998). Infection with lymphocytic chronomeningitis or stomatitis virus also stimulates Th1 cytokine responses by CD4 T effector cells isolated from the spleen. Furthermore, IL-12 deficient mice are protected from a challenge immunization with the virus (Oxenius et al. 1999). The lack of necessity of IL-12 for Th1 immune responses has also been illustrated for an autoimmune disease, specifically type I diabetes. Type I diabetes is an auto-immune disease characterized by the destruction of the insulin producing beta cells in the pancreas by the body’s own cytotoxic CD8 T cells. Trembleau et al showed that type I diabetes onset was not delayed in IL-12 deficient NOD mice, hence
illustrating that IL-12 deficiency did not prevent host CD8 T cells from becoming activated to beta cell specific antigens (Trembleau et al. 1999). Results presented in Fig 4 extend these findings to epicutaneous immunization with peptide plus adjuvant. IL-12p40 knockout mice developed a reduced but functionally active effector CD8 T cell response to ECl, as illustrated by their ability to lyse target cells, which was on par with the percent lysis achieved in wild type mice (Fig 4C). Thus, although IL-12 is required for optimal Th1 immune responses, its lack does not prevent the generation of functionally active Th1 immunity.

This indicates that other mechanisms, possibly other cytokines, are able to promote Th1 immune responses compensating for a deficiency in IL-12 production. The ability of IL-12 to promote Th1 immunity results from stimulating activated CD4 helper T cells to produce IFNγ. Until recently IL-12 was recognized as the only cytokine able to initiate IFNγ secretion. More recently, other cytokines such as IL-15 (Carson et al. 1995), IL-18 (Curry et al. 2003, Freudenberg et al. 2002) and type I interferons have been shown to stimulate IFNγ production. It remains to be determined whether any of these cytokines are present in the microenvironment of the epidermis in response to CT and or TS. The fact that CTL were achieved with CT alone and enhanced with TS suggests they may play a role. This theory is supported by a recent study which illustrated that CT treated bone marrow (BM) derived DCs were able to enhance IFNγ production by CD4 helper T cells (Jang et al. 2003). Therefore, the fact that CT inhibits IL-12 production from DCs does not necessarily disqualify it from promoting Th1 immune responses.

The toxin’s role in affecting CTL induction is further strengthened by studies which have shown it to be an effective adjuvant for promoting HIV specific CTL in mucosal immunity. Porgador et al used intranasal immunization with a class I epitope of HIV-1 glycoprotein 120 plus CT. Splenocytes recovered from these mice contained HIV specific CTL (Porgador et al. 1997). Belyakov et al used an intrarectal route of administration of multideterminant HIV peptides plus CT and demonstrated the presence of CTL recognizing HIV epitopes in Peyers patches, lamina propria, as well as the spleen. Furthermore, the immunization was found to be immunologically effective as it protected mice from an intrarectal challenge with a vaccinia virus expressing HIV-1 IIIB glycoprotein 160 (Belyakov et al. 1998). More recently, the effect of CT treatment on the type of response stimulated by BM derived DCs was investigated. It was found that CT treatment increased DC cell surface expression of B7.1, a co-stimulatory molecule believed to deliver signals important for the acquisition of effector function by activated CD8 T cells. In addition,
treated DCs from Balb/c mice were able to stimulate CTL induction in vitro when cultured with allogeneic spleen cells (Jang et al. 2003). The results presented in Fig 3 thus extend our knowledge regarding the adjuvant like properties of CT by demonstrating that 1) it can be used to enhance CTL through the epicutaneous route of immunization in addition to the mucosal route and 2) it may not be an adjuvant that exclusively enhances type 2 responses but may also effect Th1 responses, specifically by promoting CTL generation. A mechanism important for CTL induction by CT may involve increased cyclic adenosine monophosphate (cAMP) levels. Elevated cAMP levels are seen only as a result of treatment with the holoenzyme. Treatment with subunit B does not result in increased cAMP levels and immunization with peptide + subunit B fails to prime naïve CD8 T cells (Kahlon et al. 2003).

4.3 Cholera toxin and tape stripping enhance cellular immune responses partly by affecting dendritic cell migration

Having established that both TS and CT are effective means of enhancing cellular immunity to epicutaneously administered peptides we next wanted to explore the underlying mechanisms responsible for their adjuvant effects. Most adjuvants enhance adaptive immunity by stimulating the effector mechanisms of innate immunity. Two important effector mechanisms associated with innate immunity are release of pro-inflammatory cytokines and up-regulation of certain cell surface molecules on APC. Both mechanisms function to activate APC thus initiating adaptive immunity.

*Putative mechanisms of action of CT as an adjuvant*

Up-regulation of co-stimulatory molecules

Some mechanisms of action of CT as an immune adjuvant have already been elucidated. BM macrophages treated with CT up regulate expression of B7.2, an important co-stimulatory molecule (Cong et al. 1997). Human monocyte derived DCs up-regulate expression of MHC class II, B7.1, B7.2, CCR7 and CXCR4 in response to CT (Gagliardi et al. 2000, Gagliardi et al. 2002). Finally, as mentioned earlier, BM DCs treated with CT show increased B7.1 expression (Jang et al. 2003). Since these are signature molecules for and expressed by activated APC the potency of CT as an adjuvant may in part be explained by its ability to enhance their expression.
Effect on antigen processing and cytokine production

The maturation signals delivered by CT seem to be limited to its ability to up-regulate key antigens on APC surfaces. It has not been found to activate the other effector mechanism of innate immunity, namely inflammatory cytokine production. Indeed, unlike most conventional adjuvants, which promote secretion of pro-inflammatory cytokines, CT is decidedly anti-inflammatory. It does not induce the production of the cytokines IL-6, IL-10, IL-12 or TNFα. Moreover, it inhibits production of IL-12 and TNFα (Gagliardi et al. 2002).

Previous knowledge regarding the mechanism of action of TS

Effect on antigen presentation and expression of co-stimulatory molecules

As mentioned earlier, although not an adjuvant in the conventional sense of the word, TS has been shown to promote certain immune responses. While not used extensively in the literature to enhance adaptive immunity, TS has demonstrated effects on APC activation and maturation. Langerhans cells isolated from tape stripped epidermal tissue show increased expression of MHC class II molecules, B7.2, and ICAM-1 (Katoh et al. 1997).

Release of pro-inflammatory cytokines

The most significant contribution of TS in terms of immune activation is its ability to enhance the secretion of key pro-inflammatory cytokines within the epidermis and to a lesser extent the dermis. Tape stripping has been shown to increase levels of the cytokines IL-1α, TNFα, IL-1β, and IL-6 within murine epidermis (Wood et al. 1996, Wood et al. 1997). In humans TS elicits the production of all of the above cytokines plus IL-8, IL-10, TGFα, TGFβ, and IFNγ (Nickoloff and Naidu 1994). Recent literature suggests that the presence of cytokines in the microenvironment where APC are actively taking up and processing antigen and undergoing maturation may dictate the type of adaptive immune response stimulated in the secondary lymphoid organs in terms of Th1 or Th2 immunity (Vieira et al. 2000). Thus, the fact that TS induces the expression of important Th1 promoting cytokines such as IL-12 and IFNγ within the epidermis may in part explain its ability to enhance CT stimulated CD8 T cell responses (Fig 3) in the skin draining LN. This hypothesis is confirmed by the reduced number of CD8 T cells stimulated in IL12p40ko mice (Fig. 4).
Results of this study

CT and TS differentially effect DC migration in the two strains

Since one limiting factor in the number of naïve T cells which become activated in tissue draining LN is the number of APC available to present antigen, it stands to reason that mechanisms designed to enhance DC migration to the DLN would enhance adaptive immune responses. As such, DC migration may be another innate mechanism by which adjuvants enhance adaptive immunity. Since both CT and TS enhanced CD4 and CD8 T cell responses we investigated the ability of these two adjuvants to enhance DC migration. We found that both CT and TS enhanced the number of CellTracker (antigen) positive LCs within the ear draining auricular LN of Balb/c mice (Fig 2). CT was observed to provide a somewhat stronger stimulus for DC migration than TS although the difference was not statistically significant. In contrast, neither CT nor TS enhanced DC migration above the control group in B6 mice (Fig 5). However, in contrast to Balb/c mice, basal levels of DC migration were found to be higher in B6 mice. Furthermore, LN of B6 mice showed two distinct populations of migratory DCs as opposed to one population identified in Balb/c (Fig 2 and Fig 5).

Possible molecular events responsible for the induction of LC migration by TS and CT in Balb/c

Tape stripping

The fact that TS is able to stimulate LC migration in Balb/c mice is supported by the immunostimulatory effects associated with TS within the literature. Langerhans cell emigration from the epidermis is known to require signaling by IL-1α, TNFα and IL-1β (Cumberbatch et al. 1997, Cumberbatch et al. 1999a, Cumberbatch et al. 1999b, Cumberbatch and Kimber 1995, Wang et al. 1996a). All three of these cytokines are up regulated in the murine epidermis following TS (Nickoloff and Naidu 1994, Wood et al. 1996, Wood et al. 1997). Thus, the pro-inflammatory cytokines released upon TS would allow one to predict just such an effect on LC migration. This is the first study to formally demonstrate the initiation of LC movement in response to TS.

Cholera Toxin

The ability of CT to also promote LC migration in the Balb/c can be attributed to its effect on two essential chemokine receptors. CT treated DCs show increased expression of CCR7 and CXCR4 (Gagliardi and De Magistris 2003, Gagliardi et al. 2000, Gagliardi et al. 2002). CCR7 is a receptor expressed by activated APC that allows the cell to respond to a chemokine gradient
attracting DCs to the secondary lymphoid organs, including LN. CXCR4 is more important for directing DC movement within the LN in that it promotes DC localization within the T cell rich zones of the LN (Rossi and Zlotnik 2000). Thus, in addition to the characterized effects of CT, this thesis has demonstrated for the first time that CT can also enhance LC migration. During the course of writing this thesis one study was published that showed a similar affect of CT on the movement DCs from the subepithelial layer to Peyer’s patches (Shreedhar et al. 2003). Thus, in addition to the characterized mechanisms of action attributed to CT and TS in the literature, the findings in this thesis demonstrate for the first time a novel mechanism through which they both act, namely the induction of LC emigration in Balb/c mice. An enhanced baseline emigration rate of epidermal and dermal DCs in C57BL/6 mice when compared to Balb/c mice was an unexpected finding in my work. This enhanced baseline migration may have limited our ability to detect the effect of CT and TS on this parameter. Strain-related differences in DC behavior are a newly recognized phenomenon. A recent study suggests that differences in DC behavior between the two strains may in part reflect differences in TLR expression on DCs (Liu et al. 2002). The study confirmed that DCs isolated from C57BL/6 mice expressed TLR9 while those from Balb/c mice showed increased levels of TLR2, 4, 5 and 6. They reasoned that these differences were responsible in part for the distinct cytokines produced by DCs from the two different strains upon stimulation with the same microbial stimuli. In my study both CT and TS are believed to provide danger signals that potentially signal through TLRs. As such, it is possible that CT and TS effect DC emigration differently in the two strains due in part to differences in TLR expression on skin resident DCs in C57BL/6 and Balb/c mice.

**Differential DC subset migration in C57BL/6 and Balb/c**

An interesting observation with regards to DC migration in the C57BL/6 mouse, in addition to the apparent lack of effect of CT and TS, was the presence of two distinct migratory DC populations in the draining LN (Fig. 5A). The two populations can be defined as CD40^{hi}CD11c^{hi} (population III Fig. 5A) and CD40^{hi}CD11c^{int} (population II Fig 5A) based on the classification established by Reudl et al (Ruedl et al. 2000). In their study Ruedl et al postulate that the former population consists of Langerhans cells originating from the epidermis while the latter population houses DCs of dermal origin. The presence of two distinct DC populations in the draining LN could be significant taking into account studies that illustrate that different DC subsets promote CD4 and CD8 T cell responses differentially (Constant et al. 1995, Ingulli et al. 2002, Pooley et al. 2001, Smith et al. 2003). A fluorochrome labelled CD40^{hi}CD11c^{int} population
of DCs was present in the ear draining LN of C57BL/6 mice (the strain used by us to study the CD8 T cell response) but not Balb/c mice (used to study the CD4 T cell response). It is possible that this particular DC subset (possibly of dermal origin) may be involved in the induction of CD8$^+$ T cell responses.

4.4 TLR4 signaling in response to epicutaneous immunization

Rationale

It seems that part of the reason why CT and TS are effective means of promoting cellular immune responses through ECI is their ability to effect APC activation. It also demonstrates, as acknowledged previously, that adjuvants enhance adaptive immunity by modifying APC function through innate immune mechanisms. Given this novel finding we wanted to explore the noteworthy innate mechanisms that may be initiated during ECI with adjuvants. One of the most important innate immune mechanisms, conserved in insects as well as in mammals, is the activation of the toll like receptor (TLR) signaling pathway (Kearney et al. 1994, Trinchieri and Scott 1999). To date 10 TLR have been identified in humans. Each TLR expresses a distinct extracellular domain but a common cytoplasmic signaling domain that is homologous to the signaling domain of the IL-1R. As such the TLR signaling domain is referred to as the Toll/IL-1R (TIR) domain (Appendix A.2) (Dunne and O'Neill 2003, Martin and Wesche 2002). Ligands that activate either family of receptors can potentially stimulate innate immunity. The literature shows that TS can initiate this pathway through the release of the cytokine IL-1 which signals through the IL-1 receptor (Wood et al. 1996, Wood et al. 1997). We wanted to determine if other endogenous ligands that signal through the Toll/IL-1 receptor pathway are released within the epidermis upon TS.

The ideal method to determine whether TS activates the TLR signaling pathway within the epidermis would be to effectively eliminate signaling downstream of all 10 TLR and determine the effect on elicitation of adaptive immune responses to ECI. A molecule which is involved downstream of all ten signal transduction pathways is Myeloid differentiation primary response gene 88 (MyD88) (Janssens and Beyaert 2002). As such, ECI with CT and after TS in MyD88 knockout mice would be ideal to investigate the consequences of TLR signaling. As these mice are currently not commercially available, we chose to study a specific TLR pathway.
Rationale for choosing TLR4

The effect of TS on activation of TLR4 was studied based on some previously published observations. First, epidermal resident KCs and LCs have been shown to express TLR4 thus making it potentially relevant to the cutaneous immune system (Curry et al. 2003, Song et al. 2002). Second, several endogenous ligands that signal through TLR4 have been identified (Akira et al. 2001, Kaisho and Akira 2002). This is important because TS will not introduce exogenous, but rather may release endogenous ligands into the epidermal microenvironment. Endogenous ligands reported to be released within inflamed or otherwise distressed tissues include heparin sulfate (Johnson et al. 2002), hyaluronic acid (Termeer et al. 2002), fibronectin (Okamura et al. 2001) and heat shock proteins (Asea et al. 2002, Breloer et al. 2001, Bulut et al. 2002, Ohashi et al. 2000).

Heparin sulfate is a polysaccharide usually found within cell membranes and in the extracellular matrix. It is shed from the cell surface under conditions of tissue distress and general inflammation. Hyaluronic acid is a glycosaminoglycan and a constituent of the extracellular matrix. The intact acid is of a large molecular weight, however smaller fragments of HA have been found to participate in pro-inflammatory responses including activation of DCs. One mechanism through which these fragments are generated in vivo is by damage to the tissue in which they reside (Fraser et al. 1997). Fibronectin is another component of the extracellular matrix that is degraded into its signalling fragments by activated proteases (Clark 1983). The enzymatic action of matrix metalloproteases has been found to be important in enabling LC migration through the subcutaneous layers of the skin (Ratzinger et al. 2002). Since TS promotes LC migration it is reasonable to assume that it may induce activation of these proteases and as such facilitate the generation of fibronectin fragments. Heat shock proteins are generally confined to the cell cytosol and involved in chaperoning protein folding (Karlin and Brocchieri 1998, Leroux et al. 1997). Tissue injury or damage, however, can release these proteins into the extracellular space where it can bind to TLR4 (Strobel and Mowat 1998). Given that all four of these endogenous TLR4 ligands seem to be associated with tissue damage and or general inflammation it is plausible to consider that they may be released subsequent to TS within the epidermis.
Hypothesis and Objective

Thus, we hypothesized that TS, by inducing epidermal tissue injury, may cause the release of some or all of the above mentioned ligands and thus activate TLR4 signaling within the epidermis. Based on this hypothesis our objective was to determine whether TLR4 signaling played a role in cellular immune responses to ECI with a peptide + adjuvant by comparing the response between wild type and TLR4 deficient mice. The CD4 T cell response as well as the antibody response was characterized in Balb/c congenic C.C3H/HeJ mice (Vogel et al. 1994). C3H/HeJ mice developed a spontaneous mutation between 1960 and 1965 while being bred at the Jackson Laboratory that rendered them hyporesponsive to the gram-negative bacterial endotoxin LPS. The mutation consists of a proline to histidine substitution at position 712. Since LPS is the main ligand for TLR4, hyporesponsiveness to the bacterial endotoxin is considered to result from a defect in TLR4 signaling. The C.C3H/HeJ strain has been used successfully by several groups to study the effects resulting from a lack of TLR4 signaling in various experimental models (Dabbagh et al. 2002, Eisenbarth et al. 2002, Guillot et al. 2002, Haeberle et al. 2002, Rescigno et al. 2002). Balb/c mice that do not harbor this mutation and express the functional form of the lps gene were used to characterize the wild type response. The CD8 response was characterized in mice with a deletion of the TLR4 gene (C57BL/10ScCr). The background C57BL/10ScN strain without the deletion was used to characterize the wild type CD8 T cell response.

Results of this study

Humoral immune response

Results presented in Fig. 6 indicate that TLR4 signaling is not involved in the elicitation of antibody responses to peptide + adjuvant administered epicutaneously (Fig 7). No difference was observed between wild type Balb/c and the TLR4 deficient C.C3H/HeJ mice in terms of Ova IgG, IgG1, IgG2a or IgE responses.

Cellular immune responses

Similar results were obtained for the cellular arm of the adaptive immune response in that neither the CD4 (Fig. 7) nor the CD8 (Fig. 8) T cell response suffered from a lack of TLR4 signaling in the respective TLR4 deficient strains.
We conclude from these findings that TS does not activate TLR4 signaling within the cutaneous microenvironment. Furthermore, TLR4 signaling does not seem to be involved in the generation of humoral or cellular immune responses to ECI with peptide. These findings are confirmed by a recent study (published in abstract form) that found cutaneous Th2 responses to ovalbumin to be TLR4 independent (Herrick et al. 2003) while reporting a need for TLR4 signaling in pulmonary responses to inhaled allergens (Eisenbarth et al. 2002, Herrick et al. 2003). Future studies trying to delineate the role of TLRs in cutaneous immunity may want to consider TLRs 1, 2, and 5 all of which are actively involved in an inflammatory skin disease, psoriasis (Baker et al. 2003). Hence the main findings of this thesis are as follows: 1) ECI can stimulate CD8 T cell mediated immunity, 2) CT and TS are effective agents for promoting both CD4 and CD8 T cell responses, 3) IL-12 is not required for functional CD8 T cell responses in the skin, and 4) TLR4 signaling is not involved in cutaneous immune responses to peptide plus adjuvant.

4.5 Impact of described work

One of the more important findings of this thesis is the demonstration that the cutaneous environment can facilitate the generation of CD8 T cell mediated cellular immunity to exogenously administered peptide. As discussed, this may be due in part to the unique ability of skin resident APC to present exogenous peptides in the context of MHC class I molecules. This finding coupled with the already existent knowledge that the skin immune system is an efficient site for the induction of humoral antibody mediated immune responses suggests that the skin could be used successfully to activate both arms of the adaptive immune response. Coupled with the knowledge that cutaneous immune responses can be stimulated without the need for invasive procedures, ECI may provide an attractive alternative to the conventional mode of immunization involving needles (Partidos et al. 2001).

The need to find alternatives to the needle stems from the inherent problems associated with needle use. First, the use of needles requires proper personnel training to ensure used needles are disposed of properly and not reused. Even with personnel training one cannot completely eliminate needle reuse, and thus there is the risk of spreading needle borne diseases such as Hepatitis B and C and HIV (Aylward et al. 1995, Kane et al. 1999). Second, needles are perceived to be invasive and pain causing. These factors can influence compliance with
vaccination regimens, especially those requiring boosters. Third, the equipment and personnel training required increase the price of a needle administered vaccine making it less feasible for mass immunizations in developing and third world countries (Levine 2003). Epicutaneous immunization on the other hand offers a simple cost effective and practical mode of delivery of vaccines.

In addition to providing an alternative to needles, epicutaneous immunization may have another potential advantage and that is the use of certain adjuvants. Currently, the only adjuvant approved for use in humans is alum (Singh and O'Hagan 1999). Given the importance of adjuvants in ensuring potent immune responses the desire and need to find and be able to use strong adjuvants in humans exists. At present, the use of such adjuvants as CT is prohibited in humans due to their severe toxicity. However, the toxicity is due to the systemic release of these toxins through intramuscular administration and due to local tissue-specific toxicity when given orally. In contrast, adjuvants administered through the skin exert their effects locally. CT has been shown to be non-toxic when given epicutaneously as opposed to oral or mucosal delivery (Glenn et al. 1998a, Glenn et al. 1999, Scharton-Kersten et al. 1999). Thus, epicutaneous administration of vaccines may broaden the category of adjuvants that can be used in humans.
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
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<tbody>
<tr>
<td>AICD</td>
<td>Activation induced cell death</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
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<td>CpG</td>
<td>Cytosine–phosphate-guanosine</td>
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<td>CT</td>
<td>Cholera toxin</td>
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<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
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<tr>
<td>DBT</td>
<td>Dibutylthlate</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<td>ECI</td>
<td>Epicutaneous immunization</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetraacetic Acid</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>Hsp</td>
<td>Heat shock proteins</td>
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<td>IFNγ</td>
<td>Interferon gamma</td>
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<td>IL-1</td>
<td>Interleukin-1</td>
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<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
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<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
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<td>KC</td>
<td>Keratinocyte</td>
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<tr>
<td>LC</td>
<td>Langerhans cell</td>
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<td>LN</td>
<td>Lymph nodes</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
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<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TS</td>
<td>Tape stripping</td>
</tr>
</tbody>
</table>

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References


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55. Haeberle HA, Takizawa R, Casola A, Brasier AR, Dieterich HJ, Van Rooijen N, Gatalica


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Appendix A

A.1 Locating the auricular LN

cut the skin following this line and expose the thorax

A muscles
B 2 lymph nodes
(I am not sure about their name(s) but they are not the auricular LNs !!!)
C salivary gland
D pectoral muscles
E sternum
F thorax
G fat
H auricular LN
A.2 The IL-1R–TLR signaling pathway

Akira, S. et al Nature Immunology 2, 675-680