# THE EFFECTS OF CUTANEOUS ULTRAVIOLET LIGHT EXPOSURE ON MIGRATING OVINE DENDRITIC CELLS

by

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This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution, except by way of background information and duly acknowledged in the Thesis, and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made in the text of this thesis.

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# ABSTRACT

The exposure of skin to ultraviolet (UV) radiation can have serious consequences which may include the down-regulation of cutaneous immunity and the generation of tumours. This study examined effects of such an exposure on the dendritic cells migrating from skin to the draining lymph node in a cannulated ovine pseudoafferent lymphatic vessel. These investigations have revealed that exposure of skin to UV light increased migration of Langerhans cells (LC) from the skin. Exposure of skin to a dose of 8.1 $kJ/m^2$ UVB caused a sustained increase in both the number and proportion of LC migrating towards the regional lymph node. Higher doses triggered non-specific effects including increased lymph output and the increased migration of other lymphoid cells. This provides strong evidence that the depletion in LC numbers in the skin after UV exposure is partially due to the migration of these cells to the draining lymph node. In addition, a $CD14^+$ DC population which parallels the immunosuppressive macrophages in human and murine models, was shown to be increased in ovine afferent lymph after UV exposure of the skin.

Since DC are induced to migrate from UV exposed skin, the functional capability of these cells was analysed. Cutaneous exposure to 8.1 $kJ/m^2$ UVB was found to significantly reduce the capacity of migrating DC to present antigen to T cells. The same dose significantly impaired mitogen induced proliferation of peripheral blood mononuclear cells (PBMC), which were used to assess DC function. However, the differing time courses of these two effects indicates that the functional capability of both populations were impaired, thereby contributing to the overall down regulation of immune function after UV light exposure.

To determine whether altered cytokine production may play a role in these events, supernatants from DC:PBMC co-cultures and lymph draining treated areas of skin were assayed for the presence of variety of soluble factors. It was found that after UV exposure of the skin increased prostaglandin $E_2$ and IFN$\gamma$ production in culture supernatants, however the timing of this increase did not correlate with the reduced functional capacity of DC. A protein with the approximate molecular weight of IL-10 and IL-10-like activity was detected in lymph draining the UV exposed skin. This finding may help to explain the impaired functional ability of DC migrating from UV exposed skin. In summary,

this study has shown that DC with impaired functional capacity were induced to migrate from UV exposed skin and this effect was associated with increased levels of IL-10.

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# ABBREVIATIONS

| | |
|---|---|
| **AchE** | acetylcholine esterase |
| **APC** | antigen presenting cells |
| **ATPase** | adenosine triphosphatase |
| **BALT** | bronchus-associated lymphoid tissue |
| **BP** | benzo[a]pyrene |
| **CD** | cluster of differentiation antigen |
| **CGRP** | calcitonin gene-related peptide |
| **CHS** | contact hypersensitivity |
| **CPM** | counts per minute |
| **CSF** | colony stimulating factor |
| **CTL** | cytotoxic T lymphocyte |
| **CTLA** | cytotoxic T lymphocyte antigen |
| **DC** | dendritic cell(s) |
| **DETC** | dendritic epidermal T cell(s) |
| **DIS** | dermal immune system |
| **DLN** | draining lymph node |
| **DMBA** | 7,12-dimethylbenz[a]anthracene |
| **DMU** | dermal microvascular unit |
| **DNA** | deoxyribonucleic acid |
| **DNCB** | dinitrochlorobenzene |
| **DNFB** | 2, 4-dinitrofluorobenzene |
| **DTH** | delayed type hypersensitivity |
| **EIA** | enzyme immunoassay |
| **ELAM** | endothelial lymphocyte activation marker |
| **ELISA** | enzyme linked immunosorbent assay |
| **ELR** | epidermal cell-lymphocyte reaction |
| **EM** | electron microscope |
| **FCS** | foetal calf serum |
| **FITC** | fluorescein isothiocyanate |
| **GALT** | gut-associated lymphoid tissue |
| **G-CSF** | granulocyte colony stimulating factor |
| **GM-CSF** | granulocyte macrophage colony stimulating factor |
| **HIV** | human immunodeficiency virus |
| **HLA** | human class II major histocompatibilty complex antigen |
| **HSV** | herpes simplex virus |
| **Ia** | murine class II major histocompatibilty complex antigen |
| **ICAM** | intracellular adhesion molecule |
| **IFNγ** | interferon gamma |
| **Ig** | immunoglobulin |
| **IL** | interleukin |
| **LC** | Langerhans cell(s) |
| **LFA** | lymphocyte function antigen |
| **LNDC** | lymph node dendritic cells |
| **LPS** | lipopolysaccharide |

| | |
|---|---|
| **mAb** | monoclonal antibody |
| **MCP** | monocyte chemotactic protein |
| **MED** | minimal erythemal dose |
| **MHC** | major histocompatabilty complex |
| **M-CSF** | monocyte colony stimulating factor |
| **NFkB** | nuclear factor kB |
| **NO** | nitric oxide |
| **NOS** | nitric oxide synthetase |
| **OVA** | ovalbumin |
| **PBMC** | peripheral blood mononuclear cell |
| **PBS** | phosphate buffered saline |
| **PG** | prostaglandin |
| **PKC** | protein kinase C |
| **mRNA** | messenger ribonucleic acid |
| **SALT** | skin associated lymphoid tissue |
| **SCF** | stem cell factor |
| **SDS** | sodium dodecyl sulphate |
| **SI** | stimulation index |
| **SIS** | skin immune system |
| **SLS** | sodium lauryl sulphate |
| **SRBC** | sheep red blood cells |
| **TCR** | T cell receptor |
| **TEMED** | N,N,N',N'-tetramethyleythlenediamine |
| **TGF** | transforming growth factor |
| **Th** | $CD4^+$ T lymphocytes |
| **Thy-1** | murine T lymphocyte antigen |
| **TNF** | tumour necrosis factor |
| **TNP** | trinitrophenol |
| **TPA** | tumour promoter alpha |
| **UCA** | urocanic acid |
| **UV** | ultraviolet light (200-400nm) |
| **UVA** | ultraviolet light (320-400nm) |
| **UVB** | ultraviolet light (290-320nm) |
| **UVC** | ultraviolet light (200-290nm) |
| **UVB-R** | UVB-resistant |
| **UVB-S** | UVB-sensitive |
| **UV-UCA** | *cis*-urocanic acid |
| **VCAM** | vascular cell adhesion molecule |
| **VLA** | very late antigen |
| **[$^3$H]thymidine** | tritiated thymidine |
| **+** | positive |

# FOREWORD

This study has utilised the cannulation of ovine pseudoafferent lymphatic vessels to study the migration and function of cells after UV exposure of the skin. The Langerhans cells (LC) are epidermal antigen presenting cells (APC), whose migratory and functional properties are thought to play a crucial role in tumour immunosurveillance within the skin. Therefore these studies have concentrated on the migration kinetics, antigen presenting function of cutaneous dendritic cells (DC) and factors which may influence these events. Thus providing an insight into altered immunity, which may lead to tumour evasion after UV exposure.

Closely shorn sheep skin was irradiated with a single measured dose of UV light. The migration kinetics of lymphoid cells collected from lymphatic vessels draining the exposed area of skin was investigated using immunofluorescence and flow cytometry. Alterations in the numbers of LC which migrated from the UV exposed skin were studied and correlated with the reduction of LC noted in other models after UV exposure.

DC migrating from a defined area of skin were enriched and their capacity to present exogenous antigen to primed autologous T cells was assessed using *in vitro* culture assay. Alterations in the APC function of DC after UV exposure were compared to the functional capacity of these cells prior to irradiation. The dose of UVB used in these studies was one determined to alter the migratory properties of DC while having minimal inflammatory effects.

Since a reduction in the APC capacity of migrating DC was observed after UV exposure investigation of costimulatory molecule expression and soluble factors secretion which could be associated with this defect were undertaken. The effect of UV exposure of skin on the expression of B7 molecules by migrating LC was investigated using two-colour immunofluorescence. The secretion levels of the cytokines IL-1β, IL-5, PGE2, GM-CSF, IFNγ and IL-10 in culture supernatants was assessed using a combination of enzyme linked immunosorbent assays, bioassays and western blotting.

These investigations revealed that the loss of antigen presenting function by migrating DC was not found to be associated with the loss of expression of the B7 costimulatory molecules. The loss of functional activity was not associated with the loss of IL-1β production or the correlated to the production

of an immunosuppressive factor *in vitro*. However, temporary increases were noted in the production of IFNγ and $PGE_2$ but these changes were not correlated with the loss of DC function. A possible correlation was found with the *in vivo* production of the immunosuppressive cytokine, IL-10. Interpretation of this data with previously published findings would indicate that *in situ* exposure to ultraviolet light caused the increased migration of LC with aberrant functional capabilities. Further it is likely that this is associated with the *in vivo* production of IL-10.

# CHAPTER 1: LITERATURE REVIEW

## 1.1 INTRODUCTION

The skin acts as a protective layer reducing the adverse effects of the external environment on the body's internal milieu. Exposure of the skin to the ultraviolet (UV) rays contained in sunlight is a common occurrence and results in the beneficial synthesis of vitamin D and melanin. However, such exposure is linked to many harmful effects including inflammation, DNA, cellular and protein damage, which result in the down-regulation of the immune system and the generation of skin tumours. The interaction between UV light and the immune system has been subject of serious study, especially over the last three decades during which time decreases in stratospheric ozone have been documented. Decreased stratospheric ozone has led to increased levels of UV light reaching the earth's surface. This increase may have serious biological consequences including further increases in the rates of skin cancer and infectious disease throughout the human population.

The unique physical and biological properties of the skin enable it to act as a barrier against various noxious chemical, microbial and physical influences, including sunlight, which contains harmful ultraviolet rays. The skin possesses unique cell types that afford this protection. These include keratinocytes whose differentiation, keratinisation and eventual death make up the outer protective physical barrier of skin and melanocytes that produce the pigment melanin which provides protection against UV exposure. Immunological protection is provided by the Langerhans cell (LC), a dendritic antigen presenting cell (APC) found in the epidermis acting as an antigen trap, transporting foreign antigens to the lymph node. In turn the LC can present these antigens to T cells, resulting in the stimulation of immune responses. The keratinocyte and to a lesser extent the melanocyte may contribute to immune function by producing a variety of cytokines. These cells constitute the skin immune system and their interaction with UV light is the focus of this thesis.

## 1.2 SKIN STRUCTURE

The skin is the external barrier between man and the environment protecting man against various noxious agents including microbial, chemical and physical insults. The skin is composed of two layers, the epidermis and

the dermis, with the epidermis being epithethial in origin, while the dermis is mainly connective tissue (Hunter *et al.*, 1989).

The epidermis contains keratinocytes, a unique cell population, whose differentiation and development give rise to the barrier function of the skin. The keratinocyte is generated in the basal layer and forced upward into the outer cell layers due to mitotic activity. As the keratinocyte moves upward it degenerates and keratinisation of the cell increases until eventually the cell dies leaving a cytoplasmic shell of keratin. These cells are continuously shed in a process termed desquamation. Before their shedding, the dead cells form the final layer of the skin, the horny layer whose properties makes the skin an effective barrier against water soluble compounds and other various insults. Another protective function of keratinocytes is the ability to take up melanin produced by ultraviolet light stimulation of melanocytes which reside in the basal layer of the epidermis. This pigment is protective against some of the harmful effects of ultraviolet light.

In contrast, the dermis contains mainly connective tissue which is composed of collagen and elastin, proteins produced by fibroblasts to give the skin its elasticity and strength. Upward projections from the dermis (papillae) interlock with downward projections from the epidermis (rete pegs). Such projections not only enhance surface area but are also important in epidermal-dermal adhesion (Hunter *et al.*, 1989). Blood vessels are richly contained throughout the dermis, supplying nutrients to both the dermal and epidermal layers of the skin. These vessels together with the cutaneous lymphatics, provide a route for cell immigration and emigration from the upper layers of the skin. Some cells resident within the dermis have immunological roles. These include macrophages, dendritic cells (DC) (including veiled cells) and mast cells, which also produce soluble factors with immune function.

### *1.2.1 The Skin Vasculature*

The skin has an abundant blood supply which provides nutrients to both the dermal and epidermal layers, as well playing an important role in regulating temperature. The vasculature of the skin forms two horizontal layers, the upper horizontal plexus and lower horizontal plexus. The lower plexus exists at the dermis-subcutis interface while the upper exists at the dermal-epidermal interface. The two plexi are connected by vascular channels through which blood flows. These vessels supply capillary branches to the hair bulbs and sebaceous glands. The vascular channels join the upper horizontal plexus

from which branches of intertwining networks of arterioles and post capillary venules arise. These ascending loops are contained within the dermal papillae, which interdigitate with the epidermis. The post capillary venules are readily susceptible to histamine which cause the endothelial cells to contract leading to gaps and increased vascular permeability (Majno & Palade 1961, Majno *et al.*, 1969, reviewed in Braverman 1983). The post capillary venules are also readily permeable to dyes and serve as the site of emigration of neutrophils and lymphocytes into the skin with inflammation (Braveman 1983).

The lymphatic network within the skin parallels the structure of the vasculature with an upper and a lower dermal plexus. The cutaneous lymphatics contain unique valves which are orientated to prevent back flow of lymph fluid (Braverman and Yen 1974). Endothelial cell gaps are common throughout the cutaneous lymphatics and provide the route by which fluid and cells may be reabsorbed and then transported to the draining lymph node.

## 1.3 THE SKIN IMMUNE SYSTEM

Skin was first proposed as an organ of immune function by Streilein (1978). He proposed that LC, keratinocytes, skin specific T cells, endothelial cells of the dermal vasculature and lymphatic vessels together with the local draining lymph node (DLN) comprised the skin associated lymphoid tissue (SALT). The skin was thought to have its own specialised immune function in an organ specific manner similar to the gut associated lymphoid tissue (GALT) and to the bronchus associated lymphoid tissue (BALT). The cells found within the skin were proposed to be specialised and provide the immune protection for the skin. SALT however, did not take into account many cell types within the dermis which could also influence cutaneous immune responses.

Bos and Kapensberg (1986) included both the dermis and epidermis in their proposal for skin immunity in which they coined the phrase skin immune system (SIS), to describe the many cellular and humoral components that could influence immune responses in the skin. They also argued that Streilein's SALT bore little resemblance to GALT or BALT due to lack of compartmentalisation. Furthermore B cells, which are plentiful in these areas are absent from the skin (Bos *et al.*, 1987). The same study also found that the majority of T cells in the skin were closely associated with the post capillary venules of the dermis (90%), with those in the epidermis only accounting for 2% of the total population (the majority $CD^{1}$ $8^{+2}$). Hence little direct T cell

[1] Cluster of Differentiation, [2] Positive

keratinocyte interaction could occur. In addition there was a lack of intra-epidermal T cell differentiation which was proposed as part of SALT (Bos *et al.*, 1987). SALT was also proposed to involve the homing and maturation of skin specific T cells. However, the majority of T cells in the skin were activated and not at rest as would be expected (Bos *et al.*, 1987). More recently the expression of E-Selectin (also known as ELAM-1 or CD62E) by endothelial cells, has been proposed to act as skin homing molecule for memory T cells in response to inflammation (Norris *et al.*, 1991). The LC which Streilein proposed as unique cells for carriage of antigen to the draining lymph nodes, were proposed by Bos and Kapensberg (1986) to actually form part of a widely distributed family of dendritic antigen presenting cells and that their specificity for the epidermis did not suggest a skin specific function.

Recently the dermis has had a greatly emphasised role in immunological reactions occurring in the skin. The dermal microvascular unit (DMU) was proposed by Sontheimer (1989) to be the center of dermal immunological reactions. The DMU is comprised of dermal T cells, mast cells, vascular endothelial cells, dermal dendritic cells and monocytes. Nickoloff (1993) expanded the idea of the DMU, to include fibroblasts because of their intimate relationship with other dermal cells, this he called the dermal immune system (DIS). DIS like SIS included humoral components of the dermis, cytokines and neuropeptides.

The SIS theory provides the most complete and comprehensive picture of cutaneous immunity as it emphasises both the cellular and humoral components of both the epidermis and the dermis. However, Streilein's SALT proposal still has merit as it provides a connection between the immunological components of the skin and the DLN.

## 1.4 CELLS OF THE SKIN IMMUNE SYSTEM

### *1.4.1.1 Langerhans Cell*

In 1868 Paul Langerhans found a group of dendritic cells (DC) in human skin which stained with gold chloride. The cells observed under the microscope appeared to be in continuity with nerve cells, which also have an affinity for the stain. This led Langerhans to conclude that he was looking at an intraepidermal nerve cell system.

This hypothesis was accepted for nearly a century before it was disproved by electron microscopy (EM) which showed the cells had no structural similarities with nerve cells (Birbeck *et al.,* 1961). Birbeck described the ultrastructural features of the LC including a unique granule now named after its discoverer. The alternate hypothesis that LC were in fact worn out melanocytes that had lost their capacity to produce pigment as proposed by Masson (1945) and reviewed by Breathnach, (1991) was also disproved by Birbeck's study.

It was suggested by Silberberg *et al.*, (1973) that LC were involved in the immune process as antigen presenting cells, as after dinitrochlorobenzene (DNCB) sensitisation it was found that LC were apposed to lymphocytes. This hypothesis is now accepted as valid, as it has been established that mature LC have a capacity to present antigens to T cells with extraordinary efficiency (Bergstresser *et al.,* 1992).

#### 1.4.1.1 Lineage

Katz *et al.*, (1979) showed that LC originate from bone marrow. The transplantation of parental skin to F1 hybrid mice showed that the majority of the LC present in the graft were from recipient origin, whereas the keratinocytes were from donor origin. This was supported conclusively by Perreault, (1984) who showed that after the transplantation of human bone marrow from a male to a female, Y chromosome positive LC were found in the skin.

It is now generally accepted that LC precursors migrate from the bone marrow to the skin, via the bloodstream as $CD1a^+$ cells have been found in peripheral blood (Wood *et al*, 1984). Further evidence for the migration of precursors into the skin has been provided using a monoclonal antibody (mAb), 4F7. This labels dendritic cells in the mouse epidermis having phenotypic and functional properties in common with the LC yet they lack Birbeck granules. When mouse skin was treated with dinitrofluorobenzene (DNFB), an increase in the number of these cells (some exhibiting Birbeck granules) was found in the epidermis (Kolde *et al.*, 1992). This suggests these cells represent immature LC that have migrated via the peripheral blood to the epidermis. Indeed it has been demonstrated that DC can be generated from peripheral blood mononuclear cells (PBMC)(Romani *et al.*, 1994). Although Katz *et al*, (1979) and Perreault (1984) have shown that LC were continuously replaced in the skin from the precursor pool, it has been observed that LC in the skin can replicate, albeit slowly (Czernielewski and Demarchez, 1987). The speed at which this occurs is approximately half that of keratinocytes. These results are

supported by Kanitakis *et al.*, (1993) whose EM studies have shown LC undergoing mitosis in human skin.

It has also been suggested that LC precursors are of monocyte origin (Katz *et al.*, 1979), due to similar expression of cell surface markers. However, the point at which the lineages of LC, monocytes and other dendritic cells diverge is still not clear. It is however possible that that they are cells of the same phenotype with the differences being associated with location (Bergstresser *et al.*, 1992). This contention is supported by evidence showing that various cytokines can induce differentiation of monocytes into DC including interleukin (IL)-4 and interferon $\gamma$ (IFN$\gamma$) (Xu *et al*, 1995), IL-4 and granulocyte/monocyte-colony stimulating factor (GM-CSF) (Porcelli *et al.*, 1992). $CD34^+$ progenitor cells can also be induced to become DC after stimulation with tumour necrosis factor-$\alpha$ (TNF$\alpha$) and GM-CSF (Caux *et al.*, 1992). The resultant DC were shown to express Birbeck granules, characteristic of LC. CD40L (Caux *et al.*, 1994) and stem cell factor (SCF) (Young *et al.*, 1995) have also been shown to play a role in DC maturation. Hence there are at least two possible sources for DC, from a colony forming unit DC within the bone marrow (Young *et al.*, 1995) and the other from a monocyte origin. These "mixed" origins mean that DC and macrophages share many common markers and are hard to separate on an individual phenotypic marker, reviewed by Peters *et al.*, (1996).

#### 1.4.1.2 Distribution

LC are thought to be present in the epidermis of all mammals, while birds and reptiles lack them. They have been identified in the epidermis of man, the rhesus monkey, lorisidae, mice, guinea pigs, rats, rabbits, hamsters sheep and cows. However LC in pigs appear to lack Birbeck granules (reviewed by Breathnach *et al.*, 1991). LC appear in the mammalian stratified squamous epithelia, which includes epidermis, oral cavity, oesophagus, nasopharynx, conjunctiva and female genital and urinary tracts (Breathnach 1988). Furthermore migrating LC are found within the dermis, lymph nodes and lymphatic vessels draining the skin (Wolff and Stingl, 1983).

In sections of skin, LC occupy a suprabasal position in rodents and a basal position in the human epidermis. LC make up between 2-4% of epidermal cells (Stingl *et al.*, 1978a). LC cells in sheep are restricted to the basal layer of the epidermis and constitute about 10% of the cells in this layer of the epidermis (Hollis and Lyne, 1972). The density of LC per unit area has been reported to differ between species, with LC density in humans 460-1000

cells/mm² (Rowden, 1981), guinea pigs 800-1000 cells/mm² (Wolff and Winkelmann, 1967b) and in mice the highest density recorded is 800 cells/mm² (Bergstresser *et al.*, 1980). There exists regional variation between different areas of the body in the species studied. Examination of human epidermal sheets have shown that in general the highest LC density is found on the face and neck (976 LC/mm²), while the trunk and limbs contain medium levels of LC (740 LC/mm² and 640 LC/mm² respectively) and relatively low numbers are found in palm and sole skin (189 LC/mm²) (Chen *et al.*, 1985). Ashworth *et al.* (1986) found no significant difference in LC numbers in vertical sections of limbs, the face and the trunk skin but found significantly lower numbers of LC in the vertical sections of the sole and palm skin. The highest densities of LC in mice are found on the back and footpads whilst the lowest is present in the tail (Bergstresser *et al.*, 1980).

The LC count in normal skin may vary widely from individual to individual (Horton *et al.*, 1984) and in humans LC density decreases with age (Gilchrest *et al.*, 1982). LC enumeration of vertical sections can be expressed in a variety of ways such as LC/20 high power fields, LC/mm epidermal surface, LC/mm length of basement membrane, LC/200 basal cells, LC/mm² of epidermal section (Bieber *et al.*, 1988). The different enumeration methods and markers used to identify LC population in vertical sections leads to variable results and these can often be contradictory (Bieber *et al.*, 1988). For these reasons epidermal sheets are a more ideal way to enumerate LC in the skin.

#### 1.4.1.3 Ultrastructure

In the skin, LC possess a highly dendritic morphology. Sensitive immunolabelling techniques have shown branched dendrites extending in three dimensions, allowing one LC to cover a large area. LC are recognisable under the electron microscope (EM) they have folded nuclei, lack desmosomes, tonofilaments, premelanosomes and melanosomes. The Birbeck granule is a unique marker for LC and is regarded as the definitive morphological feature seen under the EM (Hanau *et al.*, 1987).

Birbeck granules appear as rod shaped structures of variable length with a central periodically striated lamella. When studied in ultrathin sections the rods appear tennis racket shaped due to their opening into a bleb at one side (Schuler *et al.*, 1991). The Birbeck granule has been shown to be derived from the invagination of the cytomembrane (Takahazi and Hashimoto, 1985, Takigawa *et al.*, 1985) and granule is thought to be derived from the

endocytosis of the T6 antigen (Hanau *et al.*, 1987). Cells with the same characteristics as LC but lacking the Birbeck granule, are named indeterminate cells, serial sectioning of these indeterminate cells often reveals the presence of the Birbeck granule in cells that would otherwise be classified as indeterminate cells (Tamaki *et al.*, 1979). Some indeterminate cells have been shown to react with a monoclonal antibody to CD1c (Murphy *et al.*, 1985). It has been proposed that these cells are in fact precursors of LC (Breathnach, 1991).

The numbers of Birbeck granules appear to bear no reflection on whether the LC is undergoing mitosis, as the granules have been found to be common (Konrad and Hongismann, 1973) and uncommon (Kanerva *et al.*, 1983) in this state. However, LC lose the Birbeck granule when they are cultured and is thought to provide evidence for maturation into lymphoid DC (Romani *et al.*, 1989, Teunissen *et al.*, 1990, Richters *et al.*, 1993). Normally only a small percentage of interdigitating cells have the granule (Kamperdijk *et al.*, 1978). However, an increase in Birbeck granule positive lymph node has been reported after immune stimulation (Kamperdijk *et al.*, 1985). This is thought to represent the migration of LC from the skin to the lymph node.

#### 1.4.1.4 Phenotype of Resident and Freshly Isolated LC

The phenotype of LC are varied and often dependant on the state in which they are isolated from tissue. They encompass a wide variety of markers expressed by other cell populations including B cells, T cells and especially those of the monocyte/macrophage lineage. Extensive studies in humans and mice and to a lesser extent in rats, rhesus monkeys and sheep reveal that the expression of surface molecules on LC varies from species to species. LC undergo profound changes in phenotype when cultured (Romani *et al.*, 1989) and this is thought to reflect their maturation into interdigitating cells. This is discussed in greater detail in the next section.

Like all bone marrow derived cells, LC express the CD45 common leucocyte antigen (Wood *et al.*, 1984). They also express MHC I as do all nucleated cells (Bronstein *et al.*, 1983). MHC II is expressed by LC (Rowden *et al.*, 1977, Klareskog *et al.*, 1977) including HLA-DR, HLA-DQ and HLA-DP (Sonthiemer *et al.*, 1986). LC appear the only cells to constitutively express MHC II in normal epidermis. Accordingly MHC II is able to be used as a reliable marker for the LC population in skin and expression of MHC II is essential in the presentation of antigen to T cells (Unanue *et al.*, 1984).

LC are positive for a marker of thymocytes, CD1a (Fithian *et al.*, 1981) and given that no other cell type in the epidermis is positive for this marker, it has become the most reliable marker for LC (reviewed by Romani *et al* ., 1991). CD1b does not appear to be expressed by LC in the epidermis (Furue *et al.*, 1992, Meunier *et al.*, 1993, Richters *et al.*, 1995). LC in the epidermis are also positive for CD1c, however murine homologues of the CD1 group of antigens are less well defined. Possible candidates in the murine system, were the Tla group of antigens but data suggests that this is not the case (reviewed by Romani *et al.*, 1991). A sheep homologue, which stains LC in epidermis has been identified and designated SBU-T6 (Mackay *et al.*, 1985). Another marker of T cells that is expressed on human LC is the CD4 molecule (Wood *et al.*, 1983, Groh *et al.*, 1986) which is up-regulated during inflammatory reactions (Groh *et al.*, 1986). CD4 expression is not observed on murine LC (Witmer *et al.*, 1988). The functional significance of the expression of this T cell marker by LC is not known. CD4 expression by LC renders the cells susceptible to infection by HIV (Tschachler *et al.*, 1987).

The close lineage of monocyte/macrophages and LC is evident by the LC expression of many so called macrophage specific adhesion markers, including complement binding molecules CD11b (Mac-1 (mouse)) and CD11c (gp 190,95) and the β chain CD18 (De Panfilis *et al.*, 1989). However, while the expression of CD11c appears to be substantial, the expression of CD11b is very low. LC also appear to be weakly positive/negative for CD11a (LFA-1) (Romani *et al.*, 1989). LC are negative for CD14 (Romani *et al.*, 1989) though reports suggest that some LC present in the nasal associated lymphoid tissue co-express both CD1a and CD14 (Graeme-Cook *et al.*, 1993). LC express the cytoplasmic markers non-specific esterase (Romani *et al.*, 1989) and myleoperoxidase (Dubertret *et al.*, 1982) which are two typical macrophage markers. The only monoclonal antibody so far developed which exhibits exclusive specificity to LC, binds to the LAG antigen, which is a glycoprotein associated with the Birbeck granule in human LC (Kashihara, *et al.*, 1986).

LC also express some other enzymatic and protein markers including adenosine triphosphatase (ATPase) which was one of the earliest reliable markers of LC discovered (Wolff and Winkelmann, 1967) and has been used extensively. However, Zelickson and Mottaz (1968), in Zelickson and Mottaz, 1970) found that keratinocytes and melanocytes may be reactive to ATPase staining and that some LC were negative. Sheep LC express acetylcholinesterase (AchE) (Lyne and Chase, 1966) and this was confirmed

by Hollis *et al.*, (1972) and more recently by Townsend *et al.*, (1997). Townsend's recent study suggests that in sheep AchE is more sensitive marker of LC than either MHC II or CD1. Another protein marker S100 is expressed intracellularly by LC in the skin however, it is also found in nerve cells, melanocytes and dermal dendritic cells within the skin which reduces its use as an LC marker (Cocchia *et al.*, 1981).

LC resident in the epidermis express few adhesion molecules however there exist some notable exceptions for example, LC have been shown to express the β1 sub unit of the very late antigen (VLA) group of adhesion molecules (Le Varlet *et al.*, 1991). The same group have shown that sub-populations of LC present in the skin express the α chains of the VLA adhesion molecules, VLA-1 (40%), VLA-2 (53%), VLA-3 (40%), VLA-4 (67%), VLA-5 (77%) and VLA-6 (90%). Many of these α chains are receptors for extracellular proteins such as collagen (VLA-1, VLA-2, VLA-3) fibronectin (VLA-3, VLA-4 and VLA-5) and laminin (VLA-6). VLA-2 and VLA-3 may allow the LC to attach to neighbouring keratinocytes, while VLA-6 and to a lesser extent VLA-1 may permit LC to attach to and migrate through the basal membrane. Furthermore VLA- 3 and VLA-5 may facilitate the migration of LC through the fibronectin network of the dermis (Le Varlet *et al.*, 1991).

LC also weakly express sialyl Lewis X which is a ligand for E-selectin expressed on endothelial cells (Ross *et al.*, 1994). The mAb HECA-452 which recognises a neuraminidase sensitive determinant on sialyl Lewis x has also been shown to react with LC (Koszik *et al.*, 1994). The localisation of skin specific memory T cells is thought to be mediated by adhesion to E-Selectin (Berg *et al.*, 1991, Picker *et al.*, 1993, Norris *et al.*, 1991), suggesting that perhaps the migration of LC precursor could also be mediated via this interaction. LC are shown to strongly express E-cadherin which has been shown *in vitro* to facilitate LC-keratinocyte binding (Blauvelt *et al.*, 1995).

While LC express the immunoglobulin receptor FcγRII in both the human and murine skin they do not express FcγRI or FCγRIII (Romani *et al.*, 1989). LC in normal human skin also express both the high affinity receptor for IgE, FcεRI (Bieber *et al.*, 1992a, Wang *et al.*, 1992) and the low affinity receptor, FcεRII (Bieber *et al.*, 1989). However, the latterreceptor is not expressed on murine LC even after stimulation with IL-4 a known inducer of FcεR expression (Hertl *et al.*, 1996). These receptors are thought to play a role in skin disorders such as atopic dermatitis.

40% of LC within the human epidermis have been shown to weakly express the CD40 antigen (Romani *et al.*, 1989) while 100% of HLA-DR positive, freshly isolated LC express the CD40 molecule (Peguet-Navarro *et al.*, 1995). LC have also been shown to express the CD69 molecule which is an early activation marker of lymphocytes though the significance of this is unknown (Bieber *et al.*, 1992b).

Freshly isolated LC from both human (Symington *et al.*, 1993) and murine (Razi-Wolf *et al.*, 1994) skin appear to lack expression of B7 molecules. Supporting this is a study by Yokozeki *et al.*, (1996), who could find no evidence in human skin of either CD80 (B7-1) or CD86 (B7-2) expression on LC. These molecules are arguably the most important for DC stimulation of T cells. This lack of expression suggests that LC within the skin would be poor stimulators of T cells via the CD28/B7 interaction. Freshly isolated LC also only weakly express LFA-3 and ICAM-1 which are adhesion molecules that play critical roles in APC adhesion to T cells, via CD2 and LFA-1 respectively (Teunissen *et al.*, 1990). Low expression of LFA-1 is seen on both human (Perry *et al.*, 1992) and murine LC (Simon *et al.*, 1993) in the skin.

Larrengina *et al.*, (1996) conducted an extensive study of cytokine receptor expression by both fresh and cultured LC. This group found that freshly isolated LC express various cytokine receptors, including the weak expression of IL-1 receptor 1 and strong expression of IL-1 receptor 2 The latter has been shown to be non functional and possibly acts as a trap for exogenous IL-1 (Colotta *et al.*, 1993). All LC expressed the 75kd receptor for TNF$\alpha$, while none of the cells expressed the 55kd receptor. 67% of LC were found to express the IFN$\gamma$ receptor. Although no LC expressed receptors for M-CSF, G-CSF, 15% expressed the $\beta$ chain for the GM-CSF receptor and 80% expressed the $\alpha$ chain. The expression of the IL-6 receptor was seen in 30% of LC while 27% expressed GP 130, the receptor's signal transducing element. Fresh LC were negative for the $\alpha$ and $\beta$ chains of the IL-2 receptor, as well as IL-4, IL-7 and IL-8 receptors.

#### 1.4.1.5 Maturation of LC in Culture

After culturing LC undergo a dramatic change in phenotype until they closely resemble lymphoid dendritic cells (Romani *et al.*, 1989, Teunissen *et al.*, 1990). This is reflected by a 30 fold increase in their ability to stimulate T cells (Schuler and Steinman, 1985). This increased functional capacity is due in part to a dramatic increase in MHC II expression (Romani *et al.*, 1989). A

nine fold increase in expression of HLA-DR , a three fold increase for HLA-DQ and a five fold increase for HLA-DP have been reported (Teunissen *et al.*, 1990). B7-1 and B7-2 molecules appear on the surface of cultured LC (Yokozeki *et al.*, 1996). The addition of mAbs against these molecules reduces the cultured LC ability to stimulate T cells by 81% and 29% respectively and 87% when combined, thus demonstrating the relative importance of B7 molecules to antigen presenting cell function. Increases are also seen in MHC I expression (Teunissen *et al.*, 1990). The expression of ICAM-1 by LC increases after culturing (Cumberbatch *et al.*, 1992), as does LFA-3 (Teunissen *et al.*, 1990) which would increase the ability of the LC to bind to and therefore, stimulate T cells.

The expression of various markers of LC have been shown to be decreased during culture including the loss of ATPase and non-specific esterase activity (Romani *et al.*, 1989). CD1a and CD1c expression have both been shown to be markedly decreased or lost after culturing (Teunissen *et al.*, 1990). Further, the reactivity of LC with various macrophage markers such CD11b and CD11c, (Teunissen *et al.*, 1990) is lost as is expression of the FC$\gamma$RII (Romani *et al.*, 1989, Teunissen *et al.*, 1990). As mentioned earlier remodelling of the antigenic profile of LC means that they resemble the interdigitating cells found within the lymph node.

E-cadherin which is thought to mediate LC-keratinocyte adherence, is also down-regulated during culture which would be expected to facilitate LC migration from the epidermis (Borkowski *et al.*, 1994). The contact allergen 2,4,6-trinitrochlorobenzene (TNCB) and the cytokine TNF$\alpha$ enhance LC migration from the epidermis with down-regulation of E-cadherin expression (Schwarzenberger and Udey, 1996). This led the authors to propose that down modulation of E-cadherin expression occurs as a consequence of local cytokine production, facilitating LC emigration and the initiation of immune responses against antigens encountered in epidermis. In contrast sialyl Lewis X which mediates binding to endothelial cells is up-regulated upon LC culture (Ross *et al.*, 1994). This was thought to increase LC binding to L-selectin expressed by T cells within the lymph nodes, as well as facilitating the binding of LC to the draining lymphatics, which express P-selectin (Ross *et al.*, 1994).

Changes in cytokine receptors are also seen after the culturing of LC. The $\alpha$ and $\beta$ chain of GM-CSF are up-regulated as is IL-1 receptor 2, the $\alpha$ and $\beta$ chains of IL-2 receptor, the IL-6 receptor and gp130. The IL-1 receptor 1 and

the 75 kD TNFα receptor are down-regulated whilst the IFNγ receptor expression was unaltered (Larregina *et al.*, 1996).

#### 1.4.1.6 LC Function

Silberberg-Sinakin *et al.*, (1976) noted that increased numbers of LC with bound ferritin appeared in the draining lymph node (DLN) in sensitised animals. The LC were seen in direct apposition to the T cells, which led the authors to conclude that LC were presenting antigen to the T cells. Stingl *et al.*, (1978b) showed the LC like monocytes/macrophages were able to induce a proliferative response in immune T cells. They were also able to act as effective stimulators of the mixed leucocyte reaction (MLR). The LC was the only cell in the epidermis able to mediate this function.

LC are also able to act as antigen presenting cells for chemical haptens (Nishioka, 1985, Shimada *et al.*, 1987). Furthermore the ability of LC to present nickel has also been shown (Braathen *et al.*, 1980). In addition to stimulating T cells hapten modified LC are able to induce cytotoxic T lymphocytes (CTL) (Pehamberger *et al.*, 1983, Tsuchida *et al.*, 1984). MHC II expression by LC was a requirement for this to happen. However, Shimada and Katz, (1988) found that trinitrophenol (TNP) modified epidermal cells, depleted of LC were able to induce equal proliferative responses in TNP specific CTL, a secondary immune response. Since CTL are CD8 positive and need antigens expressed in association with MHC I this was an expected result.

Schuler and Steinman (1985), and Inaba *et al.*, (1986) were also able to show that cultured LC were far more efficient at stimulating MLR than fresh LC. This once again shows the importance of the phenotypic changes LC undergo during culture. Fresh LC were found to be good stimulators of secondary MLR but not of primary MLR's. This may reflect the lack of expression of the B7-2 molecule, which appears to be important for the generation of primary T cell responses (reviewed by Lenschow *et al.*, 1996). The culture of LC with allogeneic T cells results in the generation of MHC II restricted T helper cells (Inaba *et al.*, 1986). However, this can also result in the generation of CTL (Faure *et al.*, 1984). LC can act as accessory cells for CD4 (MHC II restricted) T cells which, in turn, can stimulate a T cell dependent antibody response (Inaba *et al.*, 1986).

As well as being able to present haptens and alloantigens to T cells, LC can present antigens such as haptens, alloantigens, microbial antigens and tumour antigens, which can be derived from other epidermal cells such as

keratinocytes and melanocytes (reviewed by Stingl and Shevach, 1991). LC have been shown to present cellular antigens such as xenogenic red blood cells (Halliday and Muller, 1987) and are also capable of presenting viral antigens, including HIV (Girolomoni *et al.*, 1996). LC are primarily active in capturing foreign antigenic material present in the skin and presenting it to MHC II restricted T cells resulting in clonal expansion of the relevant T cells (Kapensberg *et al.*, 1989). For example it has been demonstrated that LC in mouse skin can internalise *Leishmania major*, transport it to the lymph node and generate a specific T cell response including activation of unprimed T cells (Moll *et al.*, 1993). LC can also induce contrasuppression which overcomes suppressor cell activity (Flood *et al.*, 1991).

Efficient antigen presentation, via MHC II on LC requires efficient ingestion, processing and re-expression of the antigen. LC, DC and veiled cells were until recently thought to be non-phagocytic although there existed various contradictory reports. A definitive study has shown LC can phagocytose particles from 0.5-6 μm, including zymosan particles, latex beads, microbes including *S. aures* and *C. parvum* (Sousa *et al.*, 1993) albeit the phagocytosis of complement and immunoglobulin (Ig) coated sheep red blood cells (SRBC) was not seen. However the ability to ingest *C. parvum* was markedly increased after incubation with mouse serum, thus suggesting that Fc or complement receptors play a role. The uptake of zymosan was partially mediated by mannose receptors on LC and these appear to concentrate macromolecules in MHC II compartments and maximise the capacity of DC to present non-self antigens (Sallusto *et al.*, 1995). The majority of antigen is thought to be taken in by macropinocytosis, which occurs during the formation of ruffles in the membrane. Although it was thought that DC projections and veils were used to contact T cells they may also be used for macropinocytosis (Steinman and Swanson, 1995).

Sousa *et al.*, (1993) also demonstrated that cultured LC were not able to phagocytose and during culture LC lose the ability to process soluble antigens (Steinman *et al.*, 1991). The rate of MHC II synthesis drops, as does expression of the invariant chain which is required for efficient processing of exogenous antigen for re-expression in association with MHC II (Pure *et al.*, 1990). In addition the loss of acidic organelles which are required for efficient antigen processing also occurs (Stoessel., *et al.*, 1990). This loss of the ability to process fresh antigen after culture is thought to represent the LC changing role from acting as a sentinel in the epidermis, were it is able to process

antigens in the environment, to that of an antigen presenting role, were they can present antigens they have gathered before being induced to migrate, to T cells within the draining lymph node (Ibrahim *et al.*, 1995).

The presence of MHC II alone is not sufficient to induce sustained proliferation of T cells (Dalme *et al.*, 1992) and lack of further co-stimulatory signals may lead to the generation of T cell unresponsiveness (Weaver and Unanue, 1990). The cytokine IL-1 was originally proposed as the co-stimulator molecule and with the finding that IL-1 was present in enriched LC culture suggesting that the LC were able to provide co-stimulation (Sauder *et al.*, 1984). Since this result LC have been shown to produce IL-1β (Enk and Katz *et al.*, 1992). The presence of the interleukin 1 converting enzyme (ICE) has also been demonstrated in murine LC, which is essential for the production of functional IL-1β and LC are the primary source of this cytokine within the epidermis (Ariizumi *et al.*, 1995). The production of IL-1β by LC is thought to play an essential role in the generation of immune responses in the skin (Enk *et al.*, 1993). TCR stimulation together with the addition of IL-1 has been known to induce the proliferation of Th2 clones which has been shown to be independent of IL-4. This finding suggested that APC might be able to directly stimulate Th2 type responses (Huber *et al.*, 1996).

The cytokine IL-12 has recently been shown to play an important role in the generation of Th1 responses (Seder *et al.*, 1993) and is produced by activated macrophages, B cells (D'Andrea *et al.*, 1992), DC (Macatonia *et al.*, 1995, Heufler *et al.*, 1996) and most recently, by LC (Kang *et al.*, 1996). CD1a positive dendritic lymph cells, which are derived from LC, are also capable IL-12 of production (Yawalkar *et al.*, 1996a). IL-12 is able to stimulate the T cells to secrete IFNγ and thus leads to the generation of Th1 responses (Seder *et al.*, 1993). IL-12 is also thought to induce proliferation of the Th1 cells but not that of Th2 or Th0 (Kennedy *et al.*, 1994) and inhibit differentiation of T cells into the Th2 subtype (McKnight *et al.*, 1994). The ability of LC to produce IL-12 and therefore direct T cells to produce IFNγ without addition of exogenous IL-12, anti-IL-4 or microbes, suggests that stimulation of Th1 responses is a major role for this cell (Heufler *et al.*, 1996).

The interaction between T cells and DC is not in one direction as MHC II interaction with the TCR and CD40 ligation on the dendritic cell causes increased production of IL-12 by the DC, in turn resulting in the induction of additional IFNγ production by T cells (Koch *et al.*, 1996, Cella *et al.*, 1996). CD40 ligation on DC also up-regulates the production of MHC II, B7-1, B7-2,

and LFA-3 (Caux *et al.*, 1994). It has been shown to also enhance DC viability and in contrast to the above results, up-regulated ICAM-1 and B7-2 but not HLA-DR, CD1a, CD58, and B7-1 (Peguet-Navarro *et al.*, 1995). Accordingly it is important to remember that LC/DC-T cell interaction is one where both cell types derive signals from each other which influence their functional capabilities.

LC express adhesion molecules, some of which are thought to provide co-stimulatory signals, which increase T cell proliferation. These include ICAM-1, LFA-3 members of the VCAM family (Dalme *et al.*, 1992). The most important co-stimulatory signal provided by a cell surface molecule, apart from MHC/TCR engagement, is that provided the B7 family of molecules. The B7 family binds to both the CD28 receptor and the CTLA-4 receptor on T cells and the interaction between B7-1 (CD80), B7-2 (CD86) and CD28 is thought to be a crucial event in T cell stimulation. A third molecule has also been identified, B7-3 (Boussiotis *et al.*, 1993) although its role is clear. As previously discussed LC are negative for B7-1 and B7-2 when freshly isolated from skin but are able to dramatically up-regulate this after culture or by the addition of cytokines (Chang *et al.*, 1995).

CD28 and CTLA-4, are two counter receptors which are expressed by T cells and showed only 31% homology, although CTLA-4 is highly conserved between species (>70% homology) (Lindsten *et al.*, 1993). CD28 is expressed by the majority of T cells, whilst CTLA-4 is expressed by activated T cells (Freeman *et al.*, 1992). The distinct cytoplasmic domains of these receptors as well as their distribution suggests that they have non-overlapping roles in signal transduction. It is likely that B7-1, or B7-2 binding to CD28, may be the essential T cell co-stimulatory signal (reviewed by June *et al.*, 1994). T cells lacking CD28 could initiate but not sustain an *in vitro* antigen specific response (Lucas *et al.*, 1995). CTLA-4 crosslinking seems to inhibit T cell proliferation and IL-2 secretion which are strongly induced by the B7/CD28 interaction (Krummel *et al.*, 1995). CTLA-4 has also been shown to induce antigen specific apoptosis in T cells (Gribben *et al.*, 1995). B7/CTLA-4 and B7/CD28 interactions have been shown to enhance T cell proliferation and are key control points in the activation of T cells (Lindsey *et al.*, 1995). The interaction of B7/CD28 has been shown to protect T cells from apoptosis (Boise *et al.*, 1995). B7-2 expression by LC has been shown to play a potent role in the induction of contact hypersensitivity (CHS) and was found to be specifically up-regulated after antigen stimulation, (Nuriya *et al.*, 1996) while treatment with

anti-B7-2 mAb resulted in contact allergen specific unresponsiveness. B7-1 was shown not to play a role in the induction of CHS, while neither molecule influenced the effector phase of such responses.

The two co-stimulatory pathways of the B7 family and IL-12 do not act in total isolation from one another, with IL-12 shown to act synergistically with CD28/B7 interaction, inducing efficient cytokine production and proliferation of T cells (Kubin *et al.*, 1994, Murphy *et al.*, 1994). The proliferation of Th1 cells seems to require both B7/CD28 and IL-12 to be present (Macatonia *et al.*, 1995).

Janeway, (1992) expanded theories describing how the immune system discriminates between self and non-self to include details of how the innate immune system has developed broad spectrum receptors against molecules expressed by pathogenic agents, including lipopolysaccharide (LPS), mannans and glycans, which induce the APC to express signals (co-stimulators) to activate T cells. Thus the LC in the skin is acting as a sentinel waiting for such an interaction to stimulate it to migrate to the DLN. This idea of DC acting as an immunosurveillance mechanism has been further expanded by Ibrahim *et al.*, (1995) who proposed that APC are also stimulated by microenvironmental tissue damage, stating that the signals may include free radicals, cytokines, complement and extracellular matrix degradation products. Release of these products by necrotic cells would stimulate the APC whereas cell death by apoptotis would limit this response. The response of DC to cellular damage is similar to that of "danger" stimulating APC proposed by Matzinger (1994) who argued against self and non-self discrimination by the immune system and proposes instead a "danger" signal.

The LC has many abilities which allow it to become an efficient initiator and stimulator of immune responses. These include; expression of MHC II, complement and Fc receptors, the ability to ingest and process antigen and to subsequently migrate to the DLN carrying the processed antigens. Importantly on arrival in the DLN, the LC undergoes a maturation step, with up-regulation of B7-1, B7-2 and various adhesion/co-stimulatory molecules. This taken together with the LC ability to secrete the potent T cell stimulatory cytokines IL-1β and IL-12, gives the LC the ability to act as a supremely efficient "professional" antigen presenting cell.

### 1.4.1.7 Migration and Relationship Between LC, Afferent Lymph DC, Veiled Cells and Lymph Node DC

The presence of microfilaments and a prominent system of microtubules in LC suggest that these cells are capable of movement. Tissue culture experiments have shown that LC are capable of active pseudopodial movement using dendrites that are capable of extending and contracting (Thorbecke *et al.*, 1980). It has been postulated that LC act upon a "signal" where they are induced to migrate from the epidermis via the dermis into the draining lymphatics and from there to the lymph node. Three main methods have been used to study LC migrating from the skin, cannulation of lymphatics, the use of dyes painted on the skin to track LC in the lymph nodes and the use of skin explants. The direct cannulation of lymphatics is generally not practicable due to their small size. However the generation and cannulation of pseudoafferent lymphatics has been used in ruminants allowing the collection of large numbers of DC (Hopkins *et al.*, 1985). The cellular composition of these vessels is similar to true afferent lymph and contains 1-10% MHC $II^+$ DC (Hopkins *et al.*, 1985, Hein *et al.*, 1987, Bujdoso *et al.*, 1989). A small number of studies have been performed by cannulation of human lymphatics vessels. The use of fluorescent dyes such as fluorescein isothiocyanate (FITC) have been used to track LC from the skin to the lymph node particularly in the murine model. In addition a number of studies have used human skin explants, were LC residing in the epidermis are seen to migrate via the dermis into culture media in the skin. This has been used to study the phenotypic changes that occur during this process (Richters *et al.*, 1993, Richters *et al.*, 1995).

Interdigitating cells are found in the T cell areas of the lymph node, and are the classical antigen-presenting cells of MHC restricted responses. Human LC and interdigitating cells have very similar phenotypes, including CD4 and CD1 expression (Ralfkiaer *et al.*, 1984). Immunostaining experiments using the monoclonal antibody NLDC-145, which reacts with LC, veiled cells and interdigitating cells but not other follicular DC (Kraal *et al.*, 1986), have been used to suggest that interdigitating cells are derived from veiled cells (Breathnach, 1991). The presence of the Birbeck granule is also seen in these two cell types (Silberberg-Sinakin *et al.*, 1976). The presence of the Birbeck granule (Hoefsmit, 1982) and E-Cadherin (Borkowski *et al.*, 1994), which are both expressed on LC, is only seen in lymph node dendritic cells (LNDC) draining the skin. Afferent lymph veiled cells and LNDC have been shown to be associated with antigen after cutaneous sensitisation (Lens *et al.*, 1983, Macatonia *et al.*, 1987). The application of antigen increases the number of

DC in the draining lymph node in a dose dependent manner (Macatonia *et al.*, 1986). It has also been shown that pseudoafferent lymph DC migrating from the site of an intradermal injection of antigen are able to stimulate a significant proliferative response in antigen specific T cells when compared to control cells (Bujdoso *et al.*, 1989). Furthermore, through the use of the pseudoafferent model in sheep, enhanced migration of LC from the skin has been seen after application of the contact sensitiser TNCB as well as in response to chemical carcinogens such as 7,12 dimethylbenz(a)anthracene (DMBA), (Dandie *et al.*, 1994) benzo(a)pyrene (BP) and to the tumour promoter 12-o-tetradecanoylphorbol-13-acetate (TPA) (Ragg *et al.*, 1994). Increased numbers of Birbeck granule positive LNDC are also evident after antigen treatment of the skin (Silberberg-Sinakin *et al.*, 1976). The treatment of the skin with ultraviolet light has been shown to cause increased migration of LC to the draining lymph node (Moodycliffe *et al.*, 1994) (see section 1.4.6.5) Increased numbers of LC are also seen in lymph from human skin after application of the irritant, sodium lauryl sulphate (SLS) (Brand *et al.*, 1995). In an *in vitro* model, using a reconstituted basement membrane invasion assay, the treatment of LC with hapten (TNBS or FITC) was able to induce migration through the membrane to a dermal fibroblast culture medium (Kobayashi *et al.*, 1994). However, in contrast to the *in vivo* studies of Brand *et al.*, (1995), SLS did not appear to induce migration. This was possibly due to the lack of a local release of cytokines by keratinocytes which are absent from this i*n vitro* model. Indeed some cytokines have been proposed as stimulators of LC migration with the most likely candidates being TNF$\alpha$ (Cumberbatch and Kimber, 1992) and IL-1$\beta$ (Cumberbatch *et al.*, 1997) though their exact role is not yet fully understood.

Chemokines are a family of pro-inflammatory and chemotactile cytokines with characteristic four cysteine residues. They are divided into two sub-families, C-C (which have two linked cysteine bonds) and C-X-C (which have two linked cysteine bonds separated by an amino acid) (Tizard, 1995). DC have been recently shown to migrate in response to a set of chemokines distinct from that of monocytes/macrophages. Exposure of DC to the C-C chemokines, monocyte chemotactic protein (MCP)-3, macrophage inflammatory protein-1$\alpha$ and RANTES (an acronym for a protein which is, regulated upon activation, normal T cell expressed and secreted) caused a chemotactic response and a rise in the intracellular calcium levels. In contrast, the C-X-C chemokines, IL-8 and IP-10 and the C-C chemokines, MCP-1 and MCP-2 were inactive as chemoattractants for DC (Sozzani *et al.*, 1995). Nakamura *et al.*,

(1995) found that using a transgenic mouse model in which keratinocytes expressed MCP-1, the epidermis did not contain substantially more LC, suggesting that sites for LC in the epidermis were near saturated normally. Although Sozzani's study would suggest that perhaps LC are unresponsive to MCP-1. Accordingly the role of chemokines in the migration of LC remains uncertain.

The signal for LC migration from the epidermis seems to be mediated by protein kinase C (PKC) as the addition of an analogue of diacylglycerol, L-a-dioctanoyl glycerol (a physiological activator of PKC) applied on the skin of mice, depleted $Ia^+$ cells from the epidermis and increased the numbers of FITC positive cells in the lymph node after topical treatment with FITC(Halliday and Lucas, 1993). Further evidence supporting the hypothesis that PKC is the mediator of the migration signals comes from experiments where the addition of the PKC inhibitors, palmitoyl-DL-carnitine chloride or D-sphingosine were shown to block LC migration in response to TNCB (Halliday and Lucas, 1993).

In summary, LC are a population of cells that migrate carrying antigen from the skin to the draining lymph node via the dermis. They can be visualised in transit as veiled cells and in the lymph node as interdigitating cells. A variety of stimuli includes haptens, antigens, UV light which induce inflammation producing cytokines and perhaps chemokines. These signals subsequently induce the LC to migrate to the DLN.

#### 1.4.1.8 Summary

The LC is a bone marrow derived cell which forms a network in the epidermis. LC express a wide variety of immunological markers many of which are in common with cells of the myeloid lineage. However under normal circumstances, it is the only MHC II or CD1 expressing cell in the epidermis. LC can take up and process antigens in the skin and re-expresses them in association with MHC II. The LC can be stimulated to migrate to the draining lymph node by stimuli such as inflammation. Once in the lymph node the LC undergoes dramatic phenotypic changes to resemble a interdigitating cell, including up-regulation of expression of co-stimulator and adhesion molecules, thus enabling the stimulation of naive and immune T cells. In turn primary and secondary immune responses are stimulated. This process results ultimately in the production of effector cells including Th1 cells, Th2 cells, cytotoxic T cells or antibody producing plasma cells. These cells can then respond to foreign antigens present within the skin. Further, the ability of LC to induce and

stimulate CHS and delayed type hypersensitivity (DTH) responses antigens, reflects the LC crucial role in cutaneous immunity and immunosurveillance.

### *1.4.2 Keratinocytes*

Keratinocytes make up the majority of cells in the epidermis. They have tonofilaments which are joined to form tonofibrils and desmosomes, which are small interlocking cytoplasmic processes which maintain continuity between the keratinocytes. The major function of these cells is to produce the protein keratin which is found in the skin, nails and hair. The keratin produced is visible as granules which fill the cytoplasm of the keratinocyte, giving rise to a horny cell. These form the external protective layer of the epidermis. Keratinocytes also synthesise Vitamin D from sunlight and its pre-cursor 7-dehydrocholesterol (Hunter *et al.* 1989).

Keratinocytes have been shown to play a role in lymphocyte maturation. Co-culture of keratinocytes with bone marrow cells has been shown to induce early markers of T cell maturation, including the Thy-1 antigen (Rubenfield *et al.*, 1981). Similarly co-culture was able to induce increase maturation of human thymocytes and the induction of CD1, a thymocyte antigen in a mature leukemic cells lines (Chu *et al.*, 1987). This antigen is normally expressed in the epidermis by LC and the induced expression may therefore be a reflection of the environment and not the maturity of the T cell. Generally, however, it appears the keratinocytes are able to induce further maturation of immature T cells *in vitro*.

Keratinocytes secrete numerous cytokines constitutively, including IL-1$\alpha$, IL-1$\beta$, IL-3, natural killer (NK) cell augmenting factor, IL-6, monocyte chemotaxis and activating factor, GM-CSF, macrophage-colony stimulating factor, hepatocyte stimulating factor, lymphocyte differentiating factor, thymopotein-like-factor and IFN$\alpha$. Keratinocytes may be induced to secrete other cytokines such as IL-8 (stimulated by IL-1$\alpha$ and TNF$\alpha$) and TNF$\alpha$, which is expressed in response to UVB exposure and LPS (Matsue *et al.,* 1992). Other cytokines secreted are transforming growth factor (TGF)-$\alpha$, TGF-$\beta$ and platelet-derived growth factor (Ansel *et al.,* 1990). The immunomodulatory cytokines produced by keratinocytes are thought to play a role in regulating the antigen presentation by LC (Grabbe *et al.,* 1992) as well as other immunological events in the epidermis such as inflammation (Matsue *et al.,* 1992).

Keratinocytes have been shown to constitutively express mRNA for both the p35 subunit of IL-12 and the p40 subunit (Aragane *et al.*, 1994). Previously mRNA encoding the p40 subunit could only be detected in the epidermis after

skin was treated with contact allergen (but not irritants or tolerogens) (Muller *et al.*, 1994). Importantly up-regulation by UVB of both has been shown *in vitro* and *in vivo* (Enk *et al.*, 1996).

Keratinocytes have been shown to express the MHC II molecule in disease states (Lampert *et al.*, 1981) and may be induced to express MHC II on their surface by exposure to the cytokine IFNγ (Gaspari *et al.*, 1988a). Combined with the constitutive secretion of IL-1 (Ansel *et al.*, 1990) this is suggestive of an involvement in antigen presentation, albeit this has not been conclusively demonstrated. Gaspari *et al.*, (1988a) has shown that MHC $II^+$ keratinocytes in mice were able to induce antigen specific unresponsiveness in hapten specific T helper 1 clones. The mice used were treated with 50,000 units of IFNγ per day for six days to induce MHC II expression by the keratinocytes (Gaspari *et al.*, 1988b), possibly reflecting induction of MHC II expression induced by inflammation. MHC II expressing keratinocytes are able to support T cell proliferation following the stimulation of the T cells with superantigens or with CD3 monoclonal antibodies (Dalme *et al.*, 1992). The cytokines produced by superantigen/keratinocyte stimulated T cells were IL-4, IL-5 and IL-10 but not IFNγ (Goodman *et al.*, 1994) suggestive of a Th2 like response as it is proposed the generation of a Th1 response requires the presence of the B7 group of co-stimulatory molecules (Nickoloff and Turka *et al.*, 1994).

Keratinocytes have been shown to react with the BB1 mAb (Nickloff *et al.*, 1993, Fleming *et al.*, 1993), but not with mAbs, against B7-1 or the CTLA-4 fusion protein which binds both B7-1 and B7-2 (Nickloff *et al.*, 1993). The epitope recognised by this mAb has been designated B7-3 and appears to be a ligand for CD28. However the effects of this interaction are at this time unknown. B7/BB1 expression is found focally on keratinocytes in allergic contact dermatitis and lichen planus (Simon *et al.*, 1993). The regulation of the expression of BB1 by keratinocytes is found to be mediated via a unique calcium mediated event *in vitro*. Furthermore, loss of BB-1 expression is triggered by increasing calcium (Nasir *et al.*, 1995) but what this means *in vivo* is unknown. However the addition of mAb's against BB-1 did not reduce T cell proliferation and this is suggested to operate through other mechanisms such as the heat stable antigen or the invariant chain (Nickloff and Turka, 1994). Keratinocytes cannot therefore stimulate an allogeneic immune response normally. It has been hypothesised that this may be due to the lack of expression of the invariant chain and therefore alteration of antigen presentation by MHC II (Nickoloff and Turka, 1994). B7-1 transfected

keratinocytes have also been shown to stimulate an allogeneic immune response, albeit this was observed not to be very potent (Nickoloff *et al.*, 1995). Contradicting the suggestion that lack of stimulation was due to lack of invariant chain expression, IFNγ treated transfected keratinocytes, expressing B7-1 and B7-2, were able to generate a vigorous primary allogeneic T cell response (Vercauteren *et al.*, 1995). Further the ability of keratinocytes to present *mycobacterium leprae* antigens to T cells (Mutis *et al.*, 1993), shows that MHC II expressed on keratinocytes is functional. Therefore the inability to induce allogeneic responses is due to the lack of B7 expression (Otten *et al.*, 1996). The considerable weight of evidence is strongly suggestive that keratinocytes are "non professional" antigen presenting cells.

Keratinocytes have been shown to mediate antigen specific tolerance (Gaspari *et al.*, 1988), possibly through their lack of expression of B7-1 or B7-2 (Nickoloff *et al.*, 1995). Transgenic mice with B7-1 expression by keratinocytes were able to amplify the primary immune response to oxalozone (Williams *et al.*, 1994). The ability of B7-1 transfected keratinocytes to stimulate an immune response is also supported by Nasir *et al.*, (1994).

Constitutive expression of another adhesion/co-stimulatory molecule, ICAM-1 is observed on keratinocytes in normal human skin (Lontani *et al.*, 1996) Higher levels of ICAM-1 can be induced in inflammatory disease (Singer *et al.*, 1989) and its expression is increased by TNFα (Krutmann *et al.*, 1990, Barker *et al.*, 1990), IFNγ treatment (Dustin *et al.*, 1988) or UV treatment (Krutmann *et al.*, 1990). The ability of keratinocytes to respond to IFNγ by up-regulation of ICAM-1 is progressively lost during differentiation (Little *et al.*, 1996). The ability of ICAM-1 to be induced by TNFα and UVR is highly variable from individual to individual (Middleton and Norris, 1995). Keratinocytes can also be induced to express the Fas antigen by IFNγ treatment (Sayama *et al.*, 1994). Addition of mAb against Fas, led to apoptosis in 32% of keratinocytes.

Keratinocytes play an important role in the skin immune system due to their secretion of numerous cytokines and expression of immunological cell surface proteins, including adhesion and co-stimulatory molecules. This gives the keratinocyte the ability to influence the outcomes of immune responses in the skin. The keratinocyte acts as a "non-professional" antigen presenting cell due to its lack of expression of essential co-stimulators such as B7-1 or B7-2 needed to induce primary immune responses. However, the production of both IL-12 and IL-10 indicates that the keratinocyte can directly influence the nature of the immune responses generated within the skin, thus determining whether it

is driven towards a Th1 or Th2 like response. Nickoloff and Turka, (1994) propose that the keratinocyte is able to induce active responses of the Th2 type which can be mistaken for anergy if Th1 functions are only examined.

### *1.4.3 γδ T cells*

A population of dendritic cells, that were found to form a network within the murine epidermis were shown to express the Thy1 antigen. The Thy1 antigen is mainly expressed in lymphocytes in the thymus (Bergstresser *et al.*, 1983, Tschaler *et al.*, 1983). It was shown by Tschaler *et al.*, (1983) that these Thy1$^+$ dendritic cells contained a lobulated nucleus, dense cytoplasm, and abundant filaments, but did not contain keratin filaments, desmosomes, melanosomes, Birbeck granules or Merkel cell granules which separated them from other epidermal cells. It was proposed by Bergstresser *et al.*, 1983) that these cells may be a population of intra-epidermal T cells and it was later shown by Breathnach *et al.*, (1984) that these cells were derived from the bone marrow. Thy1 is thought to act as a signal transducing molecule and is shown to trigger a rise in intracellular calcium in transfected B cells and T cells (Kroczek *et al.*, 1986). Thy1$^+$ dendritic cells were shown to express the γδ T cell receptor (TCR 1) Kuziel *et al.*, 1987 as well as CD3, which is required for effective TCR signalling (Shimada *et al.*, 1990b). Dendritic epidermal T cells (DETC) were found to express the Vγ3/Jγ1-Cγ1 and Vδ1/Dδ1/Jδ2-Cδ genes almost exclusively, suggesting a limited repertoire of antigen recognition (Kuziel *et al.*, 1987, Asarnow *et al.*, 1988 Asarnow *et al.*, 1989, Havran and Allison, 1990). Other subsets, such as Vγ4, are present (Ota *et al.*, 1992) and TCR αβ$^+$ DETC have been identified in chimera studies and were found to be Thy1$^+$ (Shiohara *et al.*, 1993). DETC were shown not to express either CD4 or CD8 but did express CD45, reviewed by Bergstresser *et al.*, (1993). γδ T cells in the intraepithelial cells of the intestine of the mouse (Goodman and Le Francois, 1988) and sheep (Gyorffy *et al.*, 1992) and in human splenic sinuosoids have been shown to express CD8. The network of DETC which appear in the mouse epidermis appear not to be present in humans (Cooper *et al.*, 1985a). However, γδ T cells seem to be associated with the pathogenesis of various human diseases, including some that involve skin (reviewed by Bergstresser *et al.*, 1993) and they also show a strong preference for the epidermis and papillary dermis in various disease sates (Horn and Farmer, 1990).

One of the major functional capabilities of γδ T cells appears to be cytotoxcity as they have been shown to act as non-MHC restricted killer cells

similar to that of LAK cells and NK cells (Bensussan *et al.*, 1989). Koide *et al.*, (1990) demonstrated that some γδ T cells are positive for CD57, an NK cell marker. They have also been shown to contain cytoplasmic granules similar to that of αβ CTL and NK cells as well as perforin and serine esterase mRNA, which is also typical of cytotoxic cells (reviewed by Taylor and Cohen, 1992). γδ T cells have been shown to kill numerous tumour cell lines including YAC-1, a leukemia cell line (Nixon-Fulton *et al.*, 1988), UV light transformed fibrosarcoma cells (Okamoto *et al.*, 1988) and K562, a natural killer cell target (Koide *et al.*, 1989). DETC have the ability to recognise and lyse transformed keratinocytes and melanocytes (Kaminski *et al.*, 1992). This suggests that one of the roles of DETC is to eliminate transformed cells *in vivo*. γδ T cells may also play a role in recognising and lysing virus infected cells as they have been shown to lyse virus infected cells in the rhesus monkey (Malkovsky *et al.*, 1992). In addition, human γδ T cells have been shown to recognise and destroy herpes simplex virus (HSV)-1 and HSV-2 infected cells (Maccario *et al.*, 1993). The expansion of γδ T cells in response to viral infection is seen *in vivo* (De Paoli *et al* ., 1991, De Maria *et al.*, 1992). Murine γδ T cells have also been shown to lyse xenogenic rat cells (Okumura *et al.*, 1995). Freshly isolated DETC fail to exhibit cytotoxic activity but it acquire it in culture after mitogen stimulation in the presence of IL-2 (Nixon-Fulton *et al.*, 1988). Freshly isolated γδ T cells from the murine intestine did exhibit cytotoxicity (Goodman and Le Francois, 1989). However DETC, which are freshly isolated from the skin express the mRNA for perforin (Kobata *et al.*, 1990) suggesting that once activated DETC can act as killer cell. Hence their role in the skin may be to "eliminate stressed, damaged or transformed cells" (Bergstresser *et al.*, 1993).

γδ T cells play a protective role in various infections, accumulating in disease lesions (Modlin *et al.*, 1989). In addition γδ T cells have been shown to play a protective role in *Listeria monocytogenes* infection in both primary and secondary infections albeit, they appear to be less important than αβ T cells (Mombaerts *et al.*, 1993). A unique lesion was found in the liver of γδ T cell deficient mice in response to this pathogen. Rakhmilevich, (1994) found that neither γδ T cells or αβ T cells were required in resolving a primary infection but both contributed to a secondary response, although γδ T cells were of less importance. γδ T cells are activated *in vitro* by *Mycobacterium tuberculosis* (Janis *et al.*, 1989, O' Brien *et al.*, 1989) and appear to accumulate at sites of infection *in vivo* (Griffin *et al.*, 1991). This activation of γδ T cells results in their secretion of large amounts of IL-2, however a lesser proliferative response than that with $CD4^+$ cells was observed. The response by these two cell types to the

same infection despite differences in activation produces similar effects such as IFNγ secretion and cytotoxicity. γδ T cells have also been shown to play a role in resistance to *Toxoplasma gondii* and have role mediating the expression of the 65kD heat shock protein (HSP) on macrophages, an important resistance factor (Nagasawa *et al.*, 1994). *L. monocytogenes* induces Th1 cytokine secretion in γδ T cells (IFNγ) and *Nippostrongulus brasilensis* induces Th2 cytokine secretion (IL-4), the same response as $CD4^+$ αβ T cells, showing that γδ T cells may contribute to the overall direction of the immune response. Freshly isolated DETC express mRNA for IFNγ, and upon concanavalin A (Con A) stimulation express mRNA for IL-2 and when expanded by further addition of IL-2 expressed mRNA for IL-4, IL-2, IFNγ, IL-1α, IL-3, IL-6, IL-7, TNFα, TNFβ and GM-CSF (Matsue *et al.*, 1993). This wide variety of cytokines that DETC produce suggest γδ T cells may influence the pathogenesis of various diseases, including intracellular bacteria, protozoan and viral infections. A clear role of DETC and γδ T cells in immune responses to pathogens is unknown as the ligands γδ T cells recognise are not yet clearly defined.

The lack of diversity in TCR expressed by DETC suggests that the ligands recognised are restricted, thus Bergstresser *et al.*, (1993) propose that they may recognise a set of molecules expressed commonly in the epidermal micro-environment. Some candidates include heat shock proteins (Asarnow *et al.*, 1988), MHC II (Matis *et al*, 1989), MHC I (Spits *et al.*, 1990) and non classical MHC I, including TL antigens in mice (Van Kaer *et al.*, 1991), CD1 (Porcelli *et al.*, 1992) and T22 (Weintraub *et al.*, 1994) in humans and indeed DETC can be activated by self antigens (Huber *et al.*, 1995a). Unlike conventional αβ T cells, transgenic γδ T cells were able to be preferentially stimulated by αβ T cells rather than dendritic cells (Spaner *et al.*, 1995). This also illustrates that γδ T cells appear to utilise a different co-stimulatory pathway which is independent of CD28/B7 interactions in allogeneic reactions (Penninger *et al.*, 1995). Although both αβ T cell and γδ T cell alloreactivity is mediated by foreign MHC bound peptides.

γδ T cells are also involved in autoimmune disease with increased numbers seen in the CSF and peripheral blood of multiple sclerosis. 43% of these cells reacted with 70 kD HSP compared to 7% of controls (Stinissen *et al.*, 1995). γδ T cells have also been isolated from the synovial fluid from patients with rheumatoid arthritis. Bergstresser *et al.*, (1993) proposes that DETC play a suppressive role by competing with αβ T cells that act as effector cells in autoimmunity.

The suppressive role of γδ T cells in other immune responses has been shown, with hapten coupled DETC able to induce hapten specific unresponsiveness (Sullivan *et al.*, 1986) and infusion of allogeneic DETC leads to recipients failing to respond to subsequent alloantigen challenge (reviewed by Bergstresser *et al.*, 1993). This is in direct contrast to LC which also form a network in murine skin and are potent inducers of CHS and DTH. The DETC density in the epidermis of various mouse strains has been shown to inversely correlate with the capacity of the animals to mount a CHS response (Bigby *et al.*, 1987). Cytokines produced by keratinocytes after contact sensitiser treatment have been shown to activate γδ T cells (Huber *et al.*, 1995b), and it appears that DETC migrate into the epidermis in response to TNFα, possibly produced by keratinocytes (Tamaki *et al.*, 1994). This provides further evidence of their importance in skin immune responses.

It was proposed recently by Love-Schimenti and Kripke (1994), from evidence gathered *in vitro* during a CHS response that DETC are activated by IL-2 and then eliminate autologous hapten specific T cells in the skin. This would limit the effector phase and the destruction of hapten modified cells. The authors suggested that this cytotoxic activity against antigen reactive αβ T cells may able to account for the ability of DETC to induce tolerance *in vivo*. While LC appear to induce immune responses within the skin, DETC appear to down-regulate skin immune responses. The γδ T cell has also been proposed to play a role in the control of inflammation and tissue necrosis, prevent exaggerated inflammatory responses that may be harmful to the host by interacting with neutrophils or other inflammatory cells (Fu *et al.*, 1994).

The role of the γδ T cells in immune responses is still not clear. They appear to be important for providing immune protection against intracellular pathogens, including bacteria, protozoa and viruses. Cytotoxicity appears to be an important functional role, as does the production of cytokines. DETC in mice have been shown to be important in skin immune responses, possibly playing a down-regulatory role in CHS responses and inflammation. While a similar network appears not to be present in human skin, the γδ T cells association with disease occurring within the skin suggests that γδ T cells may still partially influence skin immune responses.

### *1.4.4 Melanocytes*

Melanocytes are the only cells in the body that can produce the pigment melanin. They are dendritic in nature and are closely associated with

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### *1.4.4 Melanocytes*

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keratinocytes in the epidermis. Melanocytes contain unique organelles, the melanosomes, in which tyrosine is converted to Dopa and then dopaquinone by the enzyme tyrosinase. The eumelanins, which are black/brown pigments are formed by the polymerisation of dopaquinone. This compound can also react with cysteine groups to give rise to the phaeomelains which are reddish pigments (ie red hair). Fully melanised organelles are passed via the dendritic processes into the surrounding keratinocytes where they become distributed throughout the keratinocytes cytoplasm (Hunter *et al.*, 1989).

Melanogenesis is controlled by a variety of stimuli, including hormones, however, the most important stimulator of this process is UV light. After exposure to UVA (320-400nm) tanning was shown to occur in two stages immediate and delayed tanning. Immediate tanning is due to the oxidation of pre-formed melanin, while delayed tanning is due to the synthesis of new pigment (Pathak *et al.*, 1962 in Kaidbey and Kligman, 1979). Melanin migrates upwards from the epidermis within the keratinocytes were it is shed during the process of desquamation.

Negroes have dispersed melanosomes and are more intensely pigmented, where as in Caucasians they are aggregated and are degraded more readily by lysosomes. This results in higher concentrations of melanin in Negro skin (Olson *et al.*, 1970 in Olson *et al.*, 1973). Olson *et al.*, (1973) found that dark Negro skin was 33 times less susceptible to erythema than Caucasian and this was due to the size and density of the individually dispersed melanosomes. These authors proposed that the resistance to UV light induced erythema, may be due to the ability of melanosomes to absorb free radicals which, even today are believed to be a mediator of UV damage.

Melanocytes are also able to produce a variety of cytokines including IL-1, IL-6, GM-CSF and PDGF (Ansel *et al.*, 1990). However, production of these factors by the more numerous keratinocytes, means that melanocytes provide only a minor contribution to the cytokine milieu in the epidermis.

### *1.4.5 Merkel Cells*

Merkel cells are neuroendocrine cells found in the epidermis. They contain spherical granules and sparse desmosomes by which they are connected to keratinocytes They are also found adjacent to non-myelinated nerve endings (Hunter *et al.*, 1989). These cells have been shown to secrete the neurotransmitter, vasoactive intestinal peptide (VIP) (Ansel *et al.*, 1990) which is found in the granules within the cell. VIP alters immune functions,

including inhibition of lymphocyte proliferation in peyers patches (Roitt *et al.*, 1991). Infusion into the afferent lymphatics of sheep, reduced the output of small and blast lymphocytes via the efferent lymphatic (Moore *et al.*, 1988). Further the neurotransmitter calcitonin gene-related peptide (CGRP) has been shown to play a role in inflammation where it is found in association with nerve fibres and Merkel cells. CGRP causes vasodilation and inhibits antigen presentation by LC (Hosoi *et al.*, 1993). LC were shown in Hosoi's study to be intimately associated with the nerves containing this peptide.

CGRP together with nitric oxide have been demonstrated to be released by sensory nerve fibres after UV exposure. This occurs when the levels of other epidermal cell derived mediators have returned to normal (Gillardon *et al.*, 1992, Benrath *et al.*, 1995). The topical administration of the CGRP receptor antagonist restored the capability of the immune system to respond to haptens applied through UV exposed skin (Benrath *et al.*, 1995). The soluble factors produced by cells of neuroendocrine origin in the skin may play a role in the inflammatory and possibly immunological responses within the skin microenvironment, especially after UV exposure. However, the exact role of the Merkel cell and its interaction with the skin immune system is not fully understood.

### *1.4.6 Monocytes/Macrophages*

Cells of the monocyte/macrophage lineage are known to occur in the human dermis at around 30% of the density of LC in the epidermis, though if total numbers are compared the macrophages of the upper dermis clearly outnumber the LC (Weber-Matthiesen and Strerry, 1990). These authors have shown that the dermal macrophage population is heterogenous with the morphology varying from moderately dendritic, to flat cells, to large intravascular cells. The phenotype of these macrophage populations studied using mAbs, revealed that the cells had the characteristic phenotype of phagocytic macrophages and lacked features of immune macrophages, of which only the LC was phenotypically similar (Weber-Matthiesen and Strerry, 1990). $OKM5^+$ cells were identified as occurring at the dermal/epidermal junction but were not found to be MHC $II^+$ and were not of the phagocytic phenotype. After UV exposure a population of $OKM5^+$ macrophages appears in the epidermis which are proposed to originate from the dermis (Baadsgaard *et al.*, 1987). The role of dermal macrophages in the skin immune system is not clear but with multiple phenotypes they are likely to have multiple roles.

### 1.4.7 Dermal Dendritic Cells

A population of dermal dendritic cells has been shown to express MHC II antigens in both mouse (Sonthiemer *et al.*, 1989) and human skin (Cerio *et al.*, 1989). Dendritic cells in the murine skin that were Factor XIII- and Thy1.2- were capable of providing accessory cell function in Con A stimulated T cells. They were able to present alloantigens, process and present large protein antigens (Cooper *et al.*, 1987); initiated DTH responses (Tse and Cooper, 1990). Further characterisation by Duriswamy *et al.*, (1994) showed that the LC like APC function of dermal DC was due to a population of interstitial DC that were MHC $II^+$ $CD11b^-$. These cells, due to their position adjacent to the vasculature are likely to be the first APC which T cells emigrating into the skin would come in contact. Optimal sensitisation studies by Kurimoto and Streilein (1993) demonstrated that excess hapten painted on the skin was taken up by both LC in the epidermis and dermal DC, both of which could then initiate CHS. Further, the dermal DC from UV irradiated CHS resistant mice were able to stimulate CHS responses, while those from CHS sensitive mice induced tolerance (Kurimoto *et al.*, 1994). This suggest that DC from the dermis are capable of acting in a similar manner to LC in the skin immune system.

To reach the lymph node, LC must migrate through the dermis. Therefore is likely that a portion of APC function provided by the dermis may be due to these migrating cells. Richters *et al.*, (1995) found a population of cells migrating from human skin explants that expressed CD1b. The kinetics of the migration suggests this marker may be induced transiently in dermis in LC migrating from the epidermis to the DLN.

### 1.4.8 Mast Cells

Mast cells are bone marrow derived cells that are found in the loose connective tissue of the dermis as well as many other tissues within the body. mast cells upon activation release a variety of preformed mediators including histamine, serotonin, neutral proteases, acid proteases, proteoglycans (eg heparin) and cytokines including TNFα (reviewed by Van Loveren *et al.*, 1997). After receiving an appropriate stimulus, mast cells synthesise prostaglandins $D_2$, $E_2$ and $I_2$, as well as various leukotrienes and platelet activating factor. Mast cells *in vitro* have been shown to increase mRNA levels or secrete *de novo* synthesised IL-1, 3, 4, 5, 6, 10, 13, TGFβ, stem cell factor, GM-CSF, IFNγ, MIP-1α and β, T-cell activation factor 3 and TNFα. Stimuli that lead to mast cell activation include complement proteins (C5a and C3a), neuropeptides

including substance P and CGRP, UV light and the binding of immunoglobulins such as IgE and IgG (reviewed by Van Loveren *et al.*, 1997). The same reviewers also highlighted the ability of mast cells to influence inflammatory responses occurring within the skin including immediate and delayed hypersensitivity reactions.

## 1.5 ULTRAVIOLET LIGHT

### *1.5.1 Introduction*

Ultraviolet light forms part of the electromagnetic spectrum with wavelengths between 200-400nm. Ultraviolet light is broken into three parts UVA (320-400nm), UVB (290-320nm) and UVC (200-290nm)(Kochevar, 1983, Spikes, 1983). Solar UVC is absorbed by ozone in the stratosphere and is not relevant in biological systems. The division between UVC and UVB was established at 290nm for this reason. Discussion of UVC effects on the skin therefore, are kept to a minimum is this review. The energy carried by each portion of the spectrum is inversely related to its wavelength so UVC>UVB>UVA. The depth of penetration of ultraviolet light into the skin increases with increasing wavelength.

The skin is the organ most susceptible to damage by UV light as it is directly exposed. UV exposure of the skin has a number of biological effects, many of which are detrimental. The following sections deal with the effects of UV light on the skin and its constituents, concentrating on immununological changes in a variety of animal, human and cellular models.

The peak of UV induced carcinogenicity has been shown to lie within the UVB portion of the UV spectrum (reviewed by de Gruji., 1996). The primary consequence of ozone depletion is the increased amount of UVB on the earths surface, it may, in turn lead to increased incidence of skin cancer and eye damage (Lloyd *et al.*, 1993). Combined with the possibility that UVB also alters immune responses to infectious agents (reviewed by Ward *et al.*, 1995), this has brought the detrimental effects of ultraviolet exposure to the forefront of scientific discussion. This is especially relevant in Australia, where the highest incidence of any single cancer, a skin cancer (basal cell carcinoma), is reported (Green *et al.*, 1997). Australia also lies within or near the "hole in the ozone layer", perhaps, further increasing the risk of developing such cancers in this country (Osterberg and Szarfmen, 1996).

### 1.5.2 *Ultraviolet Light Methodology*

A variety of light sources has been used to study the effect of UV light on skin. These light sources vary considerably in their emission spectra, advantages and disadvantages relative to each experimental model used. A summary of different UV sources used in research, is presented in Table 1. Generally most groups currently use sunlamps in studying the effects of UV light on the immune system. However, this and the way they are used often varies from laboratory to laboratory. The use of sunlamps is widespread due to their relatively low cost and high UVB output. It has been suggested that the UVC output (non solar UV) by FS40 sunlamps is sufficient to affect MLR's and epidermal cell-lymphocyte reaction (ELR) experiments (Hurks *et al.*, 1995) However, Cooper *et al.*, (1996) have shown that the immunosuppression arising from 4 minimal erythemal doses (MED) of both FS40 sunlamps and solar simulator lamps are identical, thus validating the extensive use of sunlamps in research.

Usually UV treatment is given as a dose per unit area. However, this is often referred to in terms of minimal erythemal dose (MED), with little or no reference to the actual dose received. Urbach and Stern (1972) stated that " Furthermore it is not always clear that "minimal" refers to the shade of erythema produced or to the least amount in energy in one of series of increasing doses that produces erythema". Urbach subsequently stated that variables such as time to generate MED was rarely mentioned or spectral output of the light source used. Recent publications have begun to correct these oversights, allowing easier comparison between different studies. In humans the dose/exposure time to reach MED varies from individual to individual due to genetic differences reflected in the amount of melanin produced and stored. Table 2 gives a brief summary illustrating the varying doses required to generate MED in a variety of species and different sources of UV irradiation.

### 1.5.3 *Inflammatory Response*

#### 1.5.3.1 Vascular Events

One of the earliest observed inflammatory responses of the skin to ultraviolet irradiation was vasodilatation of cutaneous blood vessels (Finsen, 1899) resulting in the production of erythema. He postulated this was caused by exerting a direct effect on the vessels. An alternate theory by Partington, (1954) was that this effect was produced by soluble vasoactive mediators, although no direct evidence was provided for either hypothesis. The time

**Table 1.1 Ultraviolet light sources used for research**

| Light Source | UV Emission spectra | Erythemal dose (time) | Advantages | Dis-advantages | Peak Output |
|---|---|---|---|---|---|
| Terrestrial Sunlight | 290-400 nm | 20 min at lat 40° June midday (nthn hemisphere) | "Natural" Economical Large field | Variable output, Hard to predict | ~400 nm |
| Carbon arc | 200-400 nm | 5-30 sec at 25 cm | Emission spectra close to sunlight | Variable output, Large and bulky | ~400 nm |
| Cold quartz | discontinuous 253.7 predominantly | 30 sec at 25 cm | No increase in pigment, inexpensive | Not relevant to sunlight as totally UVC | 253.7 nm |
| Hot quartz | discontinuous 254,265,297, 303,313 and 365 nm | 30-60 sec at 45 cm | Inexpensive, relatively intense UVB | Warm up, discontinuou s emission (Not Natural) | 365 nm |
| Fluorescent sun lamps | 270-390 nm | 60-120 sec at 25 cm | Large field size when used in banks, intense UVB, Inexpensive | Lamp output decreases with time, (70% after 500 hrs) (Not natural some UVC) | 313 nm |
| Fluorescent black light | 310-400 nm | 15-16 min at 25 cm | Stimulation of Pigment production | Long exposure time to generate erythema | 365 nm |
| Narrow Band (UVB) | 311-312 nm | 5-9 min at 25 cm | Exposure to is less hazardous, Study of specific wavelengths | Not Natural, Long exposure time to generate erythema | ~312 nm |
| Xenon arc | 290-400 | 12 sec at 55 cm | Emission spectra close to natural sunlight | Expensive, Technical, Bulky, Small field size | 400 nm |

Adapted from, Anderson, (1993)

**Table 1.2 Minimal Erythemal Dose for a Variety of UV sources and species**

| Species | Average Dose for MED ($J/m^2$) | Main UV range | Source of UV irradiation | Reference |
|---|---|---|---|---|
| Guinea pigs | 900 | UVB | Monochromatic 295nm | Eaglstein *et al.*, 1979 |
| Guinea pigs | 260 | UVC | Monochromatic 250nm | Eaglstein *et al.*, 1979 |
| Guinea pigs | 246 | UVB | Sunlamp | Morison *et al.*, 1981 |
| Human Dermal microvascular endothelial cells | 30 (10% UVB reaches the dermis) | UVB | Sunlamp | Cornelius *et al.*, 1994 |
| Humans | $5x10^5$ | UVA | | Gilchrest *et al.*, 1983 |
| Humans | $5.15x10^5$ | UVA | | Parrish *et al*, 1981 |
| Humans | $5.52x10^5$ | UVA | Xenon arc(ss) (335 filter) | Arbabi *et al.*, 1983 |
| | | | | |
| Humans | $6.6x10^5$ | UVA | Xenon arc(ss) (335 filter) | Paul and Parrish, 1982 |
| Humans | $8.6x10^5$ | UVA | Xenon arc(ss) (345 filter) | Kumakiri *et al.*, 1977 |
| Humans | $1.2x10^6$ | UVA | UVA sunlamp (330nm &UG1 filter) | Baadsgaard *et al.*, 1987 |
| Humans | 184 | UVB | Sunlamp | Kaidbey and Kigman, 1979 |
| Humans | 200 | UVB | Monochromatic 300nm | Olson *et al.*, 1966 |
| Humans | 220 | UVB | Sunlamp | Paul and Parrish, 1982 |
| Humans | 200-300 | UVB | Monochromatic 300nm | Norris *et al.*,1991 |
| Humans | 264 | UVB | Hot Quartz | Tanenbaum *et al.*, 1976 |
| Humans | 264 | UVB | Xenon arc(ss) (345 filter) | Kaibey and Kligman, 1975 |
| Humans | 291-327 | UVB | Sunlamps | Cooper *et al.*, 1992 |
| Humans | 300 | UVB | Sunlamp | Cornelius *et al.*, 1994 |
| Humans | 400 | UVB | | Parrish *et al*, 1981 |
| Humans | 437 | UVB | Sunlamp | Arbabi *et al.*, 1983 |
| Humans | 450 | UVB | Hot Quartz | Gilchrest *et al.*, 1981 |
| Humans | 975 | UVB | T12(307nm filter) | Baadsgaard *et al.*, 1987 |
| Humans | 992 | UVC | TUV(286nm filter) | Baadsgaard *et al.*, 1987 |
| C3H Mice | 1600 | UVB | TL20 | El-Ghorr *et al.*, 1995 |

course seen for the production of erythema is biphasic with an initial erythema observed before the completion of the treatment. This is followed by a further increase usually of greater magnitude occurring an hour later and lasting for 24-48 hours. The reddening that occurs at this stage is also believed due to an increase in vasodilation (Logan and Wilhelm, 1963, Kaidbey and Kilgman, 1979). Later studies made used more sensitive instruments that measured reflectance, were able to show that vasodilation preceded visually perceptible erythema (Diffey and Oakley, 1987). The degree of erythema observed after treatment with UV light increases proportionally with dose until at least 15 MED (Farr and Diffey 1984). Eventually, at higher doses blistering, and other signs of gross damage are seen. Erythemal responses are also influenced by environmental factors, including heat, wind, humidity and water immersion (Freeman and Knox, 1964, Owens *et al.,* 1974 Owens *et al.,* 1975).

Logan and Wilhelm (1966a) also investigated the erythemal reaction in a variety of animal models including rats, rabbits and guinea pigs. They looked at various aspects, including vascular permeability, tissue leucocytosis and the production of erythema and found variation in the exposure times needed to provoke erythema in the different species.

Adaptive changes are also observed in the skin after exposure to UV light. These include epidermal thickening (hyperplasia) and melanogenesis (tanning)(Rosario *et al.*, 1979) which provide some degree of protection against further UV insult. Increased melanin production provides increased protection against the harmful effects of UVR (Shono *et al.*, 1985), to the extent that Negroes with a high melanin content, have a MED 33 times higher than their Caucasian counterparts (Olson *et al.*, 1973). Increases in melanin production correlating with increasing UV dose are also observed in some human populations (Shono *et al.*, 1985).

Many factors have been suggested as potential mediators for the erythemal response of skin to UV light however, most of these have been found to have no or only a minor effect. Logan and Wilhelm (1966a) thought the early erythemal response to UV light was similar to that of thermal injury which had been shown to be mediated by histamine. However, the addition of anti-histamines did not effect the induction of erythema in either the guinea pig or rabbit models used. The early response seen in rats was mediated by serotonin (5-HT) as the addition of an antagonist (BOL-148) blocked the induction of erythema, while the addition of anti-histamines had no effect. Greaves and Sondergaard (1970) have shown UVB erythema in humans is

linked with the action of a smooth muscle contracting agent. This agent was clearly different from histamine, bradykinin, serotonin or acetyl choline and was shown to be a fatty acid, possibly a prostaglandin. Indeed, an increase in the production of the prostaglandins $PGE_2$ and $PGF_{2\alpha}$ and their precursor arachidonate was evident 24 hours after UVB exposure of the skin (Black, *et al.*, 1978). Inhibitors of prostaglandin synthesis including aspirin (Miller *et al.*, 1967) and indomethacin (Snyder and Eaglstein, 1974, Greenberg *et al.*, 1975, Kaidbey and Kurban, 1976, Eaglstein and Marsico, 1975) had been previously noted to decrease the erythemal response but not eliminate it, providing indirect evidence for the involvement of prostaglandins. The presence of increased amounts of prostaglandins could not, however, be demonstrated at 48 hours post-treatment thus suggesting that these compounds are only responsible for the early erythemal response. The application of indomethacin topically, intradermally or orally, has been shown not to effect UVA or PUVA induced erythema (Morison *et al.*, 1977) Thus UVA induced erythema is not due to a prostaglandin mediated event.

Recently, nitric oxide (NO) has been shown to be produced by keratinocytes after UVB irradiation. This NO release is dose dependent and the enzyme required to synthesise NO is constitutively expressed by these cells (Deliconstantinos *et al.*, 1995). The same authors demonstrated that in, the guinea pig, an inhibitor of NO synthetase (L-NMMA) was shown to have a UV protection factor (PF) of 8.71. The authors concluded that this may be a major part of the integrated inflammatory response to UV light leading to vasodilation and erythema. Current evidence suggests that DNA is the chromophore for erythema, as the action spectrum for the frequency of pyrimidine dimers *in vivo* closely matches the action spectrum for erythema up to 334nm (Freeman *et al.*, 1989).

The vasodilation and subsequent erythema produced after exposure of the skin to ultraviolet light, is not due to the production of any one substance but is multifactorial with NO and prostaglandins possibly playing key roles. Other inflammatory substances including inflammatory cytokines may play minor but still important roles in the complicated reaction to UV light. The inflammatory response and its mediators cannot be considered in isolation. These, in turn, effect the immune response that may be generated from the affected area by altering adhesion molecule and cytokine expression of resident cell populations and the induction of non-resident cell types to migrate

into the area, resulting in the generation of an "inflammatory" linked immune response.

### 1.5.3.2 Morphology and Ultrastructural Changes

One consequence of UV exposure of the skin is damage to epidermal cells (Logan and Wilhelm, 1966a, Daniels *et al.*, 1961). This becomes evident as early as two hours after UV exposure. The earliest indicator of damage is a decrease in keratinosomes which results in the formation of dyskeratotic cells (Wilgram *et al.*, 1970). 16-18 hours after exposure intracellular oedema can be seen, followed at 30-48 hours by intercellular oedema which develops in the spaces around damaged keratinocytes. Sunburn cells (damaged keratinocytes) appear shortly before the presence of oedema is observed (Logan and Wilhelm, 1966a, Daniels *et al.*, 1961, Woodcock and Magnus, 1976, Obata and Tagami, 1985).

The sunburn cell was proposed as one of the early examples of apoptosis (Kerr *et al.*, 1972). UV induced apoptotic cells are rapidly phagocytised by the surrounding keratinocytes (Olson and Everett, 1975). Macrophages are also known to bind to and phagocytose apoptotic cells (Duvall *et al.*, 1985) and are known to increase dramatically after UVB treatment (Cooper *et al.*, 1992). All wavelengths of the UV spectrum including UVC are able to induce apoptosis rather than necrosis in Ly-5 murine lymphoma cells (Godar *et al.*, 1994). There appears to be two types of UV induced apoptosis, an immediate type apoptosis and a more classical delayed apoptosis (Godar *et al.*, 1995). The delayed apoptosis is thought to be induced by UV-induced damage of DNA rather than free radical damage (Godar *et al.*, 1995). Furthermore it was found that immediate apoptosis was due to constitutive factors which did not require protein synthesis, while the delayed type (inducible) apoptosis does require protein synthesis (Godar *et al.*, 1996).

Similar apoptotic changes are noted in epidermal LC. Which are especially sensitive to the effects of UV light (Aberer *et al.*, 1981, Obata and Tagami, 1985), with damage observed at lower doses than that observed in keratinocytes. LC are more sensitive to UV induced damage in comparison to keratinocytes for both UVA and UVB wavelengths (Aberer *et al.*, 1981). The degenerative changes for LC and keratinocytes include mitochondrial swelling and rupture, condensation of the cytoplasm and the appearance of pyknotic nuclei and nuclear fragments (Aberer *et al.*, 1981, Obata and Tagami, 1985, Daniels *et al.*, 1961, Nix *et al.*, 1965). The cellular damage is maximal at 48-72

hours for the total UV range (Rosario, 1979). *In vitro* exposure of an epidermal derived cell line to both UVA and UVB, has been shown to induce cytoskeletal blebbing, this being a characteristic of oxidative stress (Malorni *et al.*, 1994).

Another consequence of ultraviolet irradiation is the infiltration of leucocytes, predominantly neutrophils, into the damaged area. This becomes apparent 1/2 to one hour after treatment, with peak infiltration at 4-6 hours and this response concluded after 48 hours (Logan and Wilhelm, 1963). These studies conducted using guinea pigs showed that the peak in dermal leucocytosis occurred before both maximum erythema and vascular permeability, suggesting a cause and effect relationship. Later studies by the same authors in the rat and rabbit (Logan and Wilhelm, 1966a) suggested that lymphocyte infiltration was not as important in these animal models and peaked after both maximum erythema and vascular permeability. Eaglstein *et al.*, (1979) suggested that leucocytes have a role in UVB induced inflammation in guinea pigs but not that of UVA or UVC. The presence of neutrophils is seen in dermal inflammation due to UVB irradiation in humans. These cells were observed to be present a short time after irradiation increasing in number gradually until at least 48 hours post treatment (Hawk *et al.*, 1988). The onset was observed to be more gradual and less marked than other acute inflammatory responses. Neutrophils have the potential to add to the physical damage caused by UV light, as they are capable of releasing a variety of substances, including reactive oxygen intermediates which are damaging to cells and structures of the body (Darr and Fridovich, 1994). However, the role of neutrophils in UVB inflammatory responses is unclear (Strickland *et al.*, 1997), although they may have a role in "repair" processes such as increased elastin production (Starcher and Conrad, 1995).

The mast cell is a resident cell which has a role in UV induced inflammation. Physical changes in mast cells have been noted after UVB exposure, including the presence of hypogranulated and degranulated cells and the presence of extracellular granules in the surrounding tissue (Walsh *et al.*, 1995). It was also noted that a decrease in mast cell numbers occurred. In contrast mast cells in the lower dermis have been shown to increase in number after chronic UVB exposure of hairless mice though whether this a protective event is unknown (Kligman and Murphy, 1996).

### *1.5.4 UVA/UVB*

The erythemal responses of both UVA and UVB are thought to act through similar mechanisms, however the differences seen in skin changes which result are thought to be due to the variation in the depth of penetration associated with the different wavelengths through skin. Energy from UVB irradiation is absorbed over a short distance of the skin (mainly the epidermis), while greater amounts of light in the UVA region can penetrate to the dermis and beyond and so can disperse their energy over a greater depth (reviewed by Anderson, 1993

The wavelength of ultraviolet light influences the cytotoxicity seen, with UVB>UVA. Generally UVB acts mainly in the epidermis, while UVA injury tends to cause necrosis of the endothelial cells thus damaging the dermal blood vessels (Willis and Cylus, 1977, Rosario *et al.*, 1979, Zheng and Kligman, 1993). However, it should be noted that 10-15% of UVB penetrates to the dermis and hence it is possible for UVB to have a direct affect on microvascular endothelial cells (Everett *et al.*, 1966, Strickland *et al.*, 1997). *In vitro* studies by Cornelius *et al.*, (1994) also support a direct effect of UVB on endothelial cells.

Direct cytotoxic effects on cells in the skin are evident after UV exposure. Sunburn cells are visible in the epidermis after UVA irradiation, though fewer in number than the equivalent dose of UVB (Woodcock and Magnus, 1976, Kumakiri *et al.,* 1977). In contrast, a study using erythemal doses of UVA only, showed that while sunburn cells were absent from the epidermis perinuclear vacuoles were present in LC (Gilchrest *et al.*, 1983). The same study demonstrated the recruitment of neutrophils into the UVA irradiated skin. UVB doses that cause similar amounts of erythema, did not appear to induce this change (Gilchrest *et al.*, 1981), although this has been reported from rodent based experiments (Eaglstein *et al.*, 1979). Detailed studies by Hawk *et al.*, (1988) were also in direct contrast to those of Gilchrest *et al.*, (1981); the authors suggested that the lack of similar findings previously was due to "failure to obtain biopsy material at times of significant neutrophil infiltration". However, it must be noted that this study used a sunlamp which does contain approximately a third UVA.

A study by Tyrell and Pidoux (1987) suggests that UVA induced cytotoxicity may be just as, if not more important than UVB. When the sun is at its zenith (10am-2pm) and UVB maximal, UVA still makes up 50-60% of the

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phototoxic wavelengths found and this is increased at other times. UVA induced changes also include elastic fibre proliferation, randomly deposited microfibrils and duplication of vascular basement membrane, while after UVB some degradation of fibres is present. Microfibrils are also increased but the organisation remains; some collagen degradation also occurs (Zheng and Kligman, 1993).

### *1.5.5 UV Damage of DNA*

The heterocyclic bases of nuclear deoxyribonucleic acid (DNA) have been shown to be a major UV absorbing chromophore in the skin (Ely and Ross, 1949). The absorption (which is maximal between 260-265nm) leads to damage of the DNA which may result in the generation of tumours. DNA strongly absorbs energy in the UVB region of the spectra. The main lesions induced are pyrimidine dimers and pyrimidine-pyrimidone photoproducts, which make up 65% (Mitchell *et al.*, 1992) and 35% (Lippke *et al.*, 1981) of UVB induced DNA lesions respectively. Of the pyrimidine dimers formed 50% are thymine-thymine dimers (Vink *et al.*, 1993). These lesions have been shown to occur in the basal and suprabasal layers and can be found in Langerhans cells (Vink *et al.*, 1994). Pyrimidine dimers are proposed as an initiation step in mutagenesis and tumour formation (Hart *et al.*, 1977). Mutations in the *ras* oncogene were mostly found opposite dipyrimidine sequences (Pierceall *et al.*, 1992) providing more evidence for the role of pyrimidine dimers in tumour genesis. The exposure of skin to ultraviolet radiation also increases the proto-oncogene c-*fos* (Roddey *et al.*, 1994).

Exposure of human skin to two MED UVB induces keratinocyte and fibroblast induction of metallothionein genes *in vivo* (Anstey *et al.*, 1996). The metallothionein proteins are proposed to play a possible role in protecting the skin against photo-oxidative stress (Hanada *et al.*, 1992), which has been shown to induce strand breaks in DNA (Ghosh *et al.*, 1993). Thus expression of high levels of metallothioneins by basal keratinocytes may protect cells from UV induced DNA damage (Anstey *et al.*, 1996).

UVB light has been shown to induce nuclear factor κB (NFκB), although without the need for chromosomal DNA damage (Simon *et al.*, 1994). The gene is associated with IL-6 promoter region. Also found recently are two proteins, 40 kD and 68 kD in size, which bind, specific ultraviolet responsive elements on DNA, with the 40 kD protein playing a role in DNA replication (Yang *et al.*, 1993). The exact function of these proteins is unknown.

Ultraviolet light induces mutations in p53, an anti-oncogene (Brash *et al.*, 1991, Kanjilal *et al.*, 1993). This has been demonstrated in squamous cell carcinoma (Brash *et al.*, 1991). However mutations in p53 seem to be absent from melanomas (Lubbe *et al.*, 1994). p53 stops progression of cells through the normal cell cycle after UV exposure, thus allowing time for the cell to repair DNA damage. As p53 can also lead to the induction of apoptosis, it is not surprising that in p53 knockout mice, the formation of sunburn cells is an order of magnitude lower (Pierceall *et al.*, 1991). The heterozygote in this study displayed an intermediate phenotype suggesting that the alteration of one p53 gene in a cell is enough to cause an effect. Therefore sunlight may, by mutating a p53 gene, cause tumour initiation (DNA mutation) and by further exposure delete the normal cells (which undergo programmed cell death), allowing the p53 mutant to dominate leading to tumour promotion (reviewed by Ziegler *et al.*, 1996).

DNA damage (pyrimidine dimer formation) has also been proposed as a chromophore for the initiation of systemic immunosuppression in mice after UV irradiation (Kripke *et al.*, 1992). Photorepair of DNA damage is seen in the marsupial *Mondelphis domestica.* This animal contains photo-repair enzymes which are activated by visible light and prevent the induction of UV induced immunosuppression (Applegate *et al.*, 1989). Treatment of the skin with liposomes containing the excision repair enzyme T4 endonuclease V (T4N5), increases the rate of thymine dimer repair (Yarosh *et al.*, 1990) thus preventing the UV induced immunosuppression of DTH and CHS (Kripke *et al.*, 1992). Kripke *et al.*, proposes that DNA damage leads to the release of immunoregulatory cytokines. Indeed preliminary unpublished data by this group found that down-regulation of IL-10 production by a UV damaged keratinocyte cell line (PAM212) was seen to occur after treatment with T4N5 liposomes thereby providing a role for DNA damage in UV induced systemic immunosuppression (reviewed by Vink *et al*, 1996). Local immunosuppression is also manipulated by T4N5 treatment and prevents LC loss from the epidermis and partially reduces local immunosuppression (Wolf *et al.*, 1995). Normally, cells with UV damaged DNA can be observed in the DLN after UV treatment of the skin. Treatment of these UV damaged cells with the photolyase photorepair enzyme is able to restore the ability of these cells to induce a normal immune response. This treatment also prevents the induction of suppressor cells (reviewed by Vink *et al.*, 1996)

UV light is capable of directly damaging DNA causing mutation which may give rise to tumours, especially as mutations in oncogene and anti-oncogenes are shown to occur. DNA damage is the primary change required for the initiation of tumours, however for the tumour to become established a tumour promotion step is required. This secondary change may be provided by alteration of immune function and immunosurveillance within the skin after subsequent exposure to UV light.

## 1.6 IMMUNOLOGICAL EVENTS

### *1.6.1 UV Induced Immunosuppression*

Hanisko and Suskind (1963), observed that the contact hypersensitivity response seen in skin sensitised to DNCB was reduced if skin was previously exposed to sub-erythemal doses of UVB. Kripke (1974) investigated the antigenicity of UV induced tumours and showed that the induced tumours were highly immunogenic and were only transplantable to immunosuppressed hosts. Kripke and Fisher (1976) found that UV induced tumours were able to grow in the primary host due to a systemic alteration in the hosts immune response to the tumour. This was due to the UV exposure received, as UV irradiated recipients were unable to reject the tumours. UVB exposure has been shown to result in suppression of many immune parameters including lymphocyte function (Morison *et al.*, 1979), CHS responses (Haniszko and Suskind, 1963, Greene *et al.*, 1979, Toews *et al.*, 1980, Morison *et al.*, 1981a, Noonan *et al.*, 1981, De Fabo and Noonan, 1983, Elmets *et al.*, 1985, Elmets *et al.*, 1988) and DTH responses (Morison *et al.*, 1981b, Ullrich *et al.*, 1986, Glass *et al.*, 1989). However, little effect was shown on antibody production (Kripke *et al.*, 1977, Spellman *et al.*, 1984, Ullrich *et al.*, 1986, Chen *et al.*, 1997). Suppression of cellular immune responses was transferable with T lymphocytes and was specific for syngeneic tumours induced by UV irradiation (Fisher and Kripke, 1978). UVA exposure appears less immunosuppressive (De Fabo and Kripke, 1980). While UVB wavelengths greater than 315nm are also weakly immunosuppressive. (Noonan *et al.*, 1992). The contrasting effects of UVA and UVB have recently been confirmed by a study, in which human skin was exposed to equivalent erythemal doses, UVB was shown to depress sensitisation to the contact allergen diphenylcyclopropenone while UVA did not result in decreased immunisation rates (Skov *et al.*, 1997).

Many investigations have been undertaken since the early discovery of UV induced suppressor cell activity, aimed at identification and characterisation

of these cells. $CD8^+$ cells increased in the circulation of humans exposed to 1 hours sunlight per day, while CD4 levels decreased and suppressor cell activity was seen in T and B pokeweed mitogen stimulated cells (Hersey *et al.*, 1983). Thy $1.2^+$ cells have been shown to abrogate suppressive activity after UVB exposure. This was found to be specific for the hapten applied through the irradiated skin (Elmets *et al.*, 1983). Treatment with $Lyt1^+$ antibodies was shown to remove suppression and allow rejection of tumours, suggesting these cells are required for immune suppression (Ullrich and Kripke, 1984). However, Schwarz *et al.*, (1987) showed that serum from UVB irradiated mice was able to induce systemic suppression and this suppression was due to the generation of suppressor cells. This indicates a role for cytokines in UV induced immunosuppression.

The ability of UVB exposure, followed by hapten painting to induce specific suppressors, was assessed on strains of UV resistant and UV sensitive mice and it was found that suppressor cells were induced regardless of UV-CHS phenotype. This indicated that the difference was due to interruption of effector mechanisms (Glass *et al.*, 1989). This hypothesis is supported by the fact that UVB exposure converts LC from an immunologically potent APC, to a tolerogenic APC; were stimulation of Th1 cells is reduced while the ability to stimulate a Th2 response remains intact (Simon *et al.*, 1990). Clonal anergy appears also induced in the Th1 cells in response to UV exposed LC (Simon *et al.*, 1991). This provides a mechanism where by a "suppressor" cell is induced (Th2 cell) and the effector cell (Th1) is eliminated or anergized. Simon *et al.*, (1994) demonstrated that draining lymph nodes cells from normal and hapten treated murine skin, IL-2 and IFN$\gamma$ were able to be produced by Th1 cells. However, after UVB exposure and hapten treatment of the skin these cytokines could not be detected. This implies that the UV irradiated skin immune system would lose immunosurveillance against tumours or infectious agents requiring a cellular response (Simon *et al* ., 1992). This alteration of the immune response may be due to the action of IL-10 which has been shown to alter the immune response after UV exposure (Rivas and Ullrich, 1992).

Brown *et al.*, (1995) investigated the effect of UV treatment on the Th1/Th2 balance using the murine antibody response to *Borreilia burgdorferi* as a model. Brown *et al.*, (1995) showed that Th1 and Th2 cells differentially regulate antibody isotype production. Following UV exposure and by immunisation with *B. burgdorferi*, IgG2a and IgG2b isotypes were down-regulated (Th1 isotypes) while IgG1 was unaffected (Th2 isotype). There was

also a loss of DTH response to the organism, in comparison to immunisation without UV exposure. Injection of an anti IL-10 antibody into UV irradiated, immunised animals restored the IgG2a and IgG2b antibody responses and DTH to normal, providing further evidence of the role of IL-10 in altering the balance of the immune response.

Yagi *et al.*, (1996) was able to generate a line of Th2 cells expressing $CD3^+CD4^+CD8\text{-}V\beta7^+$, from the spleens of UVB pre-irradiated, tetracholorosalicylanilide photosensitised mice that had been subjected to repeated antigenic stimulation. These cells were shown to suppress contact photosensitivity, both *in vivo* and *in vitro*. This was neutralised by anti IL-10 mAb's as these cells produce IL-10, but not by anti-IL-4 mAb which they also produce. However, the author found amount of IL-4 produced by $CD3^+CD4^+CD8\text{-}V\beta7^+$ cells is sufficient to provide a microenvironment for Th2 cell generation, which in turn further suppresses Th1 cells.

The importance of UV induced immunosuppression can be seen by lowering of immunity to many microbes as evident by decreased DTH responses to *M. bovis* (Jeevan and Kripke, 1990), *M. leprae* (Cestari *et al.*, 1995), *L. monoctyogenes* (Goettsch *et al.*, 1996) and *Candida albicans* (Denkins *et al*,. 1989), as well as reducing the T cell proliferation response to tetanus toxoid (Pretell *et al.*, 1984).

Anti-viral immunity has a heavy reliance on the cellular arm of the immune system. The UV induced immunosuppression of this aspect of immunity has serious consequences for viral infection. In mice infected with murine leukemia virus, after UV exposure, mitogen induced proliferation was decreased, as were responses to SRBC and MLR's with more liver pathology, being seen due to infection (Brozek *et al.*, 1992). Infection of rats with RCMV, following exposure to 5 x 0.5 MED UVB exposure, resulted in an increased viral load and more frequent necrosis and inflammation (Goettsch *et al.*, 1994). The severity of herpes simplex virus (HSV) -2 infection was been shown to be increased after HSV-2 infects a site previously exposed to 4 suberythemal doses of UV (Yasumoto *et al.*, 1987). This study demonstrated that antigen specific T cells were generated and that these cells were able to suppress DTH and lymphoid cell proliferation in response to HSV-2 in transfer studies. The same decrease in immune responses to HSV-1 was observed after suberythemal doses of UVB (Howie *et al.*, 1986). For this suppression of immune responses to occur, the infection must occur within 2-14 days after irradiation (Ross *et al.*, 1988). Rooney *et al.*, (1991) found that 71% of patients with a history of herpes labilis

developed lesions with a 4 MED UVB exposure, while only 3% of patients whose skin was protected by sunscreen, had virus shedding and none formed lesions. However, Rooney *et al.*, (1991) suggests that increased infection after UV exposure may also be due, in part, to activation of the virus. After UV treatment of psoriasis patients it was found that ability of LC to present HSV was severely depressed. Antibody levels, lymphocyte proliferation and the ability of blood DC to present HSV were unaltered (Gilmour *et al.*, 1993). From these studies it is evident that decreased cellular immunity observed after UVB exposure of the skin can lead to alterations in the body's ability to respond to variety of viral and intracellular microbes.

While evolutionary advantage of immunosuppression remains unknown, one possibility is that the alteration of the skin by UV exposure alters the antigen profile of the cells, which in the absence of immunosuppression may lead to rejection (as suggested by Dr. George Klein, Koralinska Institute, Stockholm, reviewed by Nishigori *et al.*, 1996). Nishigori *et al.*, (1996) suggested that UV induced immunosuppression was a side-effect of the cytokines produced to restore damaged skin resulting in a temporary gap in the immune response.

UV induced immunosuppression is a serious complication arising from the bodies natural responses to UV exposure of the skin. Evidence at this time suggests that it may result from the generation of T suppressor cells, (Th2) which may inhibit the effector cells of Th1 type. It appears that UV induced immunosuppression depresses the function of Th1 cells and enhances the activity of Th2 cells via cytokines (reviewed by Ullrich, 1996) and via cell mediated immunosuppression (reviewed by Cooper, 1996). The combination of these events and others, shifts the normal cutaneous immune response away from cell mediated immunity. Which, in turn, causes decreased immunity to intracellular pathogens including bacteria and viruses. This loss of cellular immunity may lead to a loss of immunosurveillance to tumours, possibly arising perhaps from the direct action of UV on the skin, as it appears that individuals that are susceptible to UV-induced immunosuppression are more susceptible to skin cancer (Yoshikawa *et al.*, 1990).

### *1.6.2 Genetic Basis for Immunosuppression*

It is clear that genetic background, especially genes which regulate skin pigmentation, affect the susceptibility of an individual to skin cancer. However, other genes, which can be detected before cancers arise (reviewed by Streilein

,1996) play a role in UVB induced immunosuppression. Using CHS as a test of cutaneous immune response, there are distinct phenotypes within the human population, UVB resistant and UVB sensitive (UVB-R and UVB-S) (Yoshikawa *et al.*, 1990). This does not correlate with skin pigmentation as 40% of dark skinned individuals display the UVB-S phenotype (Vermeer *et al.*, 1991). Providing further evidence that UV induced immunosuppression has a role skin cancer, is the study by Yoshikawa (1990), which found that in basal cell and squamous cell carcinoma patients, 90% were of the UVB-S phenotype.

Streilein and Bergstresser (1988) originally demonstrated that the C3h/HeN and C57Bl strains of mice were UVB-S, while Balb/c was UVB-R. F1 hybrids of UVB-R and UVB-S strains were UVB-S, suggesting that this phenotype is the dominant one. C3H/HeJ were found to be UVB-R, despite differing from C3H/eN at only the LPS locus, suggestion that polymorphisms at this locus are important in producing the phenotype (Streilein and Bergstresser, 1988). This loci regulates the production of the pro-inflammatory cytokines IL-1, IL-6 and TNFα (Streilein and Bergstresser, 1980). The TNFα Loci is considered important, as there is a 100% correlation between the $TNF\alpha^d$ allele and the UVB-R phenotype (Vincek *et al.*, 1993)

Noonan and Hoffman (1994a) also investigated the control of UVB immunosuppression in a wide variety of mouse strains and found a more complex system of genetic control. Three distinct phenotypes with differing susceptibilities to UV immunosuppression were found. These being LO, (low susceptibility 50% immunosuppression with 9-12.3 kJ/m² UV) HI (High susceptibility 50% immunosuppression with 0.7-2.3 kJ/m² UV) and intermediate phenotype (intermediate susceptibility 50% immunosuppression with 4.7-6.9 kJ/m² UV). To relate this to the work of Streilein, Balb/c were classified as LO, C3H/eN intermediate and C57BL as HI. These phenotypes do not correlate with the MHC haplotype and the findings of Noonan and Hoffman, are in disagreement with Streilein's findings, as by their definition C3H/eN and C3H/eJ strains did not differ in their susceptibility, despite the differences at the LPS loci. Also, as the TNFα loci is located within the MHC loci, differences in these genes likewise failed to affect the UVB immunosuppressive phenotype seen. Noonan and Hoffman (1994b) did, however, find that the control UVB induced immunosuppression was controlled by at least three separate loci (Uvs1, Uvs2 and Uvs3), one of which was sex linked, while the other two were autosomal. None of these genes were linked to either pigment loci.

From these studies in experimental inbred animals it is clear that the genetic control of the UVB immunosuppressive phenotype is complex. This is likely to be even more so in the outbred human population and it may be some time before it is fully understood.

### *1.6.3 Adhesion Molecules*

A wide variety of adhesion molecules are expressed by cells of the skin and the blood vessels in the dermis, including integrins, selectins, addressins and those of the immunoglobulin super family. Many of these adhesion molecules play a role in inflammatory responses triggered by a variety of factors, such as ultraviolet light. E-Selectin (ELAM-1) is expressed at high levels at sites of acute UVB inflammation and act as a vascular homing molecule for neutrophils and memory T lymphocytes, (Norris *et al.*, 1991) Also thought to act in a similar way during immune responses are the ICAM and VCAM families of adhesion molecules.

E-selectin is up-regulated on dermal post capillary venules during inflammatory responses (Waldorf *et al.*, 1991) including up-regulation on vascular endothelium and polymorphonuclear cells after UV exposure (Norris *et al.*, 1991). This group also looked at VCAM-1 and ICAM-1 expression in human skin after a DTH response and after skin was exposed to 2 or 8 MED dose of UVB. The group found that there was no significant induction of either adhesin during the 72 hours post UV treatment. Slight up-regulation of ICAM-1 occurred on endothelial cells. This is supported by Cornelius *et al.*, (1994) who demonstrated up-regulation of ICAM-1 on microvascular endothelial cells of the dermis *in vitro* after UVB irradiation. A UV irradiated keratinocyte cell line has been shown to have a biphasic expression of ICAM-1 with an initial inhibition at 24 hours followed by induction at 48,72 and 96 hours (Norris *et al.*, 1990).

UVB treatment of melanocytes or a melanoma cell line shows a similar effect of UVB on ICAM-1(Kirnbauer *et al.*, 1992), although a variety of cytokines in the same study were seen to up-regulate this molecule (IFN$\gamma$, IL-1, IL-6, IL-7 and TNF$\beta$). ICAM-1 has also been shown to be down-regulated on cultured monocytes after UV exposure, thereby reducing their ability to act as accessory cells for T cell activation (Krutmann *et al.*, 1990).

ICAM-1 is an important adhesion molecule in the skin as it mediates the binding of T cells to keratinocytes (Dustin *et al.*, 1988) and is one of the major adhesion molecules involved in the interaction of T cells and antigen presenting cells. LC, constitutively express this molecule (De Panfilis *et al.*,

1990) and hence, down-regulation of this molecule could affect the ability of LC to stimulate T cells. Tang and Udey (1991) suggested that doses of UV light which are sufficient to cause down-regulation of ICAM-1 are ultimately cytotoxic for the LC.

Alterations in adhesion molecule interaction between DC and T cells do occur after UV exposure of the skin as shown by the reduced ability of $FITC^+$ APC from UV irradiated mice to cluster to T cells. Also observed was an increase in the numbers of $CD8^+$ T cells in the clusters (Muller *et al.*, 1994). The alteration in T cell subsets may mean different adhesins or a different combination of adhesion molecules is being expressed by the LC.

More recently the decreased expression of E and P cadherins have been shown to be induced in melanocytes following UV irradiation (Seline *et al.*, 1996), which may alter melanoma binding to keratinocytes. This may increase the metastatic potential of transformed cells and has an inverse relationship with disease progression in melanoma (Seline *et al.*, 1996). This demonstrates how UV induced alteration of adhesion molecules can have profound effects

### *1.6.4 Cytokines and Soluble Factors Produced after UV Exposure*

#### 1.6.4.1 Urocanic Acid

De Fabo and Noonan (1983) proposed that urocanic acid (UCA) a compound found within the epidermis was the chromophore for UV-induced immunosuppression. This substance is produced by the deamination of histidine by the enzyme histidine ammonia lyase (histidase). The enzyme is present in high amounts in only two tissues, the skin and liver (Noonan and De Fabo, 1981). UCA accumulates in the skin due to the lack of the catabolic enzyme urocanase (Noonan and De Fabo, 1981). UCA absorbs light in the ultraviolet region, undergoing photoisomerization from the *trans* to the *cis* isomer (reviewed by Noonan and Defabo 1992).

UCA was proposed as the photoreceptor mediating UV induced immunosuppression due to UCA's absorption spectra closely matching that of the immunosuppression action spectrum (more so than DNA) together with its superficial location in the stratum corneum and photochemical properties (De Fabo and Noonan, 1983). The administration of UV irradiated urocanic acid (UV-UCA) to mouse skin suppresses DTH to herpes simplex virus (Ross *et al.*, 1986) as well as increasing UV induced tumour yield and malignancy in the

hairless mouse (Reeve *et al.*, 1989). In addition *cis*-UCA impairs induction of CHS and induces tolerance (Kurimoto and Streilein, 1992). *Cis*-UCA treatment also inhibits alloantigen presentation by LC enriched epidermal cells in MLR experiments (Beissert *et al.*, 1995). UCA also increases keratinocyte production of PGE in the presence of histamine and induces weak ICAM-1 expression, which is increased when combined with TNFα (Mitra *et al.*, 1993).

UV-UCA induces defects in antigen presentation by splenic DC (Noonan *et al.*, 1988) as well as reducing the numbers, and altering the morphology of LC in mouse skin (Kurimoto and Streilein, 1992) in a manner similar to the observed effects of cutaneous UVB treatment. In contrast to these results, topical application of *cis*-urocanic acid had no effect on the number of dendritic cells within the draining lymph node (Moodycliffe *et al.*, 1992). The addition of anti-TNFα antibodies was able to stop the decrease in LC from the epidermis but this did not eliminate the changes in morphology as seen by Kurimoto and Streilein (1992). Moreover, the suppression of contact hypersensitivity by UV-UCA appears to be mediated via TNFα, although the induction of tolerance or DTH was not mediated via this cytokine (Shimizu and Streilein, 1994). However, treatment with antibodies against UV-UCA were able to restore DTH responses (El-Ghorr and Norval 1995). UCA therefore remains a strong contender for a major role in UV induced immunosuppression. However, this does not seem to exclude DNA damage also being important as both UCA and DNA mediate effects associated with immunosuppression (Reeve *et al.*, 1996).

### 1.6.4.2 Cytokines

UV light exposure of the skin induces many changes to the skin microenvironment, including the production of cytokines, which immunological effects (reviewed by Ansel *et al.*, 1990, Luger and Schwarz, 1990, Takashima and Bergstresser, 1996). In the epidermis the main secretor of cytokines are keratinocytes, with the changes in cytokine profile of this cell being the subject of intensive study (reviewed by Takashima and Bergstresser, 1996, See Table 3). LC production of cytokines after UV exposure of skin, while undoubtedly important has not been extensively investigated. Further investigations on many of these cytokines are required to define if they have role in altering the skin immune system.

#### *1.6.4.2.1 Interleukin 1*

IL-1 system consists of interaction between IL-1α, IL-1β, the IL-1 receptor antagonist (IL-1RA) and the IL-1 receptors, type 1 and type 2. Exposure of the

**Table 1.3 Keratinocyte derived cytokines up-regulated by UVB radiation (From Takashima and Bergstresser, 1996)**

| Cytokine | Species | References |
|---|---|---|
| IL-1 α/β | Human | Luger *et al.*, 1981, Ansel *et al.*, 1983, Kupper *et al.*, 1987, Gahring *et al.*, 1984 |
| IL-1α | Mouse | Ansel *et al.*, 1988, Ansel *et al.*, 1983 |
| IL-3 | Mouse | Gallo *et al.*, 1983 |
| IL-6 | Human | Urbanski *et al.*, 1990, de Vos *et al.*, 1994 |
| IL-8 | Human | Kondo *et al*, 1988 |
| IL-10 | Human | Grewe *et al.*, 1995, Enk *et al.*, 1995 |
| IL-10 | Mouse | Rivas and Ullrich *et al.*, 1992 |
| IL-15 | Human | Mohamadzadeh *et al.*, 1995 |
| GM-CSF | Mouse | Gallo *et al* ., 1991 |
| TNFα | Human | Kock *et al.*, 1990 |
| TNFα | Mouse | de Kossodo *et al.*, 1995 |
| NGF | Mouse | Tron *et al.*, 1990 |
| bFGF | Human | Halaban *et al.*, 1988 |

skin to UV light may alter the natural balance of this complex cytokine system. Ansel *et al.*, (1983) found that exposure of cultured keratinocytes to UVB resulted in increased production of IL-1 both intra and extracellularly. Also UVB exposure of murine skin *in vivo* caused epidermal cells to produce IL-1. IL-1 production has been detected in the skin, post UVR in humans (Kupper *et al.*, 1987, Oxholm *et al.*, 1988). While LC production of IL-1 appears to be unaltered by UV exposure (Rasanen *et al.*, 1989), the production of a specific inhibitor of IL-1 is also observed after UV exposure of epidermal cells and keratinocytes (Schwarz *et al.*, 1987). The inhibitor was termed contra IL-1 and was found in the serum of UVB irradiated mice (Schwarz *et al.*, 1988). A possible candidate is IL-1RA, which binds to the IL-1 receptor but produces no activation (Dripps *et al.*, 1991). Hirao *et al.*, (1996) demonstrated that a hundred fold excess of IL-1RA is needed to inhibit the biological function of IL-1 and that in human skin unexposed to UV IL-1$\alpha$ dominates. However, in UV exposed skin IL-1RA is dominant (Hirao *et al.*, 1996). Furthermore in the same study, exposure to 2 MED was shown to dramatically up-regulate IL-1RA in the stratum corneum of human volunteers, leading to the suggestion that this may inhibit excessive inflammation and may account for some of the UVB induced immunosuppression.

Studies using the HeCAT keratinocyte cell line, have demonstrated that TGF$\alpha$, TGF$\beta$, IFN$\gamma$ IL-6 and TNF$\alpha$ increase IL-1$\alpha$ production, while no effect on mRNA of IL-1RA was evident. This suggests that the balance between IL-1$\alpha$ and IL-1RA is due to the levels of IL-1$\alpha$, not IL-1RA, which remains constant (Phillips *et al.*, 1995) which is in direct contrast to Hirao's hypothesis.

Interestingly while proposed as antagonistic in inflammation, immunosuppressive effects of both IL-1RA and IL-1$\alpha$ have been demonstrated. IL-1RA injection into the skin prior to sensitisation with DNFB inhibits ear swelling by 36-43% (Kondo *et al.*, 1995). IL-1$\alpha$ has been shown to suppress the ability of LC to present tumour antigens to $CD4^+$ T cells (Grabbe *et al.*, 1994), as well reducing CHS (Robertson *et al.*, 1987). Enk *et al.*, (1993a) demonstrated that LC from IL-1$\alpha$ injected mice have reduced capacity to stimulate anti-CD3 treated T cells. It appears that IL-1$\alpha$ and IL-1$\beta$, which is only produced by LC in the epidermis, have different roles in CHS responses, with IL-1$\beta$ being crucial in the induction of CHS (Enk *et al.*, 1993a). IL-1$\beta$ expression has been shown to increase LC after the contact sensitiser application to the skin (Enk *et al.*, 1992) but, as yet, no evidence has been forthcoming about IL-1$\beta$ production after UV exposure.

In the skin, interactions between IL-1α, IL-1β and IL-1 receptor type 1 all induce inflammatory reactions; IL-1RA and IL-1 receptor type 2 appear to down-regulate these responses (Kupper and Groves 1995). Further studies of the interaction of this system after UV exposure of the skin, should reveal in detail more on the role of the IL-1 family in the suppression of cutaneous immune responses.

*1.6.4.2.2 Interleukin 10*

Schwarz *et al.*, (1987) showed that serum from UVB irradiated mice was able to induce systemic suppression. Furthermore, UVB exposure of cultured human keratinocytes resulted in the production of IL-10 *in vitro*, which caused systemic immunosuppression when the supernatant's were injected into mice (Rivas and Ullrich, 1992). Further evidence of UV induced IL-10 production by human keratinocytes was shown both *in vitro* and *in vivo* by Enk *et al.*, (1995). UVB strongly stimulates IL-10 production, however, UVA-1 (340-400nm wavelengths) were most stimulatory (Grewe *et al.*, 1995). However, Teunissen *et al.*, (1994) reported that human keratinocytes were unable to produce IL-10. This was supported by Jackson *et al.*, (1996) who were unable to detect IL-10 secretion from a transformed keratinocyte cell line. Higher doses of UV were required to produce barely detectable, IL-10 mRNA production and this required more extensive PCR than Grewe's study and no protein was detected. In reply Grewe *et al.*, (1996) suggested that the reverse transcriptase-polymerase chain reaction method used by Jackson may have lacked the sensitivity of their system. Grewe *et al* , (1996) also argued that the use of non-neutralising antibodies in their ELISA's increased sensitivity.

Although keratinocyte production of IL-10 is controversial, this may be irrelevant as Kang *et al.*, (1994) only detected slight increases in keratinocyte IL-10 mRNA after UVB exposure whereas large amounts of IL-10 were produced by $CD11b^+$ macrophages, which infiltrate the epidermis after UVB exposure (Cooper *et al.*, 1992). Kang *et al.*, (1994) demonstrated that the skin infiltrating macrophages production of IL-10, far outweighed that of the keratinocytes, suggesting that infiltrating macrophages were the major source of IL-10 in the epidermis after UVB exposure.

IL-10 has been shown to down-regulate many aspects of the skin immune system including inhibition of CHS (Fergusson *et al.*, 1994, Peguet-Navarro *et al.*, 1994). Such inhibition of CHS was seen to occur at the effector phase of CHS and not during the induction of CHS (Schwarz *et al.*, 1994). However, the

same study showed that IL-10 inhibited both the initiation of DTH and its effector phase. This is supported by Rivas and Ullrich, (1994) who found that the systemic administration of anti-IL-10 antibodies to mice blocks the ability of UV suppressor cells to DTH but not the suppressor cells which suppress CHS. $CD4^+$ Th2 cells isolated from spleens of UV irradiated mice produce IL-10 inhibit Th1 cells (Yagi *et al.*, 1996), hence inhibiting DTH and CHS responses.

Local IL-10 production in the skin, by infiltrating macrophages restricts the ability of LC to stimulate an effective Th1 cell response, instead anergy of Th1 cells occurs, but the ability to stimulate a Th2 response is unaltered (Enk *et al.*, 1993b). This is supported by Ullrich, (1994) who found that adding IL-10 from UV irradiated cultured keratinocytes to splenic APC caused inhibition of antigen presentation to Th1 cells and enhanced stimulation of Th2 responses. LC also lose their ability to stimulate a primary allogeneic response when incubated with IL-10 (Peguet-Navarro *et al.*, 1994). This may be due to loss of important co-stimulators such as down-regulation of B7-2 which occurs after addition of IL-10 to human peripheral blood DC (Buelens *et al.*, 1995). The ability of LC to present tumour antigens is also down-regulated by incubation with IL-10, which overcomes the GM-CSF induced maturation of normal LC APC function. Ullrich, 1996 showed that *in vitro and in vivo* administration of anti-IL-10 mAb restores the lost antigen presenting function of splenic DC

The many down-regulatory effects of IL-10 on CHS, DTH, antigen presenting function of LC and its ability to stimulate Th2 cells while inhibiting Th1 cells has resulted in IL-10 being regarded as "the primary mediator of UVB induced local as well as systemic suppression" (Takashima and Bergstresser, 1996).

*1.6.4.2.3 Other Interleukins*

The production of a number of other interleukins have been shown after UV light exposure of the epidermis (See Table 3). While IL-3 is produced in mice after irradiation it does not appear to be generated by human keratinocytes and "IL-3 like activity "in human skin can be inhibited by antibodies to GM-CSF and IL-6 (Kondo *et al.*, 1995).

IL-6 is a pro-inflammatory cytokine which has been shown to be produced in the skin of humans; the peak serum levels occurs 12 hours after severe sunburning, correlating with fever production (Urbanski *et al.*, 1990). It was shown by Kirnbauer *et al.*, (1991) that keratinocytes were able to produce IL-6 and increased serum levels were proposed to arise from this confirming the

hypothesis developed by Urbanski *et al.*, (1990). IL-6 is involved with the production of acute phase responses but its role, in the skin immune system is unknown.

IL-8 is a pro-inflammatory cytokine produced in the epidermis and causes neutrophil chemotaxis to sites of inflammation. Furthermore, IL-8 production is greatly increased after UV exposure (reviewed by Luger and Schwarz, 1990); IL-8 production is seen within an hour and the levels of mRNA expression increased 11-13 fold (Kondo *et al.*, 1993). The accumulation of neutrophils in sunburned skin (Logan and Wilhelm, 1963, Hawk *et al.*, 1988) correlates with the production of this cytokine (Strickland *et al.*, 1997).

The induction of IL-15 has been noted after UV exposure and found to be produced by keratinocytes and dermal fibroblasts (Mohamadzadeh *et al.*, 1995). Blauvelt *et al.*, (1996) reported that freshly isolated keratinocytes and cultured keratinocytes expressed IL-15 mRNA *in vitro* which was down-regulated by UV exposure in a time and dose dependant manner. Blauvelt *et al.*, (1996) were unable to explain their contrasting results with those of Mohamadzadeh *et al.*, (1995). Blauvelt *et al.*, (1996) also showed that IL-15 mRNA was expressed by LC and blood dendritic cells, and proposed that IL-15 may contribute to the enhancement of Th1 like responses, thus down-regulation of its production after UVB exposure, may contribute to UV induced reduction of Th1 like responses. Before the role of this cytokine can be established in the immune alterations following UV exposure, further studies are required on whether its production is enhanced or suppressed by UV light.

While there is no evidence for IL-4 production by cells of the skin after UV light, $CD4^+$ Th2 cells isolated from the spleen of UV irradiated mice produce IL-4 (Yagi *et al.*, 1996). Further, antibodies to IL-4 are able to reduce the ability of UV induced suppressor cells to suppress DTH responses but no reduction for CHS responses was observed (Rivas and Ullrich, 1994). This suggests that the suppression of these responses involve different mediators, recent studies in IL-4 deficient mice confirm these findings (El-Ghorr and Norval, 1997).

#### 1.6.4.3 TNFα

Tumour necrosis factor α (TNFα) is a pro-inflammatory cytokine that is produced by a number of different cell types in the skin. These include pre-formed storage in mast cells (Gordon *et al.*, 1990) and induced expression in keratinocytes (Koch *et al.*, 1990), activated Langerhans cells (Larrick *et al.*, 1989) and dermal dendritic cells (Nickoloff *et al.*, 1991). Two TNFα receptors

are described; TR60 (Type I, 55kD) and TR80 (type II, 75kD), with lymphoid cells expressing type II and cells of epithelial origin express type I (Grell *et al.*,1994). The expression of this cytokine has been shown to be dramatically increased after exposure of the skin to UVB irradiation (Oxholm *et al.*, 1988). TNFα is considered to have major role in UVB induced changes in inflammatory and immune reactions, including down-regulation of CHS responses (Yoshikawa and Streilein, 1990). *Cis*-UCA generated by the UV exposure of the epidermis appears to mediate its effects on CHS via TNFα (Kurimoto and Streilein, 1992).

TNFα affects inflammatory and immunological responses in the body. These include the induction of other cytokines, including IL-1α, but not its receptor antagonist (Phillips *et al.*, 1995). It also has a role in the recruitment of cells to areas of inflammation by affecting adhesion molecule expression in the surrounding environment (Strickland *et al.*, 1997). Intradermal injection of recombinant human TNFα up-regulates ICAM-1, VCAM-1 and E-Selectin *in vivo* (Groves *et al.*, 1995). E-Selectin was found to up-regulated on dermal microvascular endothelial cells after release of TNFα by dermal mast cells (Waldorf *et al.*, 1991). CD44 also appears to be up-regulated, which appears to be involved with increased binding of leucocytes to the endothelial surfaces. (Osada *et al.*, 1995) The increased expression of CD44 and E-Selectin is thought to be involved with increased infiltrates of neutrophils and mononuclear cells seen after UV light exposure (Norris *et al.*, 1991, Osada *et al.*, 1995, Strickland *et al.*, 1997).

Further evidence of the importance of TNFα comes from the study by Walsh, (1995) who found that UVB irradiation *in vitro* and *in vivo* resulted in TNFα release from mast cells which, in turn, leads to increases in ICAM-1 and E-Selectin expression on endothelium, within 2 hours of UVB exposure. This was found to be independent of the epidermis, suggesting that keratinocytes only make a small contribution to the acute release of TNFα. However due to the extent of the epidermis and the fact that keratinocytes produce this cytokine after UVB irradiation, it seems likely that TNFα release by keratinocytes may contribute to the later stages of the UVB inflammatory process.

TNFα was originally named because of its ability to kill tumour cell lines. Today it is a cytokine known to be involved with the induction of apoptosis (Grell *et al.*, 1994). TCR mediated TNFα induced apoptosis has been shown for mature T cells via the TR-80 receptor. This was found to be more important in mediating $CD8^+$ T cell apoptosis than $CD4^+$ T cell apoptosis, which was

mediated mostly via Fas and Fas Ligand interaction (Zheng *et al.*, 1995). The 55kD version of the TNF receptor is expressed by keratinocytes and has a "death domain" homologous to that of Fas and this has been implicated in the induction of apoptosis in non-lymphoid cells (Grell *et al.*, 1994). TNFα has been recently shown to be involved in the formation of sunburn cells (Schwarz *et al.*, 1995), as injection of anti-TNFα reduced UV induced sunburn cell formation, although the cytokine by itself was not sufficient to induce sunburn cell formation.

TNFα also has a role in regulating the survival and migration of dendritic cells found in the skin. TNFα has been shown to support the survival of LC in the murine system but this did not result in the acquisition of accessory cell function (Koch *et al.*, 1990). In the ovine system TNFα supports survival of afferent lymph DC, whereas GM-CSF does not (Haig *et al.*, 1995).

TNFα has been proposed to stimulate LC migration from skin to the draining lymph node (Cumberbatch and Kimber, 1992). This group injected increasing doses of TNFα intradermally into Balb/c mice and found that DC accumulation in the lymph node was increased after just two hours. This group subsequently found that TNFα was required for both migration and optimal contact sensitisation (Cumberbatch *et al.*, 1995). In contrast Yoshikawa *et al.*, (1992) found that injection of 50ng TNFα (identical in amount and activity to that used by the previous group) intradermally, followed by cutaneously applied hapten, led to the retention of LC within the epidermis. However, previous studies by the Streilein had shown that there was indeed a 30% reduction of $Ia^+$ (LC) within the epidermis after 2 hours but LC numbers returned to normal within 24 hours (Vermeer & Streilein, 1990). This data suggests, that the differing time courses of the experiments may have lead to differing results for these two groups. Further evidence for the DC depleting effect of TNFα comes from systemic administration of the cytokine where DC were depleted from heart, kidney and skin (Roake *et al.*, 1995). Zanella *et al.*, (1995) recently found using skin organ culture that low doses of TNFα (50-100U/ml) enhanced LC emigration by 167%. However high doses >1000 U/ml suppressed emigration, possibly due to toxicity. At this time it is still unknown whether TNFα acts on DC directly or via cytokine cascade.

#### 1.6.4.4 Prostaglandins

Increases in prostaglandin (PG) production after UV exposure of the skin have been demonstrated (Imokawa and Tejima, 1989) and the prostaglandins

produced were $PGE_2$, $PGF_{2\alpha}$ and PG 6-oxo-$F_{1\alpha}$. Treatment of the skin with indomethacin, (an inhibitor of PG synthesis) after UV exposure leads to a decrease in erythema (Lim *et al.*, 1983, Farr and Diffey, 1986) but did not prevent decreases in the density of LC (Lim *et al.*, 1983). However, administration prior to UV exposure was shown to stop LC depletion but this was found to be due to the ability of indomethacin to absorb light in both the UVA and UVB portions of the spectra, as oral administration of indomethacin did not stop LC depletion. However, oral administration of indomethacin has been shown to partially reduce the depression of CHS by UV exposure (Chung *et al.*, 1986, Jun *et al.*, 1988). The production of PG after UV exposure may have immunodulatory effects on the skin immune system. However, it is likely that these effects are minor compared to that of IL-10 and TNF$\alpha$. Interestingly both TNF$\alpha$ and IL-1$\alpha$, which are produced in the skin after UV light exposure, up-regulate keratinocyte synthesis of $PGE_2$ (Grewe *et al.*, 1993).

### *1.6.5 Effects of UV on Langerhans Cells and Antigen Presentation*

LC are the only constituent antigen presenting cell type found within the epidermis and, after exposure to UV alterations in LC morphology and kinetics have been observed (Aberer *et al.*, 1981, Obata and Tagami 1985). LC are considered to be an important cell type in the lowering of immune function and the induction of tolerance after UV exposure (Simon *et al.*, 1990 and Simon *et al.*, 1991).

Alterations in LC where first shown by Fan *et al.*, (1959) who examined high, clear level cells (Langerhans cells) of the epidermis and found they were difficult to demonstrate in human epidermis after exposure to UV light In 1967 Wolff and Winkelmann exposed guinea pig skin to ultraviolet light which revealed no changes in LC distribution. In this study, while the exposure times were identical to those used in the previous study, however, no treatment dose was given in either study. In contrast to this, a histochemical study of the human epidermis after a single exposure to 6 MED of sunlight, undertaken by Zelickson and Mottaz (1970) revealed that the number of LC present in the epidermis was decreased. Repeated daily exposure for two weeks showed an absence of LC from the epidermis. Additional studies have revealed that LC are depleted from the human epidermis after UV treatment (Aberer *et al.*, 1981, Cooper *et al.*, 1992). This has also been demonstrated for other animal models including that the guinea pig (Lim *et al.*, 1983) and a variety of murine models (Aberer *et al.*, 1981, Obata and Tagami, 1985). Recently, UV damaged LC

expressing thymine dimers have been shown to migrate to the lymph node one hour after UV exposure (Sontag *et al.*, 1995), indicating that the depletion of LC from the skin from UV irradiated skin may be associated with an increase in migration.

While the depletion of LC from the skin would reduce the immunological ability of the epidermis to respond to foreign antigens this is not the only effect UV light has on LC. It has also been demonstrated extensively that UV light exposure affects APC function. Levis *et al.*, (1978) found that the ability of UV treated APC to stimulate hapten (DNCB) specific autologous lymphocytes in primary culture was abrogated and the ability of APC to stimulate blast cells in secondary culture was diminished but not eliminated. They suggested that this may be due to the alteration of cell surface markers, as well DNA by UV light. The effects of UV light in APC function, was also studied *in vivo* by Greene *et al.*, (1979) who reported that TNP derivatised APC from UV irradiated mice failed to induce an immune response in UV irradiated syngenic mice, when compared to normal APC. This failure to induce immunity was associated with suppressor T cells. *In vitro* studies by Stingl *et al.*, (1981) have also shown that the ability of epidermal cells to stimulate T cells was reduced in a dose dependent fashion after exposure to UV. No epidermal cell death was observed, however as LC made up only 3-5% of epidermal cells, specific cytotoxic effects on these cells could not be ruled out, although similar percentages of LC were found in epidermal cells preparations, both before and after irradiation. The loss of cell surface markers was put forward as the most likely explanation for these events. This suggestion was consistent with data showing exposure of LC to UV light lowers ATPase activity *in vivo* (Toews *et al.*, 1980). A reduction in the expression of MHC II was seen by Aberer *et al.*, (1981) using 600-800 J/m$^2$ UV, although this is a topic of debate as this may have been due to the loss of the cells expressing this marker. Exposure to milder doses of UV light *in vitro* (200J/m$^2$) had little effect on the retention of Ia (murine MHC II) antigen or the T6 antigen (Czernielewski *et al.*, 1984). Recent work suggests that no reduction in MHC II expression is seen in FITC$^+$ DC migrating after a dose of 2 kJ/m$^2$ (Muller *et al.*, 1994).

The loss of important co-stimulator expression may, in turn, provide at least a partial explanation for the reduction in LC function observed after UV exposure. This has been shown as a reduced T cell proliferative response (Stingl *et al.*, 1981) and by a loss of DTH response (Perry and Green *et al.*, 1982). Loss of CHS response after UV irradiation is also associated with

deficient antigen presentation (Noonan *et al.*, 1981). It should also be noted that splenic APC function is down-regulated after UV exposure (Kripke, 1984) which may be mediated by UV-UCA (Noonan *et al.*, 1988), suggesting a systemic reduction in APC function after UV exposure of the skin.

The reduction in the ability of enriched $T6^+$ LC to induce an ELR was shown to be reduced immediately after exposure of the skin to UV, when compared to non-irradiated controls (Cooper *et al.*, 1985b). Further, this reduction in allostimulatory activity was not reversed by the addition of indomethacin or IL-1 to the culture system. It can be speculated that the production of IL-10 by keratinocytes or UV induced macrophages may be able to down-regulate the co-stimulator B7-2 on LC, as has been shown to occur after addition of IL-10 to human peripheral blood DC (Buelens *et al.*, 1995). IL-10 is proposed to mediate the systemic suppression of APC function observed after UVB exposure (Ullrich, 1996). LC can be induced to express the B7-1 and B7-2 co-stimulatory molecules after culture, however, UVB exposure of epidermal cell suspensions, with doses ranging from 100-200 $J/m^2$, reduce functional B7-1 and B7-2 expression in a dose dependant manner. This could be partially restored with sub-mitogenic doses of anti-CD28 mAbs (Weiss *et al.*, 1995). Since LC could not be returned to their full allostimulatory capacity. Weiss *et al.*, (1995) suggests that this may be due to the presence of an inhibitory cytokine, with possible candidates being PGE, IL-1, IL-10 or TNF$\alpha$ produced after UVB exposure. Any loss of B7 expression would render the LC less able to stimulate T cells, as the interaction without B7, may lead to anergy reviewed by Linsley and Ledbetter (1993) and possibly to specific down-regulation of Th1 responses (reviewed by Constant and Bottomly, 1997). In support of this hypothesis is crucial evidence presented by Simon *et al.*, 1990 which shows that LC from UV irradiated skin are able to stimulate Th2 cells but not Th1 cells. Furthermore LC from UV treated skin can induce anergy in Th1 cells (Simon *et al.*, 1991) which appear to be essential for cellular immune responses such as DTH and CHS. Thus generating Th2 "suppressor cells" and reducing or eliminating Th1 "effector cells", both of which are required for UV immunosuppression.

### *1.6.6 UV Induced Macrophages*

UV irradiation reduces allostimulatory capacity of DC isolated from the epidermis immediately after treatment, however, three days later an increase in the allostimulatory capacity was noted. This increased allostimulatory capacity was shown to be due to $T6^-$ DC, which were seen to infiltrate the

epidermis at this time (Cooper *et al.*, 1985b). These infiltrating DC, expressed the monocyte specific marker, OKM5 (CD36) (Cooper *et al.*, 1985b). Suggesting that the cells were of the monocyte/macrophage lineage. These cells were shown to be capable of inducing T cell proliferation and were able to act as stimulators of an autologous ELR (Cooper *et al.*, 1985b) and were subsequently shown to express MHC II (Baadsgaard, 1987).

A later study by Cooper *et al.*, (1992) showed a sub-population of the infiltrating cells were non-dendritic neutrophils which is consistent with data obtained from studies on other species (Logan and Wilhelm, 1963, Logan and Wilhelm, 1966b) including humans (Hawk *et al.*, 1988). Another sub-population of infiltrating DC expressed high levels of the macrophage marker, CD11b and co-expressed high levels of MHC II. Timing of the peak infiltration of this sub-population coincided with the return of UVB epidermal APC function from 21% to 59% at three days and the maximal depletion of LC. Hammerberg *et al.*, (1994) demonstrated this sub-population of infiltrating cells when isolated from UVB exposed, hapten treated skin led to re-sensitisation, rather than tolerance. These were also shown to stimulate suppressor inducer cells (Th2) and CD8 cells with the addition of IL-2 (Baadsgaard, 1988 and Baadsgaard 1990).

It has also been shown that the UV induced macrophages are a major source of IL-10 secreted in the epidermis post irradiation (Kang *et al.*, 1994. As IL-10 is a potent down-regulator of Th1 (cellular) immune responses (Del Prete *et al.*, 1992), this provides a mechanism by which the infiltrating macrophage population can suppress cellular immune responses generated by LC. Recent work has found that infiltrating UV-induced macrophages appear to come from the expansion of a subset of dermal macrophages (Meunier *et al.*, 1995) which are $CD36^{+}$ $CD11b^{+}CD1-$.

One study by Kurimoto *et al.*, (1994) using mice, has produced data which contradict Cooper's theory that macrophages mediate the down-regulation of immune function after UV exposure. This investigation used liposomes which contained dichloromethylene diphosphonate, which selectively kills phagocytic macrophages and showed that these cells play a role in generating CHS responses in UV resistant mice, but had no effect on tolerance induction in UV resistant mice. Although they were trying to compare their results to those of Cooper's group, the UV irradiation consisted of four treatments, whereas Coopers group used a single dose. No immunohistochemical studies were performed to show that macrophages were depleted the skin, which may be important as Weber-Matthiesen and Strerry, (1990) found that $OKM5^{+}$

macrophages are non-phagocytic, so they may not have ingested the liposomes.

While strong evidence exists in humans for the infiltration of macrophages after UV, less is known about this in animal models. After UVB exposure of the skin, macrophages do appear in the lymph nodes of mice (Tang *et al.*, 1992). Evidence in mice shows an increased proportion of DC expressing CD11b (Mac-1) binding to CD4 and CD8 positive cells (Muller *et al.*, 1994). It should be noted that epidermal LC in the human (De Panfilis *et al.*, 1989) and cultured murine LC (Tang *et al.*, 1992) also express CD11b and therefore it cannot be taken as a specific marker of macrophages.

The timing of the maximum migration of UV induced macrophages into the skin coincides with the maximal depletion of LC as they secrete IL-10 (Kang *et al.*, 1994) and stimulate suppressor inducer T cells (Baadsgaard, 1988 and 1990), both which result in down-regulation of Th1 immune responses. It would therefore appear that the infiltrating macrophages may contribute to UV induced immunosuppression.

### *1.6.7 γδ T Cell Changes*

UVB exposure of murine skin can completely deplete DETC from the epidermis (Aberer *et al.*, 1986 and Alcalay *et al.*, 1989). This depletion was sustained and Thy-1 DEC did not reappear until 14-22 weeks following exposure of the skin, whereas as influx of LC was continuous (Aberer *et al.*, 1986). The doses used were 4 exposures to 700 J/m² or a single exposure of 1000 J/m². Chronic exposure, such as that used by Alcalay *et al.*, (1989) consisting of 8 kJ/m² three times a week until the mice became moribund from the tumours induced, resulted in these cells being consistently absent, after 43 weeks post treatment. LC were observed to return to the skin and infiltrate tumours (Alcalay *et al.*, 1989). This suggested to the authors that the tumours persisted, because of the lack of Thy-1 DEC and their cytotoxic potential. Love-Schmenti and Kripke (1994) found that DETC incubated in the presence of IL-2, were capable of deleting hapten specific autologous T cells. The lack of DETC network in the human and ovine systems suggests that the relevance of these cells may be limited to the murine system.

## 1.6 SUMMARY

As well as acting as a physical barrier, the skin is an immunological barrier due to the interaction of many cells in the skin. LC have an extremely important role in initiating and stimulating immune response by migrating to the draining lymph node and presenting antigens to T cells, in a manner which induces efficient proliferation and generation of effector cells. The keratinocyte is able to secrete a vast array of cytokines and soluble mediators, which in turn affect LC and attract other cells into the skin. Other minor skin cell populations may also play a role in the skin immune system.

Human skin is often exposed to ultraviolet light in sunlight. The exposure of the skin to UV down-regulates the skin immune system by inducing a number of cellular changes. The trigger(s) for these effects are thought to be direct DNA damage of cells or photoisomerization of *trans* to *cis*-UCA and its subsequent interaction with cells in the skin. These alterations induce changes in the cytokine profile of keratinocytes. Further, LC antigen presenting function appears to be altered so the Th1/Th2 balance is tipped in favour of Th2 cell function. Further, population of macrophages which secrete IL-10, thereby suppressing Th1 responses, migrates into the epidermis. These events combine, leading to the generation of suppressor T cells, which would appear to produce Th2 cytokines, thus further disrupting cell mediated immune responses by inhibiting effectors of the Th1 subtype. The induction of specific suppression of CHS and DTH responses is evident and is believed mediated by TNF$\alpha$ and IL-10 respectively. IL-10 suppresses the effector phase of both, possibly by inhibiting APC production of IL-12 via down-regulation of co-stimulators. These changes in the cellular arm of the skin immune system would increase susceptibilty to viral infection and greatly reduce any effective immune response to tumour which may arise in the skin as a result of UV induced mutation.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 ANIMALS

Outbred Polwarth/Comeback sheep, 6-12 months old, were obtained from the University of Tasmania Farm at Cambridge, Tasmania via the Central Animal House, University of Tasmania. The sheep were obtained and used with the permission of the University Ethics Committee (Animal experimentation). Sheep were kept in individual metabolism cages and had free access to lucerne chaff stock pellets (Gibson Stock feed, Cambridge, Tasmania), water and salt lick.

## 2.2 SURGICAL METHODS

All Surgery was performed by Dr. Geoffrey W. Dandie, Division of Pathology, University of Tasmania. "Pseudoafferent" prefemoral lymphatic vessels were generated by removing prefemoral lymph nodes 8-12 weeks prior to cannulation by the method described by Hopkins *et al.*, (1985). This allowed the afferent lymphatic vessels to re-anastomose with the remaining efferent lymphatic vessels. The prior intradermal injection of patent blue dye into the flank skin was used to identify during surgery which pseudoafferent vessels were cannulated by the method of Hall and Morris (1962).

## 2.3 LYMPH COLLECTIONS

### *2.3.1 Routine Lymph Collections*

Lymph draining from the cannulated pseudoafferent vessel was continuously collected in 100 ml bottles (Nalgene, Nalge Labwear, Rochester, New York, USA) containing 500 units of sodium heparin (Commonwealth Serum Laboratories (CSL), Parkville, Victoria, Australia) and 100 units penicillin (David Bull Laboratories (DBL), Mulgrave, Victoria, Australia). Collection bottles were changed at intervals of 8-16 hours. The cell concentration in the lymph was determined either using a model DN Coulter cell counter (Coulter Electronics Ltd., Harpenden, England) or by cell counts under a light microscope using an improved Neubauer counting chamber. Cell viability was assessed but uptake of 0.25% trypan blue stain (Sigma, St Louis, MO, USA).

### 2.3.2 *Sterile Collections*

Collection of lymph under aseptic conditions was undertaken when cells were required for culture or when lymph supernatants were required for analysis. Clean bottles were autoclaved at 121°C for 15 minutes with a piece of autoclave indicator tape placed over the nipple top. The bottle was opened under sterile conditions and 500IU of heparin (CSL) and 10000IU of gentamicin sulphate (DBL) was added and the lid replaced. When the bottle was tied onto the side of the sheep the cannula was wiped with 0.5% chlorohexidine (Hibitane, ICI Pharmaceuticals, Melbourne, Australia) in 70% ethanol (CSL), the tape removed from the nipple and the cannula placed inside the bottle avoiding contact with contaminated surfaces. A piece of Hibitane soaked cotton surgical gauze was then wrapped tightly round the cannula and the nipple top of the bottle and secured by two wire ties to minimise contamination.

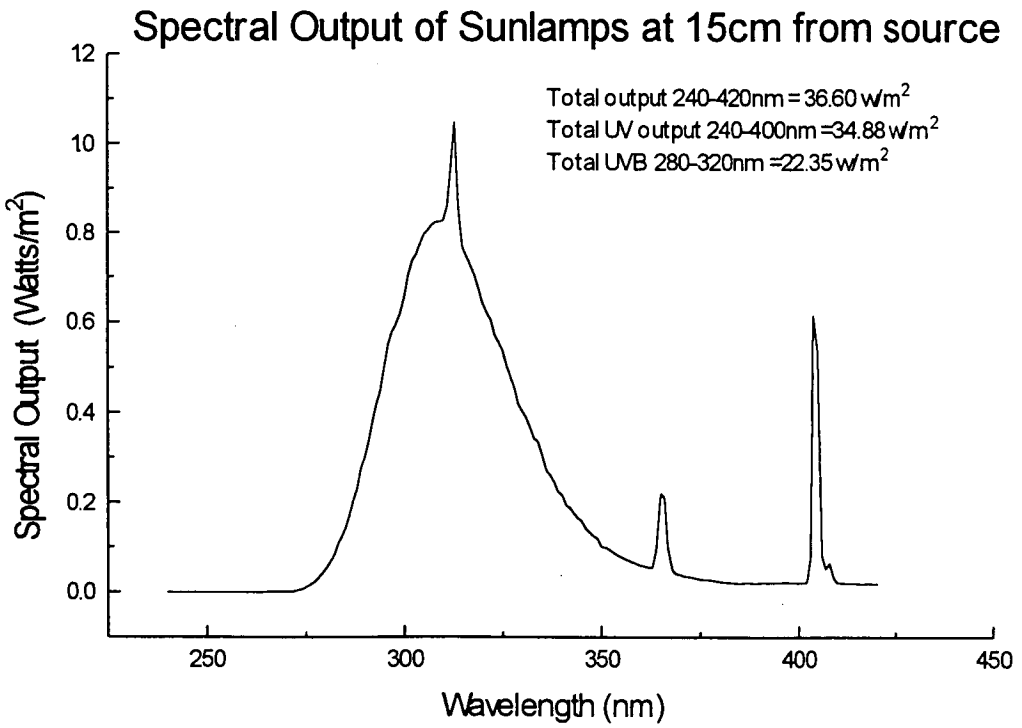
## 2.4 UV TREATMENT

The flank to be treated was shaved, exposing an area of approximately 200 $cm^2$ of the skin. The sheep was placed in a smaller metabolism cage to restrict its movement. The remainder of the body was protected by an aluminium backed reflective blanket. The bottles were removed from the sheep to prevent direct irradiation of the lymph. The sheep flank was then exposed to a bank of 6 pre-warmed (15 minutes) FS40 UVB emitting fluorescent lights (UVS, Ultraviolet Pty. Ltd., Scoresby Victoria, Australia) until the required UVB dose was reached (See Table 2.1). This dose was measured using an IL-1700 research radiometer (International Light, USA) fitted with an SED240 detector with diffuser and UVB-1 filter. Due to the nature, shape and texture of the flank skin of individual sheep this dose is approximate only. The spectral stability and total irradiance of the lamps was monitored at intervals using a SR9910 spectroradiometer (Figure 2.1). After the UV treatment new bottles were attached and collections recommenced.

**Table 2.1 UV dose regime**

| Approximate Duration of Exposure (mins) | Total UV dose | UVB dose given |
|---|---|---|
| 60 | 120 kJ/m² | 80.4 kJ/m² |
| 30 | 60 kJ/m² | 40.2 kJ/m² |
| 15 | 30 kJ/m² | 20.1 kJ/m² |
| 6 | 12 kJ/m² | 8.1 kJ/m² |
| 2 | 4 kJ/m² | 2.7 kJ/m² |

**Figure 2.1**



## 2.5 IMMUNOFLUORESCENT STAINING

### *2.5.1 Procedure*

Lymph was centrifuged at 800g for 5 minutes in 50 ml tubes (Corning, Corning, New York, USA). The supernatant was collected and stored at -80°C until cytokine assays were performed. The cell pellet was washed twice by centrifugation at 800g in phosphate buffered saline (PBS), which was prepared by a 1/20 dilution of PBS stock solution in Milli-Q water. This stock solution was prepared by dissolving 170 g NaCl (anhydrous) (BDH Chemicals, Australia Pty. Ltd. Kilsyth, Victoria), 21.4 g $Na_2HPO_4$ (anhydrous) (BDH) and 7.8 g $NaH_2PO_4.2H_20$ (BDH) in one litre of Milli-Q water. The centrifuged cells were resuspended in sterile PBS+2% bovine serum albumin (CSL) and 0.1% $NaN_3$(BDH) (PBS-BSA-AZIDE) at a concentration of $2x10^7$ cells per ml. 100µl of the washed cells were added to wells in a 96 well "v" bottom plate (Nunc, Roskilde, Denmark). The plate was centrifuged again at 800g, the supernatant discarded and the pellet resuspended in 50µl of the appropriate monoclonal antibody and incubated for 30 minutes at room temperature. The cells were then washed three times by centrifugation in PBS-BSA-AZIDE and resuspended in 50µl of sheep anti-mouse Ig $F(ab')_2$ fluorescein isothiocyanate (FITC) conjugate (Silenus Laboratories (Silenus), Hawthorn, Victoria, Australia). The CTLA-4 and MCD40 fusion proteins were detected using 50ml anti-human FITC conjugate (Silenus) and 50µl of sheep anti-mouse Ig $F(ab')_2$ phycoerytherin conjugate (Silenus) was used to identify SBU-T6 positive DC. Labelled cells were washed three times by centrifugation in PBS-BSA-AZIDE before being resuspended in 150µl of 500ml PBS with the following additions 13ml 40% Formaldehyde (BDH), 10g D-glucose (Ajax Chemicals Ltd. Sydney Australia) and 1.09 ml 15% NaN3 in PBS (BDH)(FACS FIX). The plate containing the fixed cells was covered in aluminium foil and stored at 4°C until analysed by flow cytometry. The above staining procedures were also performed on macrophages isolated from bronchial lavage fluid.

Flow cytometric analysis in Chapter 3 was performed with the assistance of Mr Mark Cozens, Department of Pathology, University of Tasmania using an EPICS 741 flow cytometer (Coulter), who also asssisted in later studies in Chapter 4, which a detailed analysis of dendritic cell populations were carried out on either a FACSCAN (Becton Dickenson (BD), Immunocytometry systems, San Jose, USA) or Elite ESP flow cytometer (Coulter). Langerhans cells were identified by their forward and 90° scatter profiles and intensity of fluorescence.

### 2.5.2 *Monoclonal Antibodies*

The mAb secreting cell lines producing anti-sheep CD4 (SBU-T4, Maddox *et al.*, 1985) and T19 (γδ T-cells, McClure, *et al.*, 1989) were used to produce specific monoclonal antibodies and were supplied by Dr C. Mackay (Basel, Switzerland) . Lyophilised supernatants against sheep MHC I (SBU 41-19, Gogolin-Ewens *et al.*, 1985), MHCII (SBU 49.1- Pan MHC II, Puri *et al.*, 1987a), CD1w1 (SBU-T6, Mackay *et al.*, 1985), CD8 (SBU-T8, Maddox *et al.*, 1985), CD4 (SBU-T4, Maddox *et al.*, 1985) and T19 (γδ T-cells, McClure, *et al.*, 1989) were obtained from Dr M. Brandon (Centre for Animal Biotechnology, School of Veterinary Science, University of Melbourne). The OM1, OM2 and OM3 mAbs were provided in the form of ascites fluid by Dr. J. Rothel (Centre for Animal Biotechnology, School of Veterinary Science, University of Melbourne) and were used with permission of Dr M. Pepin. These mAbs have been shown to react with ovine macrophages (Pepin *et al.*, 1992). A mAb against bovine CD14 (CC-G33) was also used and this was provided by (Dr. C. Howard, Institute of Animal Health, Compton, U.K.). Monoclonal antibodies were visualised using indirect immunofluorescence. The second layer antibody used was a fluorescein conjugated sheep $F(ab)_2$ portion of antibody specific for mouse immunoglobulin. Both fluorescein conjugated antibodies were obtained from Silenus Laboratories and were used at a 1/80 dilution in PBS-BSA-AZIDE.

A summary of the monoclonal antibodies used in flow cytometric studies is presented in Table 2.2

## 2.6 ASSESSMENT OF IMMUNITY TO OVALBUMIN IN SHEEP

### 2.6.1 *Preparation of Serum*

10 ml of ovine peripheral blood was collected into a 10 ml sterile container (Disposable Products) and incubated at 37°C for one hour. The clot that formed was detached from the side of the bottle and stored at 4°C overnight. The serum was poured into a tube and centrifuged for 10 minutes at 2000g to remove cells. The complement in the serum was inactivated by incubating at 56°C for 30 minutes.

### 2.6.2 *Antigen Coating of Sheep Red Blood Cells*

400µg of ovalbumin (OVA) (Sigma) was added to 10 ml of a 5% solution of washed (3 times in normal saline) sheep red blood cells containing 450µl of 0.1% chromic chloride (BDH), in normal saline. Following a 10 minute

## Table 2.2 Monoclonal Antibodies for Immunofluorescence

| Clone No. | Antigen | Dilution | Source | Reference/Remarks |
|---|---|---|---|---|
| SBU-I (41.19) | MHC I | undiluted supernatant | M. Brandon | Gologin-Ewens *et al.*, 1985 |
| SBU-T4 (44.38 + 44.97) | CD4 | undiluted supernatant | M. Brandon and C. Mackay | Maddox *et al.*, 1985 |
| SBU-T6 (20.27) | CD1w1* | undiluted supernatant | M. Brandon and C. Mackay | Mackay *et al.*, 1985 *Reclassified as CD1w1 by Nassens *et al.*, 1997 |
| SBU-T8 (38.65) | CD8 | undiluted supernatant | M. Brandon and C. Mackay | Maddox *et al.*, 1985 |
| SBU-T19 (19.19) | T-19 | undiluted supernatant | M. Brandon and C. Mackay | Mackay *et al.*, 1986 Mackay *et al.*, 1989 |
| SBU-II (49.1) | MHC II | undiluted supernatant | M. Brandon | Puri *et al.*, 1985a |
| OM1 | CD11c* | 1:80 acites | J. Rothel | Pepin *et al.*, 1992 *Reclassified, as CD11c by Nassens *et al.*, 1997 |
| OM2 | Macrophage subset | 1:40 acites | J. Rothel | Pepin *et al.*, 1992 |
| OM3 | Macrophage subset | 1:40 acites | J. Rothel | Pepin *et al.*, 1992 |
| CC-G33 | CD-14 | 1:50 | C. Howard | Sopp *et al.*, 1996 |
| CTLA4-Hγ1 | B7 molecules | undiluted supernatant | K. Shortman | Linsley *et al.*, 1991 |
| MCD40-Hγ1 | B7 control | undiluted supernatant | K.Shortman | Linsley *et al.*, 1991 |
| DDAF | mouse Ig | 1:80 | Silenus | FITC congated $F(ab')_2$ |
| HDF | Human Ig | 1:80 | Silenus | FITC congated $F(ab')_2$ |
| DDAPE | mouse Ig | 1:80 | Silenus | PE congated $F(ab')_2$ |

incubation at room temperature, coated erythrocytes were washed three times in PBS and made up to 10 ml with PBS.

### 2.6.3 *Haemagglutination Assay*

25 µl samples of sheep serum (as prepared above) were diluted using serial 2 fold dilution in PBS across wells 1-11 of a 96 well round bottom microtitre plate (Nunc). Wells in column 12 contained only 25 µl of PBS and served as a negative control. 25 µl of a 2.5% suspension of OVA coupled sheep red blood cells (SRBC) was added to each well and mixed gently. The plate was then sealed with adhesive tape and incubated at 37°C for 1 hour. Antibody titre was determined by the reciprocal of the highest dilution giving 50% agglutination of SRBC.

## 2.7 ANTIGEN PRESENTATION ASSAY

### 2.7.1 *Immunisation Procedure*

Alum precipitated OVA was prepared by the addition of 4.5 ml of 1 M $NaHC0_3$ (BDH) to a 10 ml solution of 2 mg/ml of OVA (Sigma) in PBS. This was followed by the slow addition of 10 ml of 0.2 M $AlK(SO_4)_2.12H_20$ (BDH) while stirring. The solution was left for 15 minutes at room temperature, then washed three times in PBS by centrifugation (300g for 15 minutes) and resuspended at the concentration required (method from Hudson and Hay, 1980).

Sheep were immunised twice over a 48 hour period by subcutaneous injection between the scapulae with a suspension of alum precipitated OVA (2 mg/ml). This was repeated eight weeks later and serum antibody levels were determined by haemagglutination as detailed above. Re-immunisation was performed if the titres were low.

### 2.7.2 *Collection Procedure*

Lymph from a cannulated pseudoafferent lymphatic vessel was collected under sterile conditions (see 2.2.2). 5-10 ml of peripheral blood from the same sheep was collected into a syringe containing heparin (CSL) at the same time as lymph was collected.

### 2.7.3 *Assay Procedure*

This procedure was adapted from the method of Bujdoso *et al.*, (1989) by Ragg *et al.*, (1995). Cells from sterile lymph were collected by centrifugation (5

minutes at 850g) washed twice in PBS and resuspended in 5 ml of RPMI 1640 and gentamicin (100 units/ml) (RPMI-Wash media). This was layered over a 14.5% metrizamide (Nycomed, Oslo, Norway)/15% FCS (CSL) gradient. The peripheral blood was diluted 1:1 with PBS and was underlayed with 5ml Histopaque 1077 (Sigma). The blood and washed cells were centrifuged at 850g for 30 minutes.

The peripheral blood mononuclear cells (PBMC) were collected from the Histopaque/serum interface and placed in a 10 ml tube. The DC were collected from the interface of the metrizamide gradient and placed in a 10 ml tube. The cells were washed twice in PBS and counted using an improved Neubauer counting chamber or DN coulter counter. PBMC were resuspended in RPMI 1640 (HyClone), 2mM L-glutamine (CSL), 5x10-5 M 2-mercaptoethanol (Sigma), 100U/ml gentamicin (DBL) and 5% FCS (RPMI+Supplements) and incubated at 37°C/5% $CO_2$ until required. The DC were resuspended at $2x10^6$ cells/ml in RPMI+100U/ml gentamicin (DBL) (WASH MEDIA). The resulting suspension was divided into two tubes. To one tube, 10 mg/ml OVA in PBS was added to give a final concentration of 1mg/ml, both cell suspensions were the incubated for 1 hour at 37°C. After incubation DC, were washed once with PBS and resuspended at $1x10^7$ cells/ml in PBS. DC were then incubated in a 25 µg/ml solution of Mitomycin C (Kogyo Co. Ltd., Kyowa, Haako, Tokyo Japan) for 20 minutes at 37°C. The suspension was made up to 10 ml and mixed by inversion and left to stand for 5 minutes allowing clumped dead cells to settle out. All but a small fraction of the suspension which contained clumped dead cells was removed and placed in a fresh 10 ml tube. This was washed twice by centrifugation in PBS and resuspended at $1x10^6$ in RPMI+supplements. Assays were performed in triplicate using a 96 well round bottomed plate (Greiner, Frickenhausen, Germany). 100µl of OVA pulsed DC were added to the first three wells and a 2 fold serial dilution was performed to 1:16 dilution, 100µl of PBMC were then added to each well. The same procedure was followed for unpulsed DC. Controls were used to assess proliferation of the separate cell populations which including pulsed DC, unpulsed DC or PBMC The cells were then incubated at 37°C/5% $CO_2$ for five days.

All wells were pulsed with 10µl tritiated ([$^3$H]) thymidine (Amersham Life Science, Amersham, U.K.) 16 hours prior to harvesting. The cells were harvested on to glass filter paper (ICN Biomedicals, Aurora, Ohio, USA) using a cell harvester (Skatron, Australia) and counted in 0.5 ml of scintillation fluid

for one minute in a Rackbeta scintillation counter (LKB Wallac, Turku, Finland).The result was expressed as the stimulation index as shown below which has been used as a measure of antigen specific proliferation.

$$\text{Stimulation index} = \frac{^{3}\text{H-thymidine incorporation by PBMC+antigen pulsed DC}}{^{3}\text{H- thymidine incorporation by PBMC+unpulsed DC}}$$

## 2.8 STATISTICAL ANALYSIS

The use of outbred sheep and the nature of the surgery involved, prevents direct comparison of the number of cells migrating from the skin of each sheep. Accordingly, only changes in the trend of cell migration associated with each treatment protocol have been compared.

The normal range of lymphoid cell migration for individual sheep was calculated as twice the standard deviation of the mean of at least 4 collection points prior to the UV treatment. This represents >90% confidence interval (Zar *et al.*, 1984). This was used as a guide to determine the normal range migration of cell populations or the normal range of stimulation indices. This method was also used to assess the normal range of B7 expression on migrating LC and levels of cytokines prior to UV exposure of the skin. Where only two background points were used +/- 4.4 times the standard deviation from the mean was applied to set confidence limits of >90% (Zar *et al.*, 1984). The stimulation indices for the functional studies were plotted showing the mean stimulation index +/- the error accumulated by the division of CPM of quadruplicate samples of PBMC cultured with antigen pulsed DC by mean CPM values of quadruplicate samples of PBMC cultured with unpulsed DC.

## 2.9 CYTOKINE ELISA'S

### *2.9.1 IL-1β*

This assay was based on the method described in Egan *et al.*, (1994). The antibodies used in this assay were provided by G. Barcham, Centre for Biotechnology, University of Melbourne, Australia. The mAb directed against ovine IL-1β (Clone 3.41, ascites) was diluted 1:250 in 0.05M carbonate/bicarbonate buffer pH 9.6. This buffer was prepared immediately prior to use by the addition of 16 ml of 0.2M sodium bicarbonate to 34 ml of 0.2 M sodium hydrogen carbonate and made up to 200 ml with Milli-Q water. 100

µl of the antibody/buffer solution was added to each well of a 96 well ELISA plate (Disposable products, Sydney, Australia). Plates were incubated overnight at room temperature to allow coating of the plate with antibody. Plates were washed three times with PBS-TWEEN (PBS + 0.05% Tween (Bio-Rad)) and non-specific binding sites blocked by the addition of 200 µl PBS-TWEEN+3%-FC. The plate was then incubated for one hour at 37°C and was then washed twice with PBS-TWEEN, before the addition of 100µl of duplicate samples and standards occurred. Re-incubation for one hour at 37°C was followed by four washes in PBS-TWEEN and a further two hour incubation in 100µl polyclonal rabbit anti-sheep IL-1β antibody. A further five washes in PBS-TWEEN were performed followed by a one hour incubation at 37°C five washes in PBS-TWEEN, 100µl of sheep anti-rabbit-horse radish peroxidase conjugate was added prior to re-incubation. After the final incubation the plate was washed six times in PBS-TWEEN before 100µl of 3,3',5,5' tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories (KPL), USA) per well was added. The reaction was stopped by the addition of 100µl of 0.18M $H_2SO_4$ (BDH) to each well. The absorbance of each well was measured using a Bio-Rad 3550 plate reader at a wavelength of 450 nm

### *2.9.2 GM-CSF*

The components for the GM-CSF immunoassay were obtained from Dr. Haig (Moredun Institute, Edinburgh, UK). ELISA plates (Disposable Products) were coated overnight at 4°C with 50µl per well of 8D8 affinity purified mAb (8D8) at 1µg/ml in freshly prepared 0.1 M carbonate buffer, pH 9.6 (see previous section). The wells were washed six times with PBS-TWEEN and then blocked with PBS-TWEEN+3%-BSA- for 1 hour. The wells were subsequently washed twice in PBS-TWEEN. 50µl of samples and standards were added, in duplicate, to wells. Lymph and culture supernatants were added after being diluted 1:3 in in PBS-3%-BSA-TWEEN. The plate was then incubated for one and half hours at room temperature. This was followed by a further six washes with PBS-TWEEN. 50µl of affinity purified, peroxidase conjugated, monoclonal (3C2) was added to each well and incubated for one hour at room temperature. After this incubation the wells were washed a further six times in PBS-TWEEN. 100µl of TMB (KPL) was added to each well and once the colour change had occurred 100µl of 0.18M $H_2SO4$ (BDH) was added to halt the reaction. The absorbance of each well was measured using a Bio-Rad 3550 plate reader at a wavelength of 450 nm

### 2.9.3 *Prostaglandin $E_2$ EIA*

Supernatants from DC:PBMC co-cultures were assayed for $PGE_2$ levels using a $PGE_2$ competitive enzyme immunoassay kit supplied by Cayman Chemical Co. (USA). This assay system measures $PGE_2$ levels by the competition between varying amounts of $PGE_2$ contained in samples and a known amount of $PGE_2$ tracer for a limited amount of a mouse anti-$PGE_2$ mAb. The mAb-$PGE_2$ complex binds to the goat anti-mouse polyclonal antibody that was previously attached to the well. The tracer, sample and monoclonal antibody were incubated 18 hours at room temperature. Following this incubation the plate was washed five times in a 1/500 dilution of the wash buffer provided. The activity of the acetylcholinesterase linked tracer was detected by incubation for 90 minutes in Ellman's reagent. The absorbance (ABS) was measured using a Bio-Rad 3550 plate reader at a wavelength of 415 nm. Thus the amount of tracer remaining is inversely proportional to the amount of $PGE_2$ in the sample. Standards were supplied with the kit and a 1/10 dilution was performed followed by doubling dilutions to give a range of 8-1000 pg/ml. Samples were used at a 1/5 dilution in EIA buffer (both the EIA buffer and Wash buffer concentrates were supplied as part of the kit and required dilution in Milli-Q water before use). Non specific binding (NSB) was assessed by the addition of the tracer without the addition of the monoclonal antibody, while the maximum binding was assessed by adding tracer and monoclonal antibody only. The total activity (TA) of the tracer was assessed by adding the 5µl of tracer to the wells together with the Ellman's reagent. The percentage sample bound/maximum bound (B%/B0) was calculated for samples and standards using the following formula.

$$\%B/B0 = [(Abs_{Sample} - Abs_{NSB}) / (Abs_{B0} - Abs_{NSB})] \times 100$$

A curve was constructed by graphing %B/B0 vs $PGE_2$ concentration for each standard and this was used to quantitate the levels of $PGE_2$ in samples. The detection limits for this kit were between 80% and 20% of the maximum bound. Samples outside this range were reassessed at a different dilution.

### 2.9.4 *IFNγ*

The supernatants from DC:PBMC co-cultures were assayed using the BOVIGAM™, commercial bovine interferon γ ELISA from CSL, Veterinary Division (Parkville, Victoria, Australia). This kit has been shown to react with ovine IFNγ (Rothel *et al*, 1990). 50µl of diluent was added to wells which were pre-coated with an IFNγ specific mAb. To these wells 50 µl of samples and

supplied standards and controls were added. This was incubated for one hour at room temperature followed by six washes in the diluted wash media provided. After which 100 µl of conjugated anti-IFNγ was added, followed by a further one hour incubation at room temperature. The plate was then washed a further six times, followed by the addition of 100 µl of the supplied chromogen, which was incubated for 30 minutes. The reaction was stopped by the addition of 50ml of 0.25 M $H_2SO4$ (BDH). The absorbance of each well was measured using a Bio-Rad 3550 plate reader at a wavelength of 450 nm.

## 2.10 ISOLATION OF CELLS FROM ALVEOLAR LAVAGE FLUID

The lungs and trachea were removed intact from a euthanised sheep, 1-2 years old, and flushed with 400-800 ml of sterile PBS. The lavage fluid was passed through a sterile stainless steel fine sieve to remove debris. The cells were gathered by centrifugation (800g) for 10 mins. The cells were washed twice in WASH MEDIA before being resuspend at $4x10^6$ cells/ml in RPMI+Supplements.

### *2.10.1 IL-10 macrophage inhibition assay*

The ovine assay for IL-10-like activity was based on the method described by Hein (1997) and by Martin *et al.*, (1995). Test samples were dispensed in 0.5ml aliquots to 24 well culture plates (Corning) and 0.5 ml of macrophage suspension was added. LPS (Sigma, #L2360) was added to a final concentration of 10 ng/ml. Control cultures included macrophages incubated with and without LPS. The cells were cultured for 24 hours at 37°C and 5% CO2. The cultures were centrifuged at 10,000 rpm for 2 mins to remove cells and debris, the resulting supernatants were aliquoted and stored at -80°C. The supernatants were thawed when required and analysed for IL-1β concentration (see section 2.9.1).

## 2.11 DETECTION OF IL-10 PROTEIN

### *2.11.1 SDS -Polyacrylamide Gel Electrophoresis*

A 15% SDS (sodium dodecyl sulphate)-polyacrylamide mini gel was prepared to perform electrophoresis on lymph samples which were found to be positive for IL-10-like activity from the IL-10 bioassay. A Mini-PROTEAN II electrophoresis cell (Bio-Rad) was used for both the preparation and running of

the gel. The gel was formed by adding 5ml of 30% Acrylamide/bis-acrylamide solution (Bio-Rad) to 2.4 ml distilled water, followed by 2.5 ml of 1.5 M Tris-HCl (pH 8.8) (Sigma) and 50µl of 20% SDS (Bio-Rad). This solution was degassed for 15 minutes by applying a vacuum to a stoppered conical flask. In turn 50µl of ammonium persulphate (Bio-Rad) and 5µl of N,N,N',N', tetramethylethylenediamine (TEMED, Bio-Rad) were added and the gel was poured between two glass plates. 100µl of 0.1% sodium dodecyl sulphate (SDS, Bio-Rad) was added as an overlay to prevent evaporation of the gel contents. The gel was then allowed to polymerise for approximately 1 hour, during which time a 4.0% stacking gel was prepared. To 3.3 ml distilled water 1.25 ml of 0.5 M Tris -HCl (pH 6.8), 25 µl of 20% SDS (Bio-Rad) and 670 µl of acrylamide/bis-acrylamide was added. The solution was again degassed and 50µl of APS and 5µl TEMED added. The overlay was decanted from the separating gel and the stacking gel was added. The comb which forms the lanes to which the samples are loaded, was inserted. The stacking gel was allowed to polymerise for approximately 30 mins. 20 µl of lymph was mixed with 20 µl of 2 x SDS sample reducing buffer (2.0 ml 0.5 M Tris, pH 6.8, 1.6 ml glycerol (Bio-Rad), 3.2 ml of 10% SDS, 2-β-mercaptoethanol (Bio-Rad) and 400 ml of 0.05% bromophenol blue (Bio-Rad) and the samples placed in boiling water for 5 minutes. Samples were microfuged at 12,000 rpm and placed on ice. The gel was placed into the cell, which contained approximately 600 ml of running buffer (1.8g Trizma base (Sigma), 8.64g glycine (Bio-Rad), 0.6g SDS (Bio-Rad) dissolved in 600 ml Milli-Q water) and samples (15 µl) loaded into specified lanes of the gel. 10 ml of pre-stained SDS-PAGE broad range marker (Bio-Rad), was also added to a lane in the gel. The Bio-Rad 200 power pack was used to apply current through the gel. Initially a 100 volt current was applied but when the sample began to run through the stacking gel the current was increased to 200 volts. When protein lined up on the separating gel the current was lowered to 90 volts. After the sample had finished running the gel was carefully removed from between the glass plates and the stacking gel removed from the separating gel. The gel was then western blotted to enable immunochemical probing for IL-10.

### 2.11.2 Western Blotting

The SDS-PAGE gel was soaked for 15 minutes in Towbin transfer buffer which was prepared by the dissolving of 3.03g Trizma base (Sigma), 14.4 g glycine (Bio-Rad) and 200ml of Methanol (BDH)in 1l of water. The gel was then placed on two sheets of filter paper and covered firstly by a nitrocellulose

membrane (Bio-Rad) and two further layers of filter paper. A cylinder was rolled over this sandwich to remove air bubbles, trapped between the different layers. This was placed into a mini trans-blot cell (Bio-Rad) containing Towbin transfer buffer and an ice pack. 30v of current from a Bio-Rad 200 power pack was applied through the cell overnight at 4°C. The apparatus was dismantled and the western membrane now containing protein transferred from the gel was washed twice by gently agitation for 10 minutes in TBS (2.425 g Tris (Sigma) + 9g NaCl (BDH) dissolved in 1L Milli-Q water), pH 7.5. This was followed by a 2 hour incubation of the membrane in blocking buffer (TBS + 5% BSA). Polyclonal rabbit anti-ovine IL-10 antibody was supplied by G. Barcham (Centre for Biotechnology, University of Melbourne, Australia) and was then added to give a final ratio of 1:100 polyclonal antibody to blocking buffer. This was incubated for a further hour at room temperature. The membrane was washed twice in TTBS (TBS+ 0.05% Tween-20) and once in TBS before being incubated for 1 hour in a solution containing a 1/1000 dilution of goat anti-rabbit horseradish peroxidase conjugated antibodies (Silenus) in blocking buffer. After this incubation, the membrane was washed four times in TTBS, before being placed in a 10 ml buffered solution containing 3,3-diaminobenzidine (DAB) and urea peroxide. This was prepared 30 minutes prior to use by dissolving DAB and urea tablets in 10 ml of distilled water (Sigma). The membrane was left in the DAB (peroxidase substrate) for a sufficient time for clearly visible signal to developed. The reaction was halted by washing the membrane thoroughly in distilled water.

## 2.12 IL-5 BIOASSAY

This bioassay involved culturing the BAF cell line which has a requirement for IL-5 in order to proliferate. IL-5 was provided to keep the cells growing by the addition of 5% v/vol supernatant from the IL-5 producing X63 cell line. Both cell lines were generously provided by Dr. D. Emery (CSIRO, Sydney, NSW) and used with permission of Dr. C Sanderson. BAF cells were cultured in culture media containing DMEM Hi-Glucose (HyClone), 2mM L-glutamine (CSL), 1mM pyruvate (CSL), 100 mg/ml Penicillin/ 60 mg/ml streptomycin (CSL), $5x10^{-5}$ M 2-mercaptoethanol (Sigma), 5% FCS (CSL) (DMEM + Supplements) with the addition of 5% X63 supernatant for two days. BAF cells were washed twice in an excess of medium only, and resuspend at a concentration of $10^6$ cells/ml. 100µl of cell suspension was added to each well on a 96 well round bottom culture plate (Greiner). Test wells contained the BAF cells plus 50µl of sample and 50µl of culture media. Wells also containing X63

supernatant, to give a final concentration of 5% constituted positive controls. 100µl media only was added to BAF cells to serve as a negative control. Cells were incubated for 36 hours at 37°C/5% $CO_2$ and pulsed with [$^3$H] thymidine (Amersham) over the last eight hours of culture. The cells were harvested on to glass filter paper (ICN Biomedicals) using a cell harvester (Skatron, Australia) and counted in 0.5 ml of scintillation fluid for one minute in a Rackbeta scintillation counter (LKB). The ability of samples to stimulate BAF cell growth was compared to the positive and negative controls.

## 2.13 MISCELLANEOUS METHODS

### *2.13.1 Glassware*

All pipettes used for cell culture was soaked overnight in 2% extran. Pipettes were washed in tap water in a self-dumping pipette washer for at least 4 hours. Pipettes were then rinsed in five changes of Milli-RO water followed by a further 3 Milli-Q rinses. After which cleaned pipettes were dried in a 50°C oven before being plugged with cotton wool and autoclaved at 121°C for 15 mins in a copper canister. Then the pipettes were allowed to dry overnight in a 50°C oven. All bottles were washed using a phosphate free detergent (Extran 300, BDH) rinsed ten times with tap water followed by a further five rinses with Milli-Q water. All bottles were dried and then autoclaved at 121° for 15 minutes.

### *2.13.2 Solutions and reagents*

Solutions which required sterilisation were sterilised either by filtration through a 0.2µm filter (Millipore) and transferred to a previously autoclaved 500ml bottle or by autoclaving at 121° for 30 mins. Deionised reagent grade water was use in all solutions (Milli-Q system, Millipore Corp. USA).

### *2.13.3 Preparation of Media*

A 10 litre powder pack of media (RPMI-1640 or DMEM) (HyClone, Logan, Utah USA) was added to 10 litres of Milli-Q water and dissolved by stirring. 20g of Sodium Bicarbonate was also added bring the concentration to 2g/L and the media was left to stir for a minimum of 15 minutes. A peristaltic pump was used to pump media through a 0.2µm MediKap hollow fibre filter (Microgon, Laguna Hills, California, USA) into sterile bottles, this step being performed in a Class II BioHazard Cabinet. Filled and capped bottles were stored at 37°C for

several days to check for contamination. Media that was free from contamination was stored at 4°C in the dark until required.

# CHAPTER 3 LANGERHANS CELL MIGRATION AFTER UV EXPOSURE

## 3.1 INTRODUCTION

The response of a T cell to an antigen is crucially dependant on its interaction with a dendritic cell (Ibrahim *et al.*, 1995). In the cutaneous environment, the dendritic cells which play this role are the LC. These cells form a network in the epidermis where they are believed to fulfil a role in immunological surveillance (Janeway 1992, Ibrahim *et al.*, 1995). LC can be activated by bacterial and viral products (Janeway *et al.*, 1992) or microenvironmental damage (Ibrahim *et al.*, 1995), to process antigens and migrate to the draining lymph node. There they present antigen to T cells resulting in the generation of specific immune responses (Silberberg-Sinakin and Thorbecke, 1980). Any process which deleteriously alters the migration, survival or the ability of LC to present antigen to T cells will reduce cutaneous immunity. This in turn may adversely impact on the ability of the immune system to deal with the growth of neoplastic cells within the skin.

UV light, especially UVB is able to penetrate the outer protective layers of the skin, damaging the underlying cells including LC (Aberer *et al.*, 1981). UV light has been shown to cause cell death in the epidermal layers of the skin (Kerr *et al.*, 1972, Olson and Everett, 1975). It has also been shown that the immune system is impaired in its ability to respond to antigens applied through UV damaged skin (Haniszko and Suskind, 1963, Greene *et al.*, 1979, Toews *et al.*, 1980, Morison *et al.*, 1981a, Morison *et al.*, 1981b, Noonan *et al.*, 1981, De Fabo and Noonan, 1983, Elmets *et al.*, 1985, Ullrich *et al.*, 1986, Glass *et al.*, 1990). Morphological and ultrastructural changes have been seen in LC within the epidermis (Aberer *et al.*, 1981, Obata and Tagami, 1985) and dendritic cells with UV specific damage, are found in the lymph nodes draining UV exposed skin (Sontag *et al.*, 1995). These findings pose the question of whether LC depleted from the epidermis after UV exposure, migrate to the draining lymph node or die *in situ*. If these cells do not all die *in situ* they may in turn contribute to the aberrations observed in immune function.

The sheep model is ideal for the investigation of migrating cell populations after UV exposure of the skin. Pseudoafferent lymphatic vessels were generated by the excision of the prefemoral lymph nodes and re-

anastomosis of the afferent lymphatics with what had been the efferent duct. Subsequent cannulation of pseudoafferent lymphatic vessels in this animal by the methods described by Hall and Morris (1965), allow the collection of large volumes of lymph and large cell numbers. This overcomes the problem of the small size and thus small lymph and cell output from true afferent lymphatic vessels draining the skin of small animals such as mice. The cell populations in pseudoafferent lymphatic vessels are similar to that of the true vessels and contains large numbers of dendritic cells, comprising 1-10% of the total population (Budjuso *et al.*, 1989). The study of cells migrating from the skin in pseudoafferent lymphatic vessels as compared to lymph nodes, eliminates the presence of resident DC which occur in the DLN. However it must be emphasised that the cells found in the psuedoafferent lymph would be the cells that eventually reach the lymph node. The use of patent blue lymphography dye to define the area of skin drained by the pseudoafferent vessel also minimises contaminating DC from areas not affected by treatment.

## 3.2 MATERIALS AND METHODS

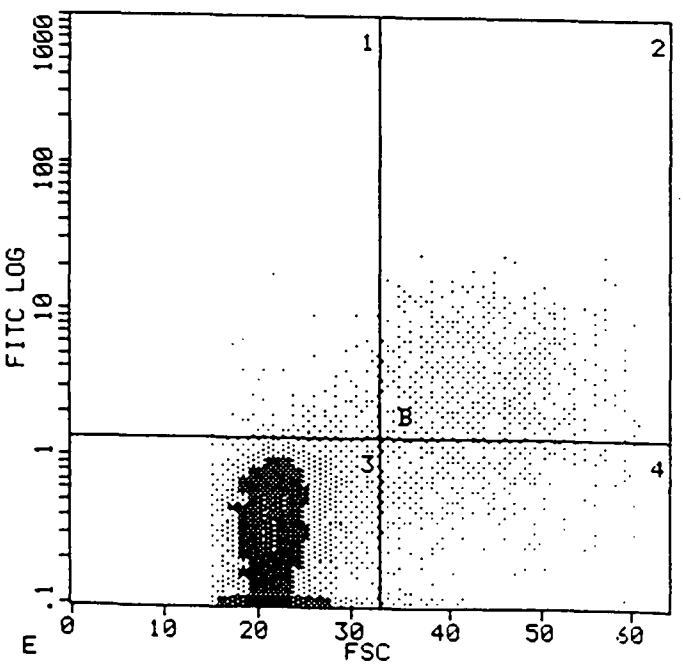
### *3.2.1 Experimental Plan*

The aim of this series of experiments was to look at changes in cell population kinetics of pseudoafferent lymph after treatment of the skin with UV light. In particular, the effects on LC were closely monitored.

After bilateral cannulation of pseudoafferent lymphatic vessels (Section 2.2.2), lymph was collected in bottles using the protocols described in sections 2.3.1 and 2.3.2. From each collection the volume of lymph flowing per hour was calculated and the cell concentration per millilitre was determined using a DN model coulter cell counter (Coulter Electronics Ltd., Harpenden, England). A surgical recovery period of usually 5-7 days was needed to ensure flow rate and cell concentration had stabilised. After this time, cells in the lymph were collected and stained using various monoclonal antibodies and immunofluorescent staining (Section 2.4). LC were identified by their forward and 90° scatter profiles and intensity of fluorescent staining with the SBU-T6 mAb (anti-CD1w1) (see Fig 3.1). $CD4^+$ T cells, $CD8^+$ T cells and $T19^+$ $\gamma\delta$ T cells were identified using the mAbs SBU-T4, SBU-T8 and SBU-T19 respectively. Flow cytometric analysis was performed using an EPICS 741 flow cytometer (Coulter). The staining of cells was repeated at least four times to establish the "normal" pattern of migration prior to further treatment. The flank skin of the

# Figure 3.1

Two parameter flow cytometric analysis of CD1 (SBU-T6) staining of pseudoafferent lymph cells. Langerhans cells are defined as large (high forward scatter) green fluorescent (FITC positive) cells. Langerhans cells are the population of cells found within quadrant B2 of this histogram. In this particular sample 1.3% were $CD1^{+}$ lymphocytes, 7.0% Langerhans Cells, 87.1% lymphocytes and 4.6% $CD1^{-}$ dendritic cells.



sheep was then treated with a single exposure UV fluorescent sunlamps as per section 2.3. The use of a single exposure avoids complications induced by multiple doses including epidermal thickening and melanogenesis (Kaidbey and Kligman, 1981), alterations in mediators involved in the production of erythema and pigmentation and thus optics of the skin (Kaidbey and Kligman, 1979). Monitoring continued for a two week period after exposure as LC numbers within the skin are shown to recover during this period (Obata and Tagami, 1985).

Various exposures were used ranging from a UVB dose of 2.7 kJ/m² to 80.4 kJ/m² (see table 2.1). The doses 2.7 kJ/m², 8.1 kJ/m², 20.1 kJ/m² and 40.2 kJ/m² UVB were examined in greater detail as they were found to be less damaging and better reflected "natural sunburning" doses in sheep.

### *3.2.2 Statistical Analysis*

The mean plus or minus two standard deviations of at least four pre-treatment points, were used to establish a normal range of migration. Points lying outside this normal range were regarded as significantly different to normal migration data (Zar, 1984).

### *3.2.3 Presentation of Data*

The nature of the outbred sheep model, means that direct comparison between individual experiments is very difficult. The parameters monitored such as afferent lymph flow, cell numbers and the percentage of individual cell populations, varies greatly from individual to another, and even from one flank to the other flank on the same sheep. Although consistent trends can be observed by exposing different sheep to the same treatment regime these factors prevent pooling data from replicate experiments. It is therefore necessary to present results from a single representative experiment. All results shown for each dose are from the same animal and are representative of the trends observed from at least three experiments. A summary table has showing the trends observed for each individual experiment has also been included for each UVB dose used. The sustained changes in migration mentioned are when two or more data points fall outside the normal range. The normal range for each parameter is represented by the area within the dotted lines in Figures 3.3.1.1 - 3.3.5.6. It should be stressed that figures from result sections 3.3.4-3.3.5 are from a previous thesis (Gavin Clydesdale, 1993) and

are used to illustrate the effects of higher doses of UV light on lymph output and cell migration kinetics.

## 3.3 RESULTS

### *3.3.1 Changes in Lymph Flow, Cell Kinetics and Composition after Exposure of Skin to 2.7 kJ/m² UVB*

#### 3.3.1.1 Effect on Total Cell Output and Lymph Flow

Lymph flow stayed within the normal range for the duration of this experiment, apart from the final point which was seen to fall below the normal range (Fig. 3.3.1.1). Cell output remained within the normal range for the duration of this experiment (Fig. 3.3.1.2)

#### 3.3.1.2 Effect on LC Migration

Initially LC migration remained within the normal range, before a slight increase above the normal range at 58 hours. This was followed by a transient return to within the normal range (Fig. 3.3.1.3). This in turn was followed by a significant, sustained increase in LC migration between 83-96 hours post-treatment. The 3.6 fold increase in LC at the 83 hour time point was the peak of LC migration. LC numbers then returned to within the normal range. After which there was a transient decrease below the normal range. A further sustained decrease below the normal range was seen between 168-191 hours post-treatment. This decrease was most significant at the 168 time point, representing a ten fold reduction in LC numbers. After this LC output was seen to increase, once again showing a sustained increase in LC numbers between 225-312 hours post irradiation. This late increase in LC numbers peaked at 273 hours post-treatment with a 2.6 fold increase in LC numbers.

Sustained increases in the proportion of LC were observed between 83-96 and 225-312 hours post-treatment (Fig. 3.3.1.4). The timing of the peak 3.4 fold increase in the percentage of LC, at 83 hours, corresponded to the peak increase seen in LC numbers (Fig. 3.3.1.3). The percentage of LC migrating between 168-191 hours was reduced. The largest reduction seen was at 168 hours which corresponded to 0.2 % LC compared to the mean pre-treatment value of 2.3%.

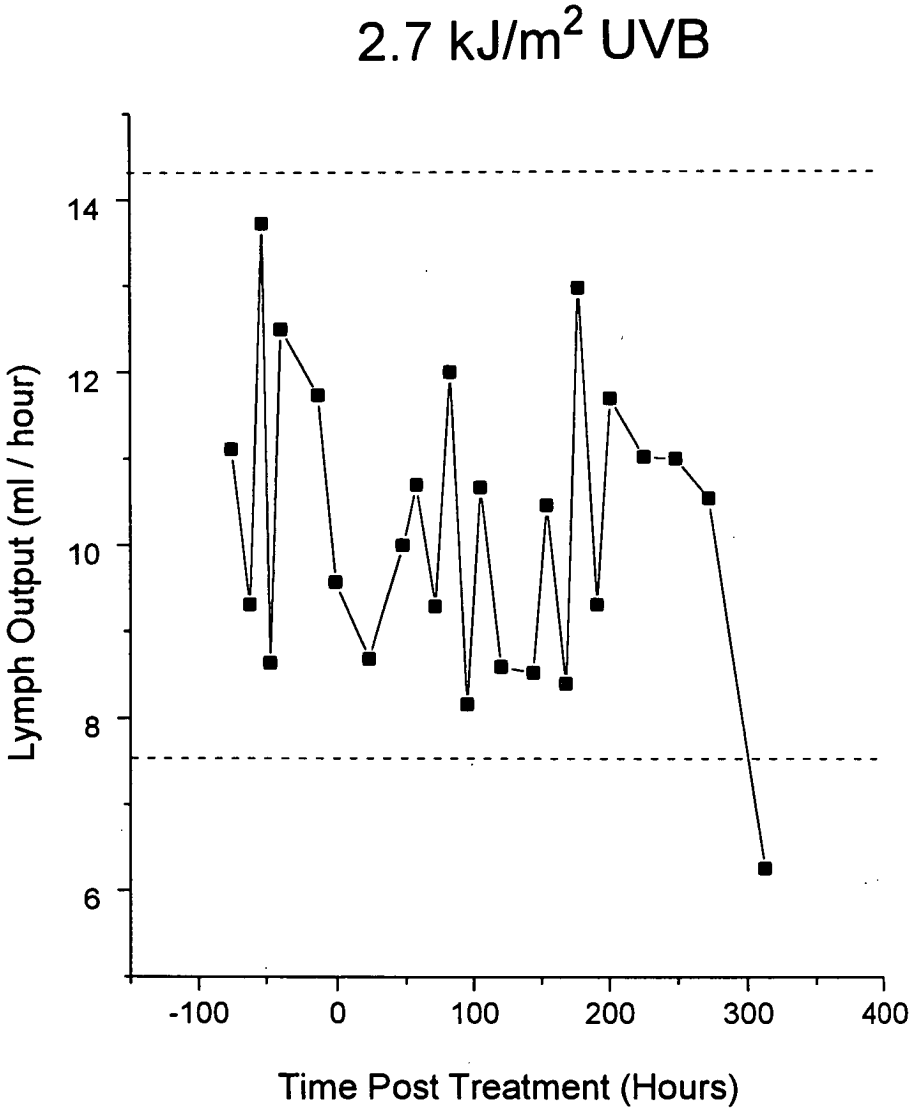



**Figure 3.3.1.1** The rate of lymph flow from skin treated with 2.7 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

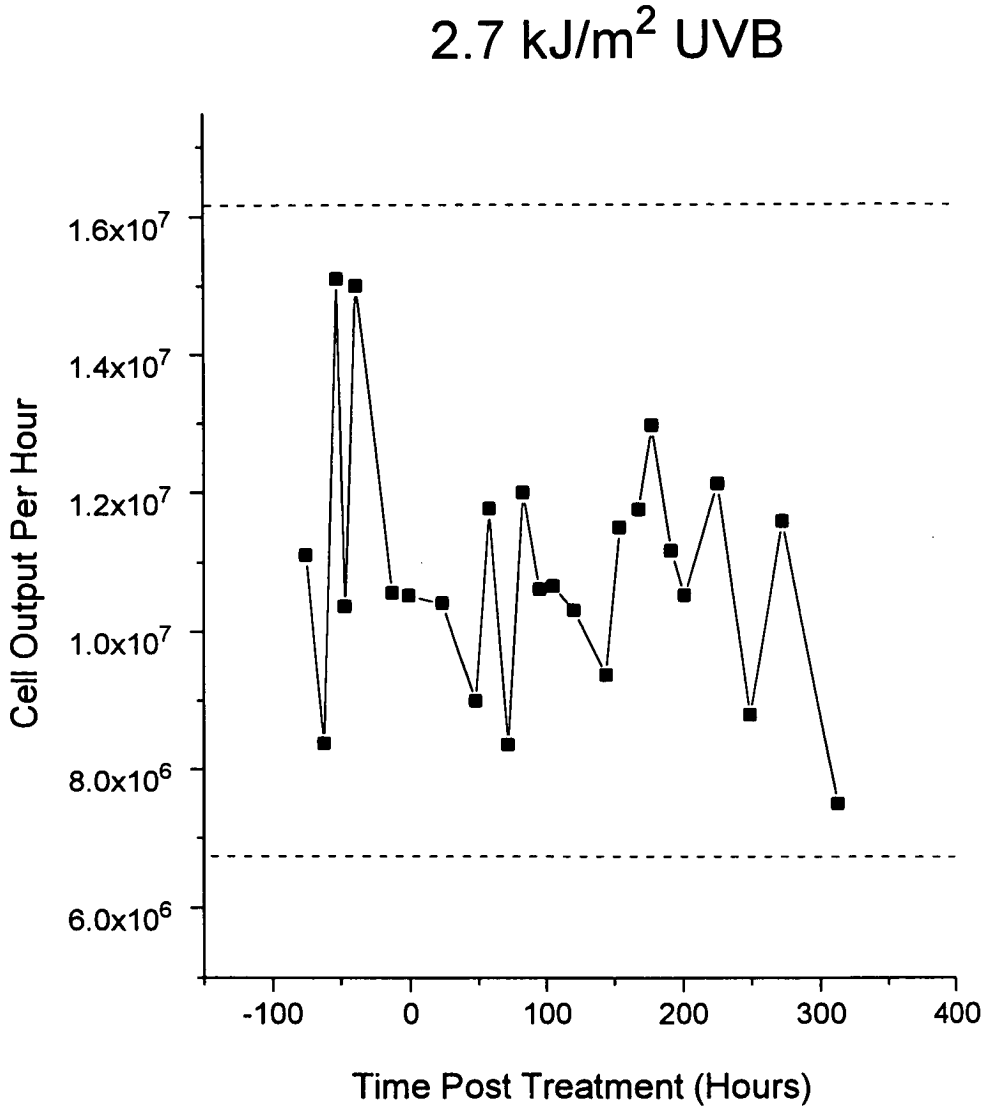



**Figure 3.3.1.2** Total cell output in lymph draining skin treated with 2.7 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

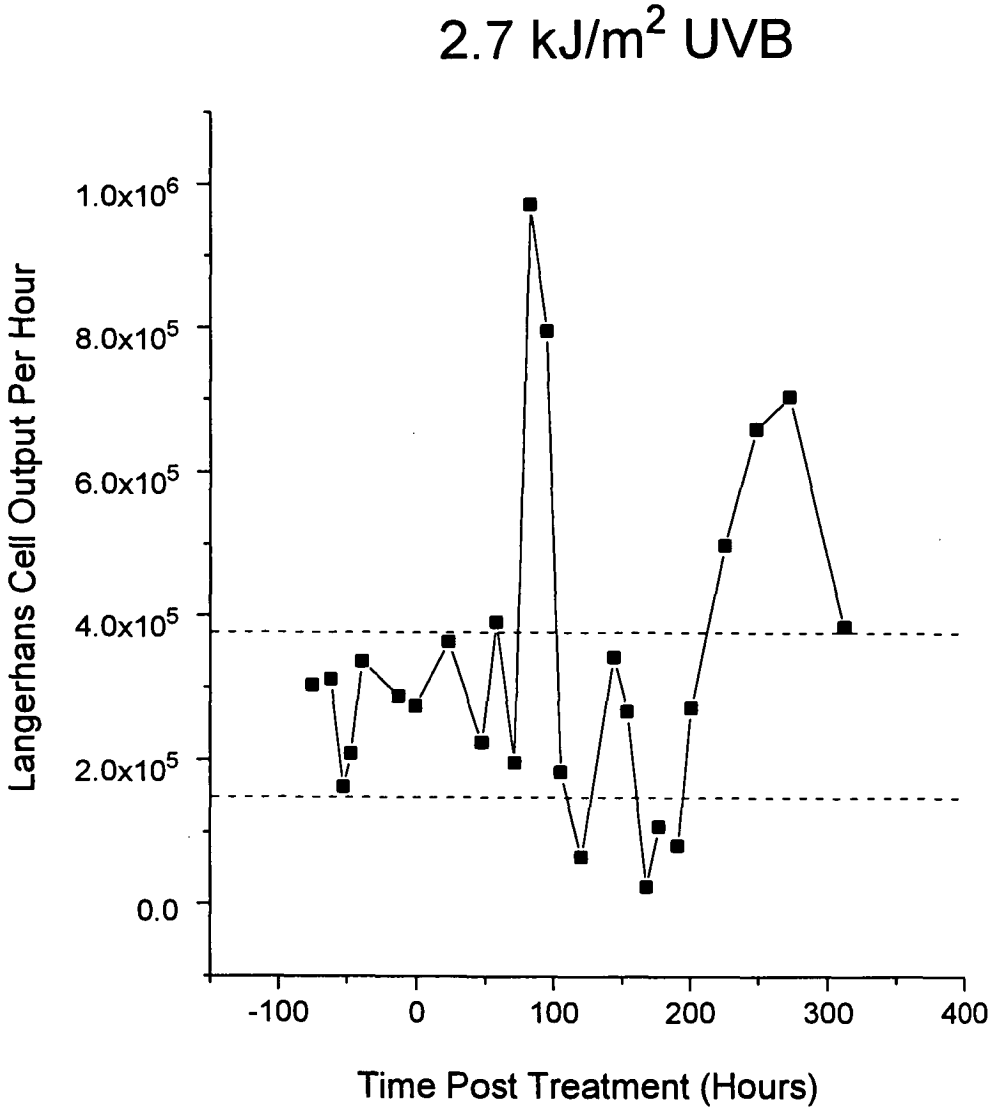



**Figure 3.3.1.3** Numbers of Langerhans cells in lymph draining skin treated with 2.7 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

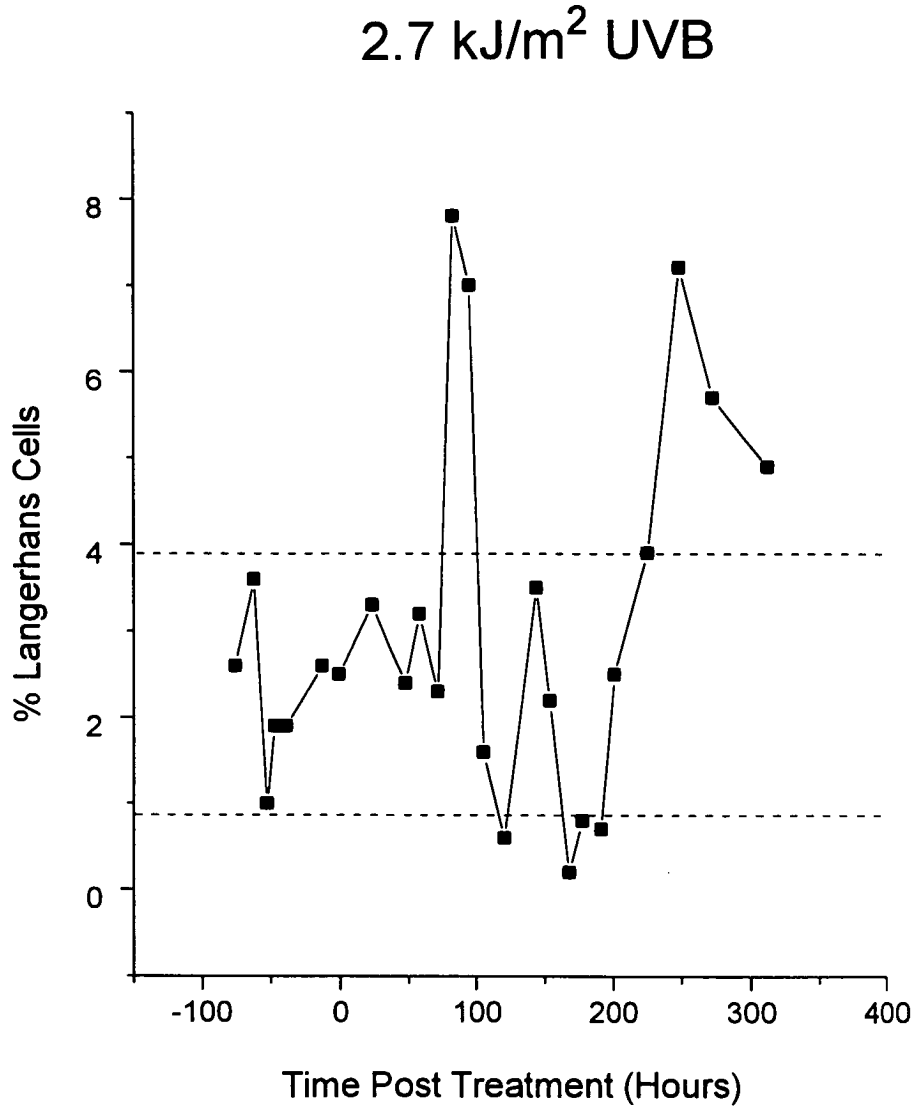



**Figure 3.3.1.4** Percentage of LC in lymph draining skin treated with 2.7 kJ/$m^2$ UVB treated skin. Dashed lines indicate the normal range for this single representative experiment.

### 3.3.1.3. Effect on T Cell Migration

The $T19^+$ γδ T cell migration remained within the normal range until 58 hours post-treatment when a slight increase above the normal range was observed. Immediately followed by a slight decrease below the normal range. A significant 1.8 fold increase occurred at 273 hours post-treatment (Fig. 3.3.1.5) and was the only major point of $T19^+$ γδ T cell migration for this experiment. $CD8^+$ T cell migration showed a peak of near identical size at the same time (Fig. 3.3.1.6). $CD4^+$ positive cells were seen at no time to increase above the normal range. A decrease in the migration of $T19^+$ γδ, $CD4^+$ and $CD8^+$ T cells was observed at 72 hours post-treatment (Fig. 3.3.1.5 and Fig. 3.3.1.6). Further decreases below the normal range were evident only in the $CD8^+$ cell population at 144 and 168 hours post-treatment, being just outside the normal range.

**Table 3.1 Summary of Effects after Exposure to 2.7 kJ/m² UVB**

| Parameter | Initial Increase (hours) | Peak increase (hours) | Magnitude of Peak Increase | Period of sustained increases (hours) |
|---|---|---|---|---|
| Lymph output | - | - | normal range | - |
| Total cell output | - | - | normal range | - |
| LC | 58 | 83 | 3.6 fold | 83-96, 225-312 |
| % LC | 83 | 83 | 3.4 fold | 83-96, 225-312 |
| $T19^+$ γδ T cells | 273 | 273 | 1.8 fold | - |
| $CD4^+$ T cells | - | - | normal range | - |
| $CD8^+$ T cells | 273 | 273 | 1.7 fold | - |

### 3.3.1.4 General

The results for the representative sheep used to illustrate trends after this experiment are summarised in Table 3.1. Exposure of sheep to 2.7 kJ/m² UVB, the lowest dose used, resulted in inconsistencies in lymph flow and cellular

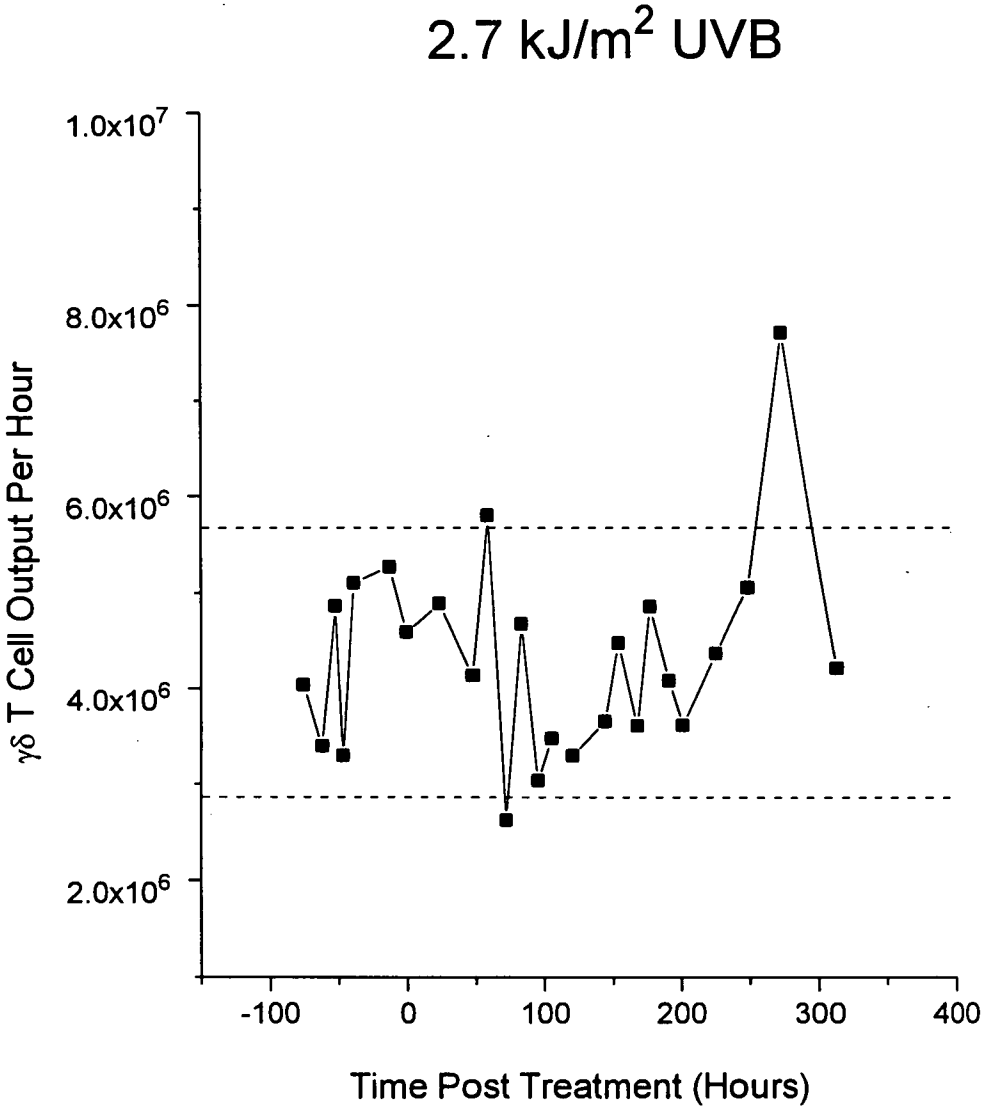



**Figure 3.3.1.5** Numbers of T19 $^{+}\gamma\delta$ T cells in lymph draining skin treated with 2.7 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

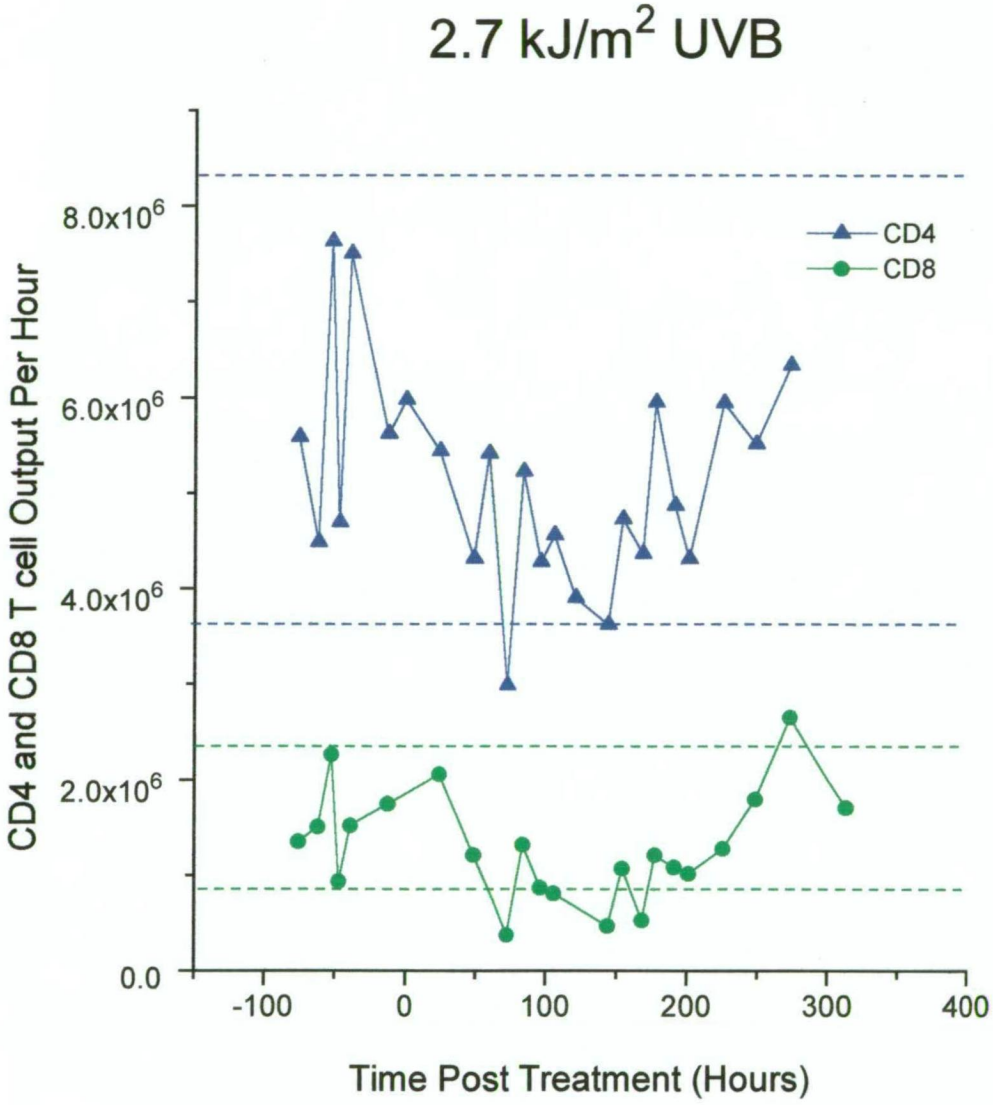



**Figure 3.3.1.6** Numbers of CD4 and CD8 T cells in lymph draining skin treated with 2.7 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

output, as well as variable effects on the LC, $T19^+$ γδ ,$CD4^+$ and $CD8^+$ T cell populations. A minority (one out of four sheep) showed sustained increases in lymph flow and total cell output. However, a majority (three out of four) showed a significant sustained increase in LC numbers, and all were approximately three fold in magnitude. However, only two out of four sheep were shown to have a corresponding increase in the percentage of LC in lymph. One out of the four sheep monitored showed a 1.8 fold increase in $T19^+$ γδ T cell output. The same sheep also showed $CD4^+$ and $CD8^+$ T cells to be increased above normal, however increases in $CD8^+$ cells were of lower magnitude. The observed increases in T cell populations in this sheep occurred in parallel with those of cellular output.

## *3.3.2 Changes in Lymph Flow, Cell Kinetics and Composition after Exposure of Skin to 8.1 kJ/m² UVB*

### 3.3.2.1 Effect on Total Cell Output and Lymph Flow

The exposure of the flank skin to 8.1 $kJ/m^2$ did not appear to cause any increase in the rate of lymph flow (Fig. 3.3.2.1). Lymph flow appeared to stay within the normal range at all times, with the exception being the final point. Similarly, cell output also remained within the normal range post-treatment (Fig. 3.3.2.2).

### 3.3.2.2 Effect on LC Migration

This treatment caused a sustained increase in the number of LC migrating in the pseudoafferent lymph from 25 hours to 148 hours post-irradiation (Fig. 3.3.2.3). This sustained increased peaked at 49 hours at a level of 2.4 fold above normal. This was followed by a further sustained elevation between 187 and 217 hours and further transient increases above normal at 240, 265, 290 and 314 hours post irradiation. Transient decreases below that of the normal range were seen a 11, 170 and 305 hours, after treatment, although the number of LC generally increased after this treatment (Fig. 3.3.2.3). Broadly speaking increases in the percentage of LC migrating paralleled the increases observed in LC numbers (Fig. 3.3.2.4). However, the first initial increase in the proportion of LC migrating was only sustained between 49 and 121 hours post exposure which is a shorter duration than that of increased LC numbers. The second sustained increase was mirrored by similar transient increases in the percentage of LC migrating at 240, 290 and 314 hours but not at 265 hours.

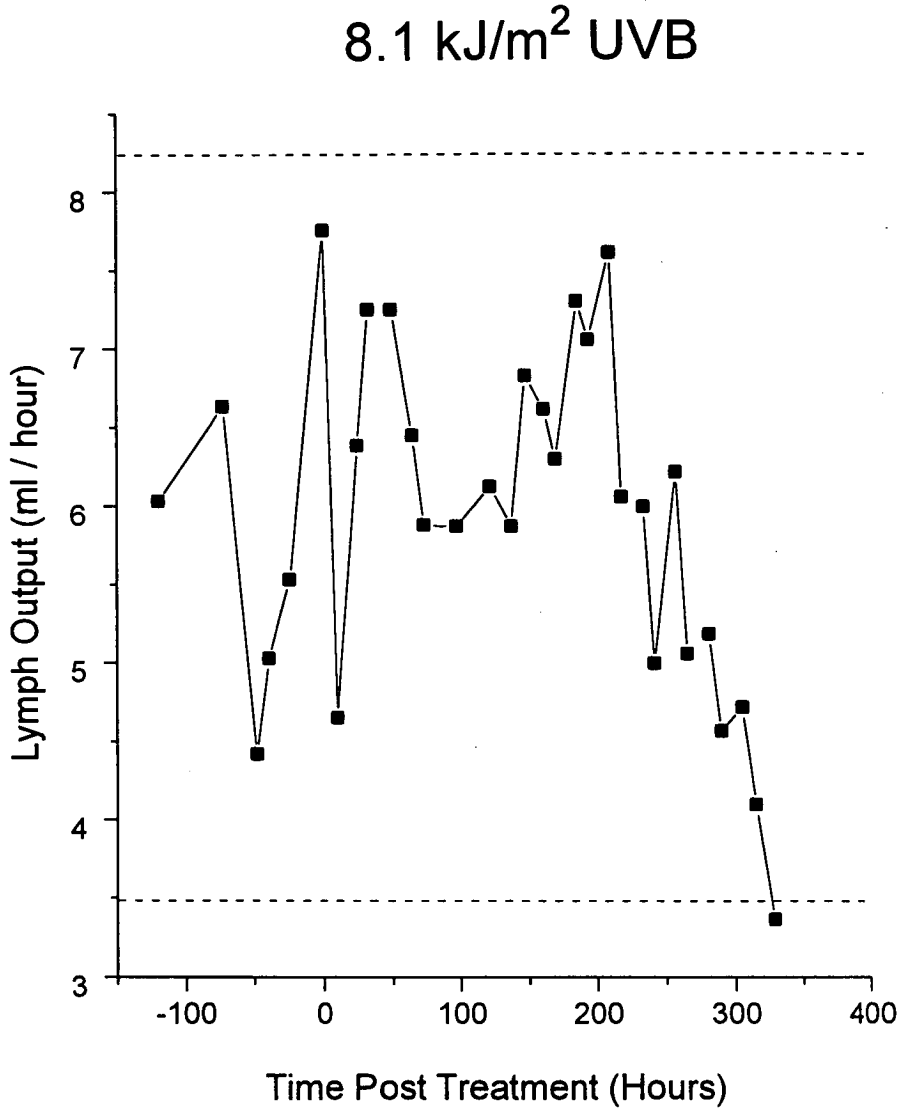



**Figure 3.3.2.1** The rate of lymph flow from skin treated with 8.1 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

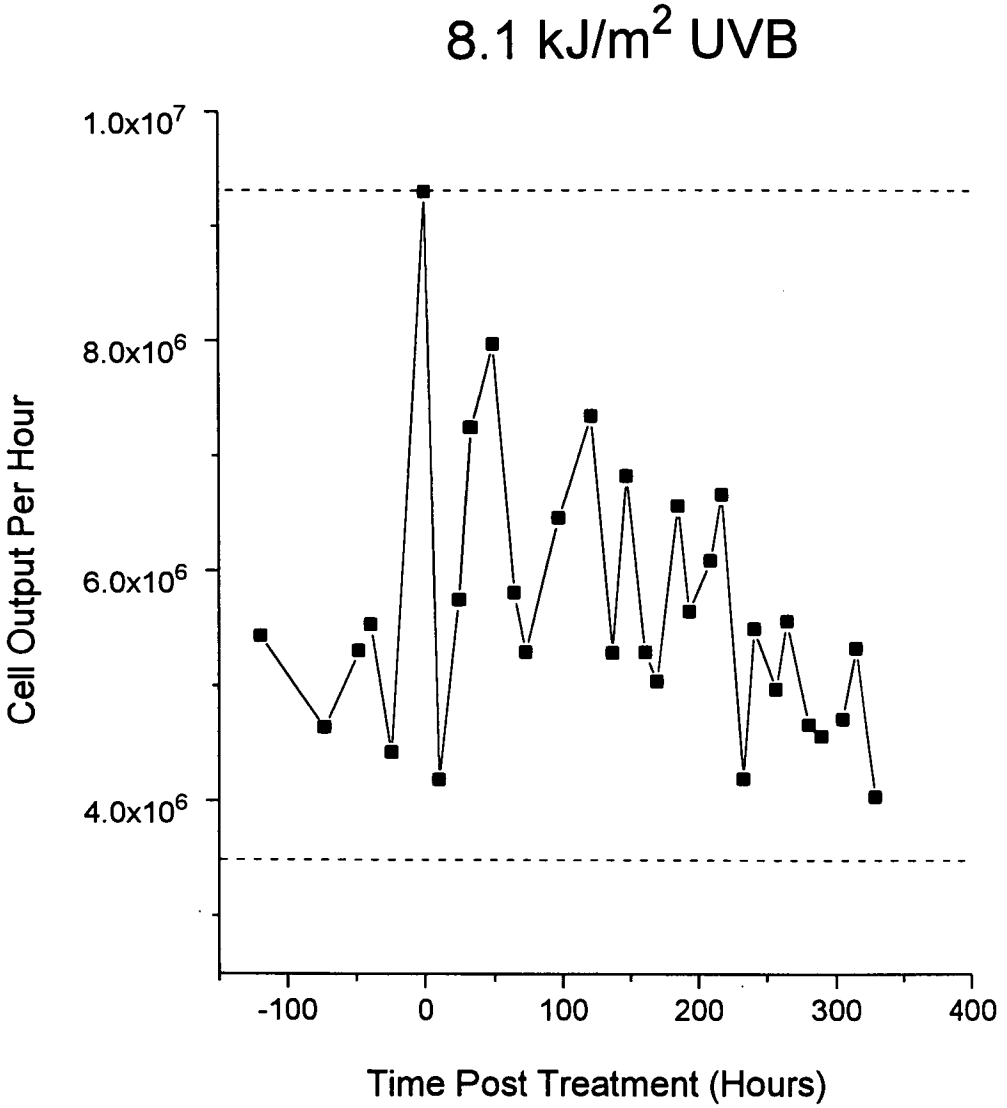



**Figure 3.3.2.2** Total cell output in lymph draining skin treated with 8.1 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

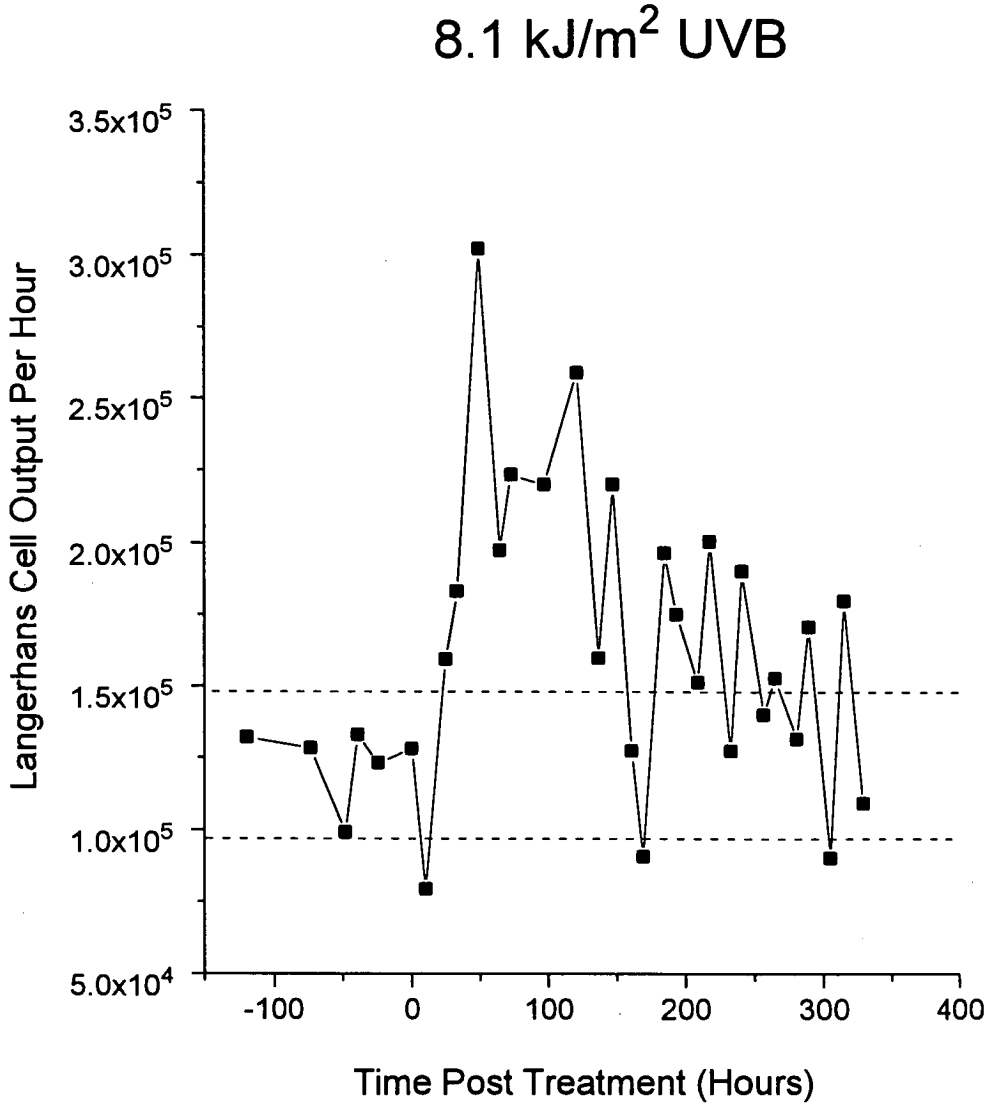



**Figure 3.3.2.3** Numbers of Langerhans cells in lymph draining skin treated with 8.1 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

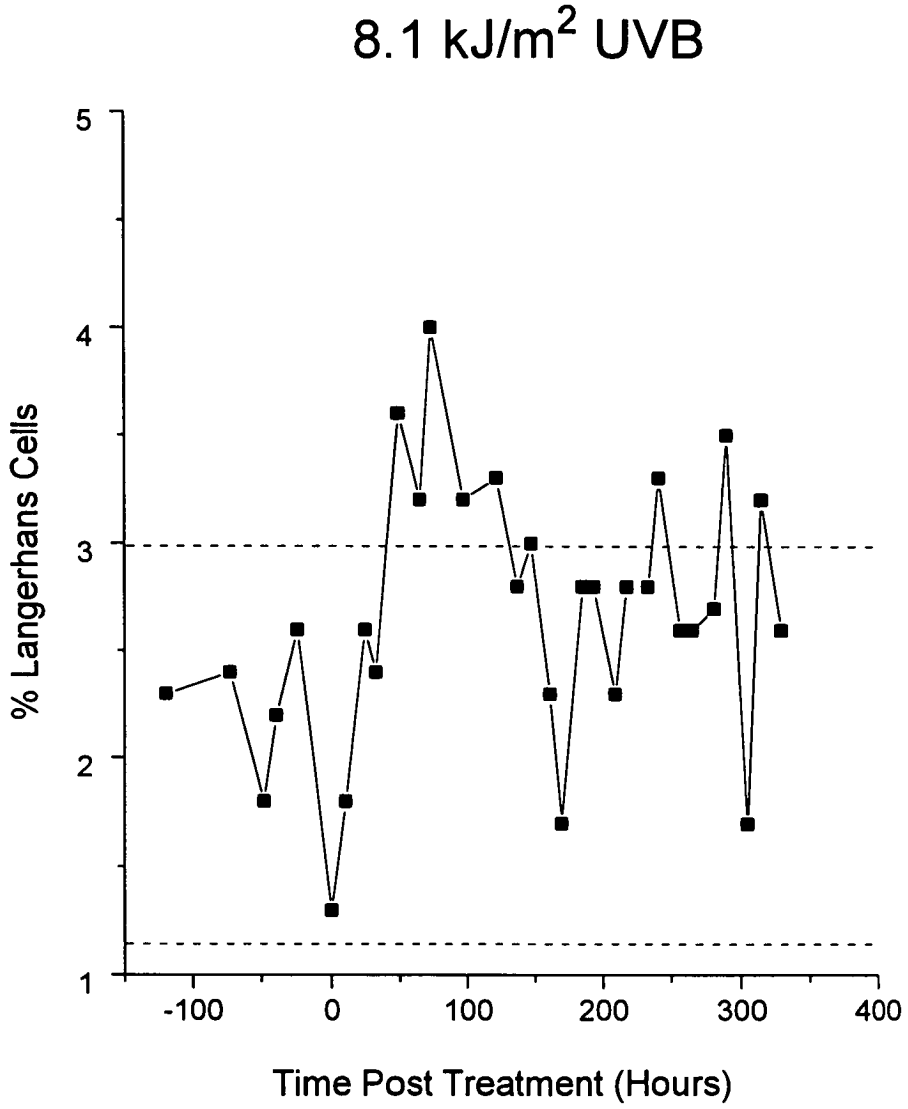



**Figure 3.3.2.4** Percentage of LC in lymph draining skin treated with 8.1 kJ/m$^2$ UVB treated skin. Dashed lines indicate the normal range for this single representative experiment.

### 3.3.2.3. Effect on T Cell Migration

The $T19^+$ γδ T cell output remained within the normal range of migration throughout the duration of the experiment (Fig. 3.3.2.5) as did the $CD4^+$ and $CD8^+$ T cell populations (Fig. 3.3.2.6). There were relatively more $CD4^+$ positive cells in this example than either $T19^+$ γδ T cells or $CD8^+$ positive cells. However, there were relatively similar numbers of $T19^+$ γδ T cells and $CD8^+$ positive cells.

### 3.3.2.4 General

The results for the representative sheep used to illustrate trends observed after multiple experiments is summarised in Table 3.2. Lymph output showed a sustained increase above the normal range in one of four sheep. While two other sheep showed increases these were not sustained and tended to alternate above and below the normal range. Cell output was similarly affected by this dose of UVB as only one out of six sheep showed any increase above the normal range. LC migration was increased after treatment in all sheep. This increased migration of LC occurred within 24 hours of treatment in all cases. Two cases showed sustained increases for 240 and 315 hours post-irradiation. The magnitude of increased LC numbers was approximately 2.4 fold for all sheep. The proportion of LC in lymph was increased in all sheep. Only one sheep out of four showed a sustained increased migration of $T19^+$ γδ T cells after treatment with this dose, another sheep was seen to produce transient increases. The timing of these increases coincided with that of increases in total cell output. Only one sheep monitored showed any significant alterations $CD4^+$ and $CD8^+$ cells above the normal range.

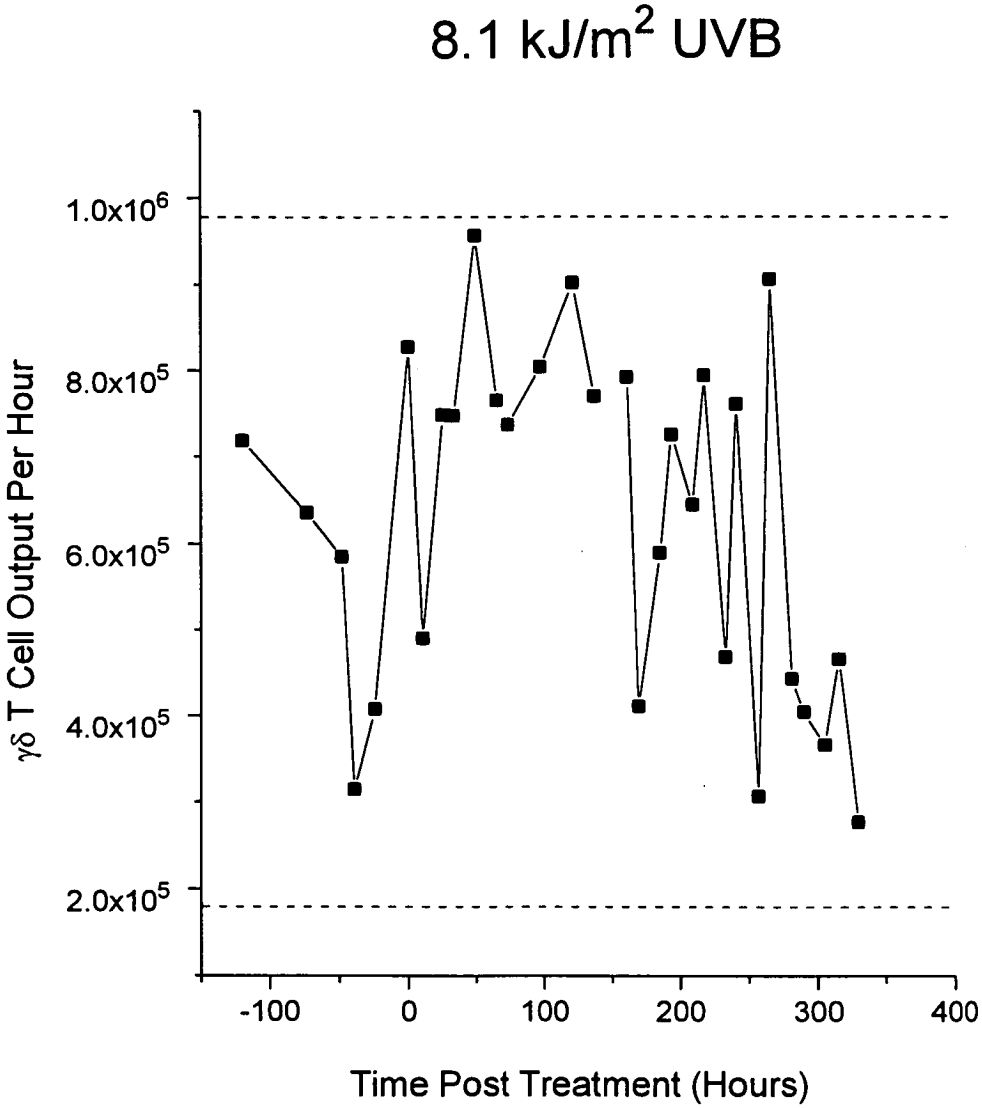



**Figure 3.3.3.5** Numbers of T19 $^{+}\gamma\delta$ T cells in lymph draining skin treated with 8.1 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

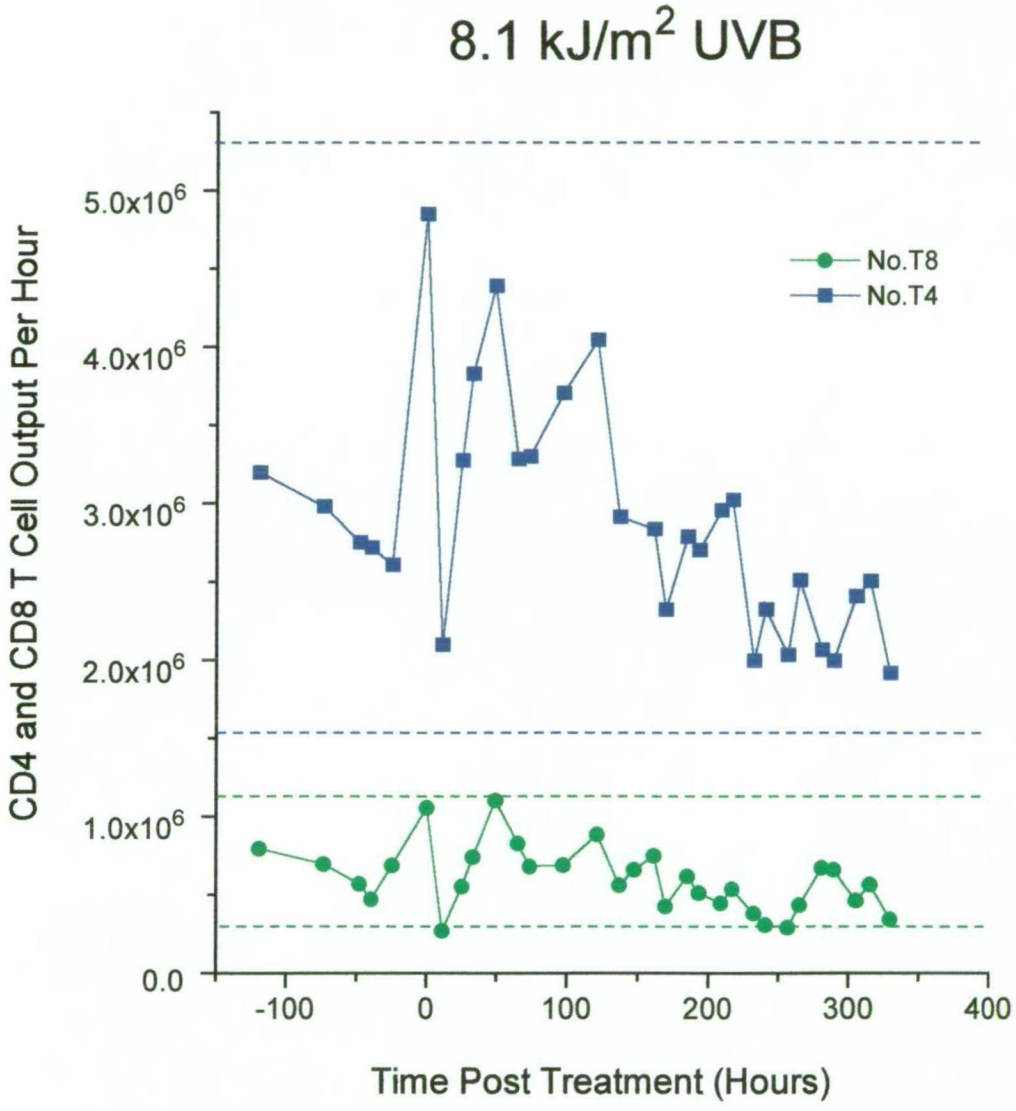



**Figure 3.3.2.6** Numbers of CD4 and CD8 T cells in lymph draining skin treated with 8.1 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

**Table 3.2 Summary of Effects after Exposure to 8.1 kJ/m² UVB**

| Parameter | Initial Increase (hours) | Peak increase (hours) | Magnitude of Peak Increase | Period of sustained increases (hours) |
|---|---|---|---|---|
| Lymph output | - | - | Normal range | - |
| Total cell output | - | - | Normal range | - |
| LC | 24 | 49 | 2.4 fold | 25-148, 187-217 |
| % LC | 49 | 73 | 1.8 fold | 25-121 |
| $T19^+$ γδ T cells | - | - | Normal range | - |
| $CD4^+$ T cells | - | - | Normal range | - |
| $CD8^+$ T cells | - | - | Normal range | - |

## *3.3.3 Changes in Lymph Flow, Cell Kinetics and Composition after Exposure of skin to 20.1 kJ/m² UVB*

### 3.3.3.1 Effect on Total Cell Output and Lymph Flow

It can be seen from Fig. 3.3.3.1 that lymph flow remained within the normal range throughout the duration of monitoring, with the exception of a period of elevated lymph flow between 24 and 80 hours post-treatment. However, this was only significantly above normal at 24 and 53 hours. Although it was observed that the other points between 24 and 80 hours fell within the in the high end of the normal range. A further increase was seen at 321 hours post irradiation. Cell output which had a rather large normal range due to variation in background points showed no deviation outside the normal range, although from 100 hours onwards, the cell output remained in the lower half of the normal range (Fig. 3.3.3.2).

### 3.3.3.2 Effect on LC Migration

From Fig. 3.3.3.3, it is clear that a sustained, dramatic increase in LC cell numbers migrating from the exposed site occurred from 11 to 81 hours post

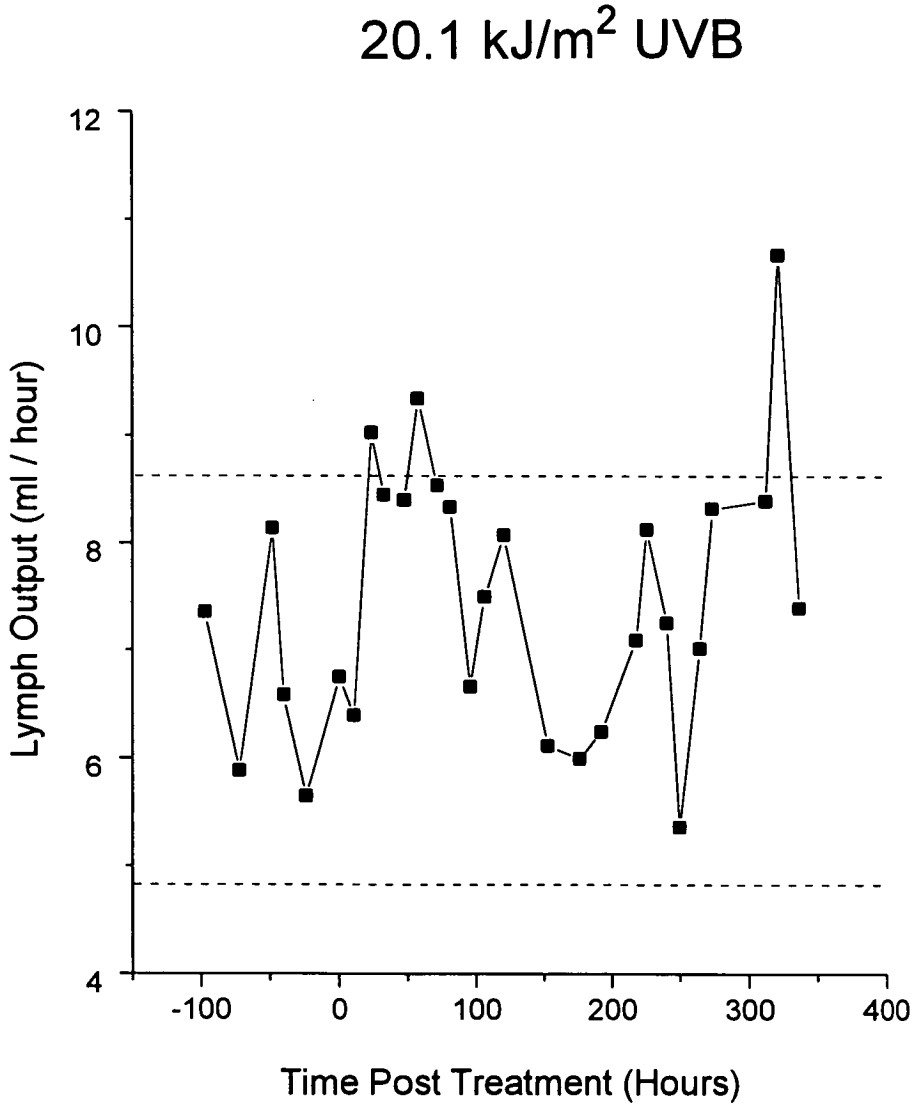



**Figure 3.3.3.1** The rate of lymph flow from skin treated with 20.1 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

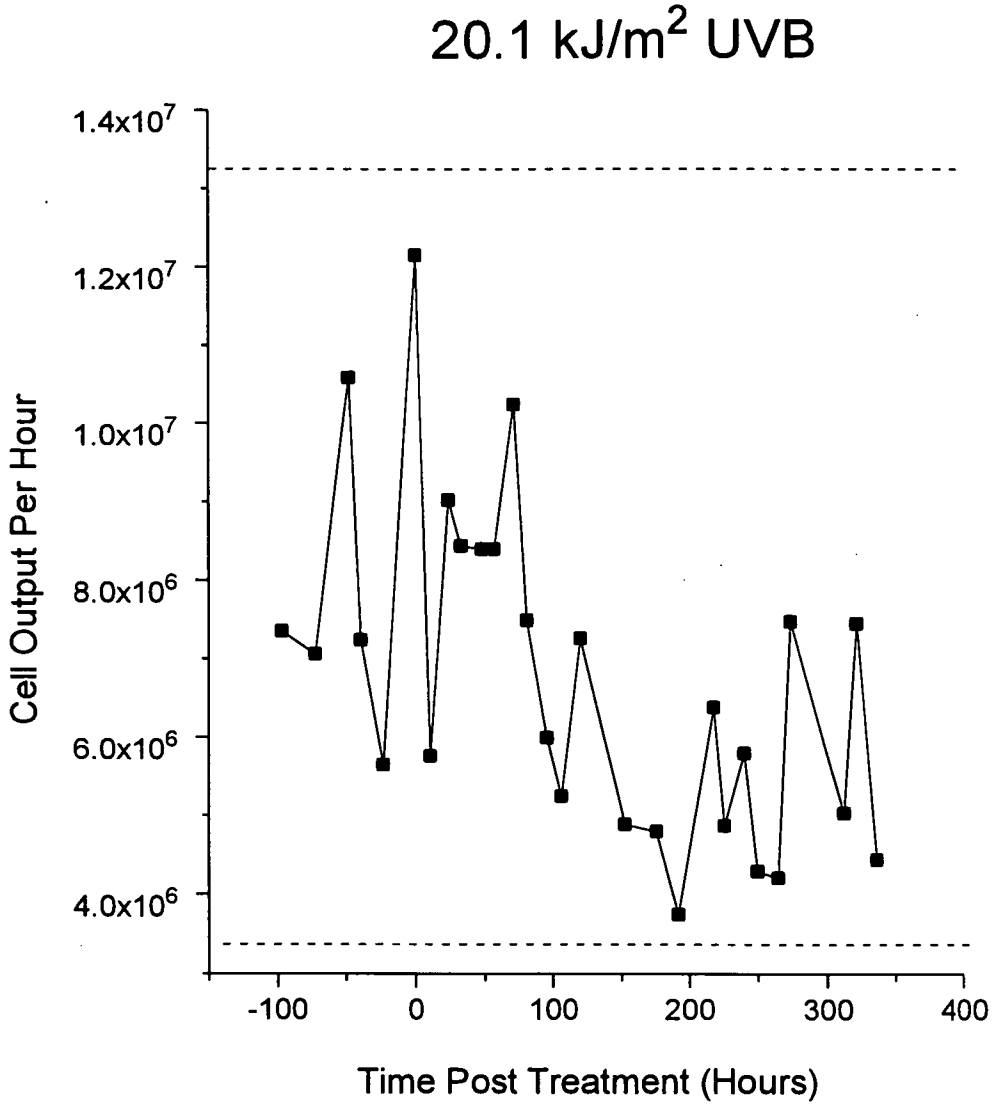



**Figure 3.3.3.2** Total cell output in lymph draining skin treated with 20.1 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

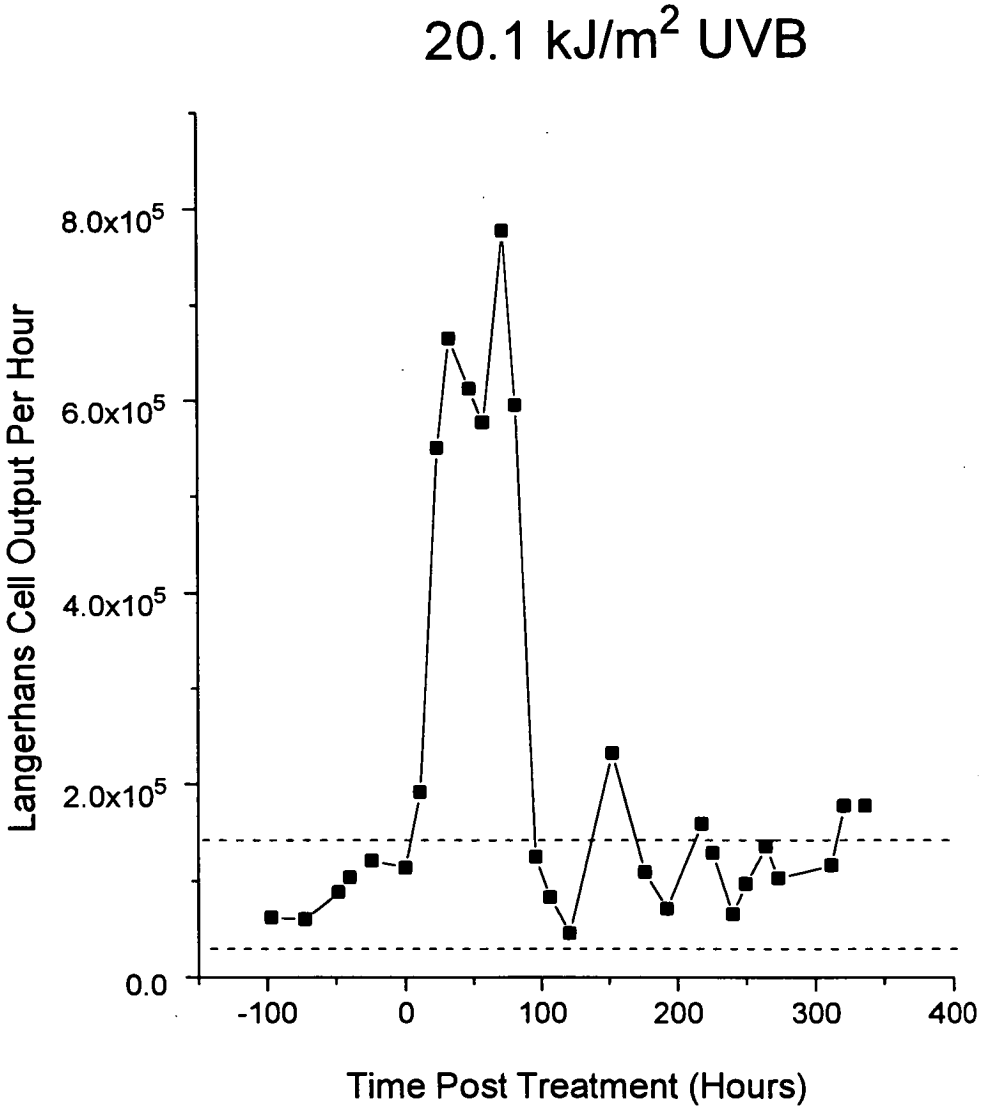



**Figure 3.3.3.3** Numbers of Langerhans cells in lymph draining skin treated with 20.1 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

irradiation, peaking at an approximately a 7.5 fold increase above the mean of normal values at 72 hours post-treatment. This was reflected in a similar percentage increase in migration of LC during the same time period (Fig. 3.3.3.4). After this, LC numbers fell back to within the normal range. Apart from transient deviations above the normal range at 150, 217 321 and 336 hours post-treatment none of these increases in LC numbers were of the same magnitude as that observed earlier, however the increases in percentage of LC at 150, 264 and 336 hours were still quite dramatic and represented a 4.5, 3.1 and 3.4 fold increases respectively (Fig. 3.3.3.4).

### 3.3.3.3. Effect on T Cell Migration

The $T19^+$ γδ T cell numbers remained within the normal range throughout the duration of the experiment. However cell numbers were seen to mainly lie within the lower portion of the normal range after 81 hours post-treatment (Fig. 3.3.3.5). No deviations outside the normal range were noted in either the $CD4^+$ or $CD8^+$ populations (Fig. 3.3.3.6). These cell populations also showed a general trend in the sense that the numbers of cells migrating fell towards the lower end of the normal range, parallelling the general trend observed in the total cell output (Fig. 3.3.3.2).

### 3.3.3.4 General

Results for the representative sheep used to illustrate trends observed after multiple experiments is summarised in Table 3.3. Exposure to 20.1 kJ/m² UVB only caused a sustained increase in lymph output in one of three sheep assessed. However, in this one case the increase consisted of only three points only one of which was substantially away from the upper limits of the normal range. Whilst the others had transient increases above normal, they generally remained within the normal range. Two out of the three sheep had sustained increases above the normal range for cell output. All sheep studied showed an increase in LC migration within the first 100 hours (7.5 fold, 3.3 fold, 3.1 fold increases) after which one sheep showed a return to normal levels but the other still showed sustained levels of migration until 408 hours post-treatment (one experiment was of shorter duration and such long term effects could not be assessed). These increases in LC numbers were mirrored by similar increases in the proportion of LC migrating in the lymph. $T19^+$ γδ T cell migration was found to stay within the normal range for two out of three sheep. However, one sheep as well as showing two transient increases above the normal range also showed sustained levels below that of the normal range,

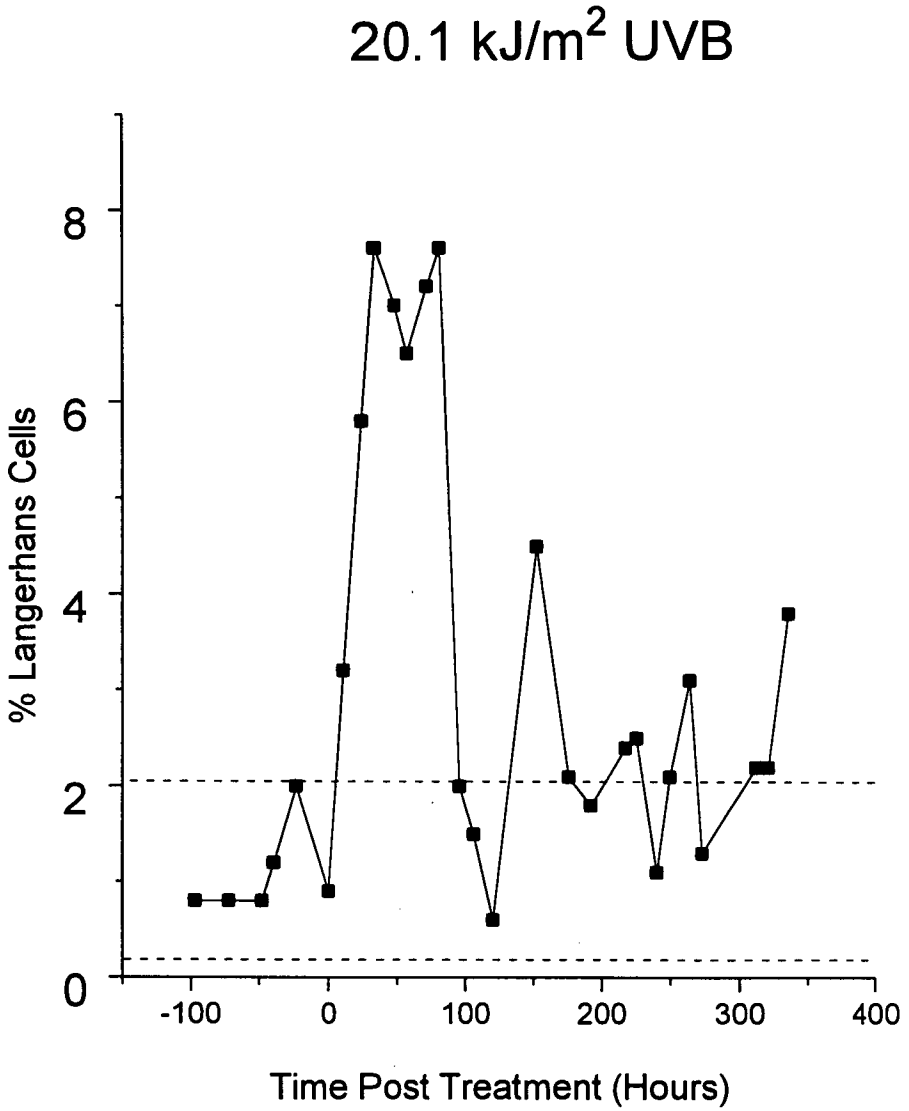



**Figure 3.3.3.4** Percentage of LC in lymph draining skin treated with 20.1 kJ/m$^2$ UVB treated skin. Dashed lines indicate the normal range for this single representative experiment.

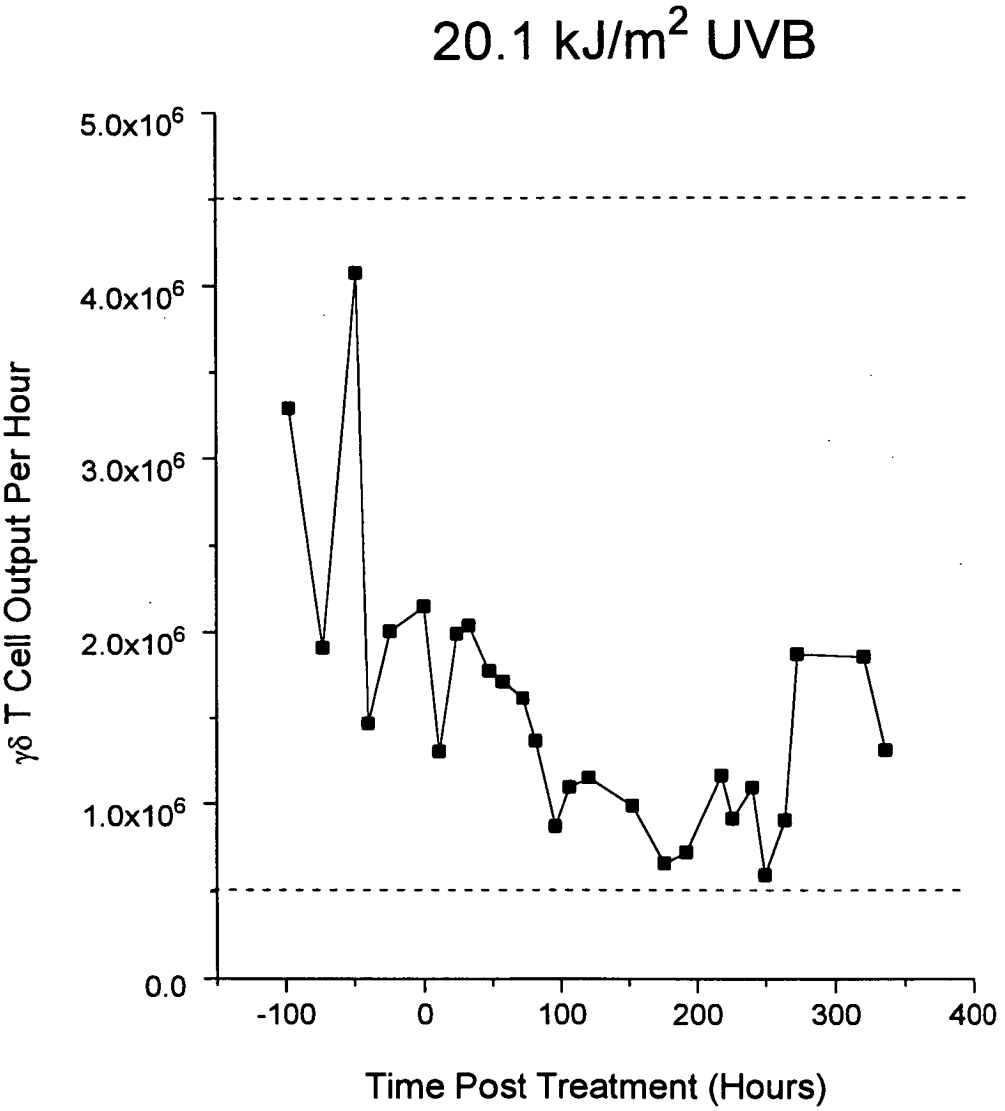



**Figure 3.3.3.5** Numbers of T19 $^{+}\gamma\delta$ T cells in lymph draining skin treated with 20.1 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

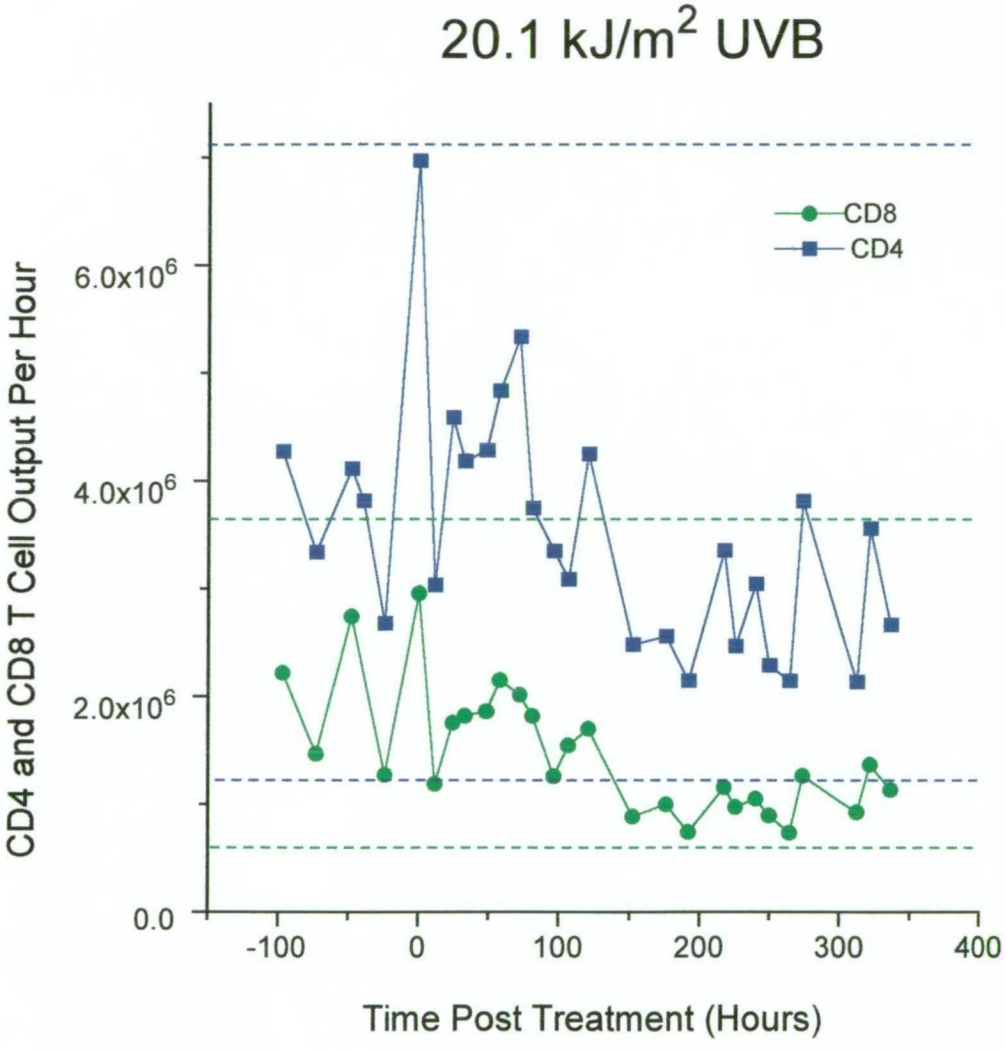



**Figure 3.3.3.6** Numbers of CD4 and CD8 T cells in lymph draining skin treated with 20.1 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

with the majority of points after treatment falling below the normal range. One of the sheep whose $T19^+$ γδ T cells stayed within the normal range, were at the lower end of the normal range for the duration of the experiment. $CD4^+$ cells were increased in two out of three experiments and the increases seen followed those of increased cell output. The migration of $CD8^+$ cells generally stayed within the normal range for all sheep, however some transient increases above normal were noted for one sheep. The same sheep that showed $T19^+$ γδ T cells decreasing towards the lower end of the normal range also demonstrated this for $CD8^+$ cells. The timing of the transient but substantial points of increased migration of $CD8^+$ cells seen in one sheep was unlike that of any other cell population. These included peaks of five fold in magnitude which were mirrored by a dramatic increase in the proportion of $CD8^+$ cells in the lymph.

**Table 3.3 Summary of Effects after Exposure to 20.1 kJ/m² UVB**

| Parameter | Initial Increase (hours) | Peak increase (hours) | Magnitude of Peak Increase | Period of sustained increases (hours) |
|---|---|---|---|---|
| Lymph output | 24 | 321 | 1.6 fold | - |
| Total cell output | - | - | Normal range | - |
| LC | 11 | 72 | 7.5 fold | 11-81 |
| % LC | 11 | 33 & 81 | 6.8 fold | 11-81,152-176, 221-226, 312-336 |
| $T19^+$ γδ T cells | - | - | Normal range | - |
| $CD4^+$ T cells | - | - | Normal range | - |
| $CD8^+$ T cells | - | - | Normal range | - |

### *3.3.4 Changes in Lymph Flow, Cell Kinetics and Composition after Exposure of Skin to 40.2 kJ/m² UVB*

### 3.3.4.1 Effect on Total Cell Output and Lymph Flow

Fig. 3.3.4.1 shows that increases in lymph flow were seen after eight hours post-treatment and this increase was sustained until 70 hours post-treatment. After a single transient decrease below the normal range at 94 hours, was observed before the rate of lymph flow returned to within the normal range, where it remained for approximately 50 hours. A further transient increase above normal was seen at 157 hours, followed again, by return to the normal range for nearly 100 hours. Again this was followed by an increase above the normal range for three out of four of the remaining points.

Cell output showed a dramatic increase above that of the normal range between 40 and 70 hours post-treatment, peaking at 60 hours with a 4.9 fold increase above the mean of the pre-treatment points (Fig. 3.3.4.2). After this time, the number of cells migrating from the treated site returned to the normal range apart from two transient increases at 157 and 335 hours which were 3.4 and 2 fold above the mean pre-irradiation levels respectively.

### 3.3.4.2 Effect on LC Migration

LC numbers showed sustained increases above normal between 40 and 157 hours with, only a single return to the normal range at 133 hours. Two distinct peaks were seen; the first with a sustained increase peaking at 94 hours with a 6 fold increase above mean pre-treatment levels and the second more transient peak 157 hours after irradiation at a level representing a 7 fold increase. After 285 hours, a further sustained increase in LC numbers outside the normal range was seen, however, this was smaller in magnitude than the earlier peaks. LC migration was greater in magnitude and longer duration than the increase in the total cell output. This was reflected by the sustained increase in the proportion of LC migrating in comparison to the total lymphoid cell numbers, throughout the monitoring period. The increased proportion of LC represented a 2 fold increase above the mean pre-treatment levels. A return to the normal range was seen between 166 and 239 hours.

### 3.3.4.3. Effect on T Cell Migration

$T19^+$ $\gamma\delta$ (Fig. 3.3.4.5), $CD4^+$ and $CD8^+$ (Fig. 3.3.4.6) all showed similar migration patterns to the total cell output (Fig. 3.3.4.2). However, the increase in $CD4^+$ T cells was of a greater magnitude (6.4 fold) in comparison to that of the $T19^+$ $\gamma\delta$ or $CD8^+$ T cell populations (4.5 and 1.9 fold increases respectively). Indeed the relative increase in $CD4^+$ T cells was greater than the peak in cell

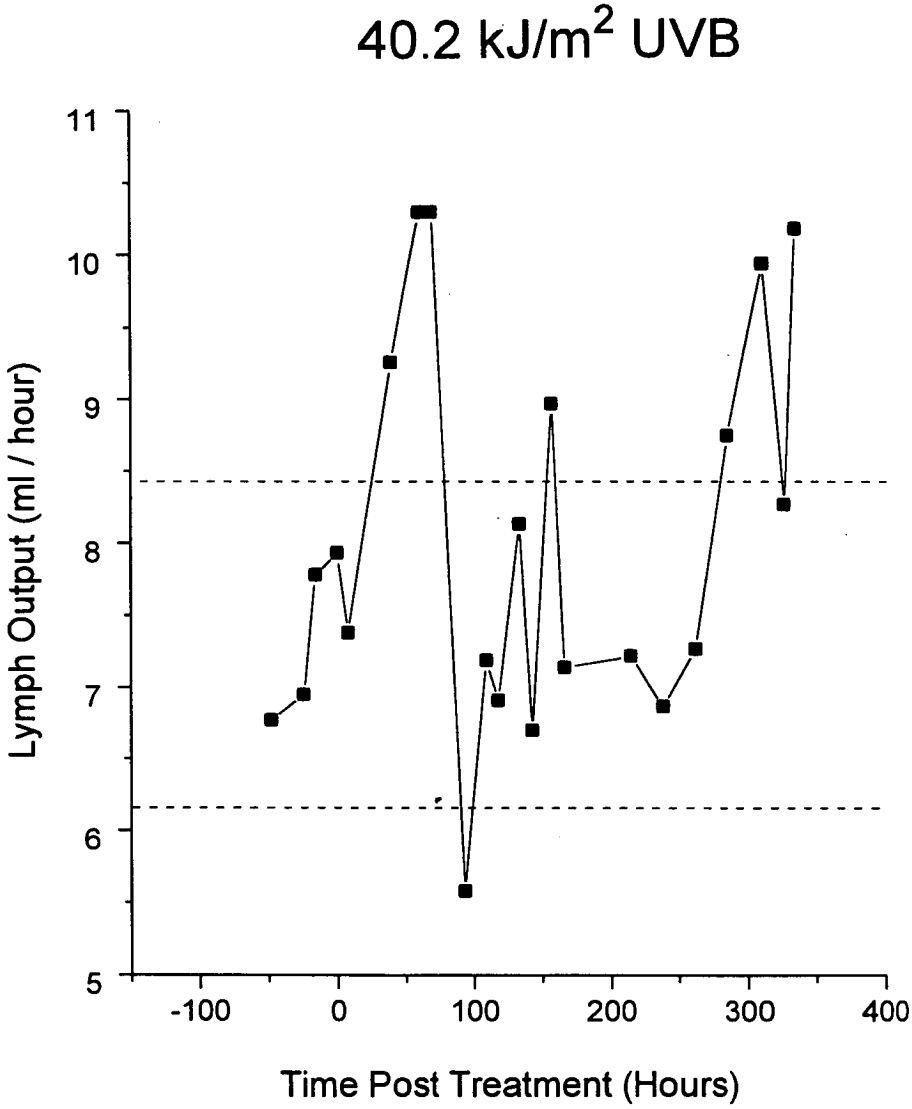



**Figure 3.3.4.1** The rate of lymph flow from skin treated with 40.2 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

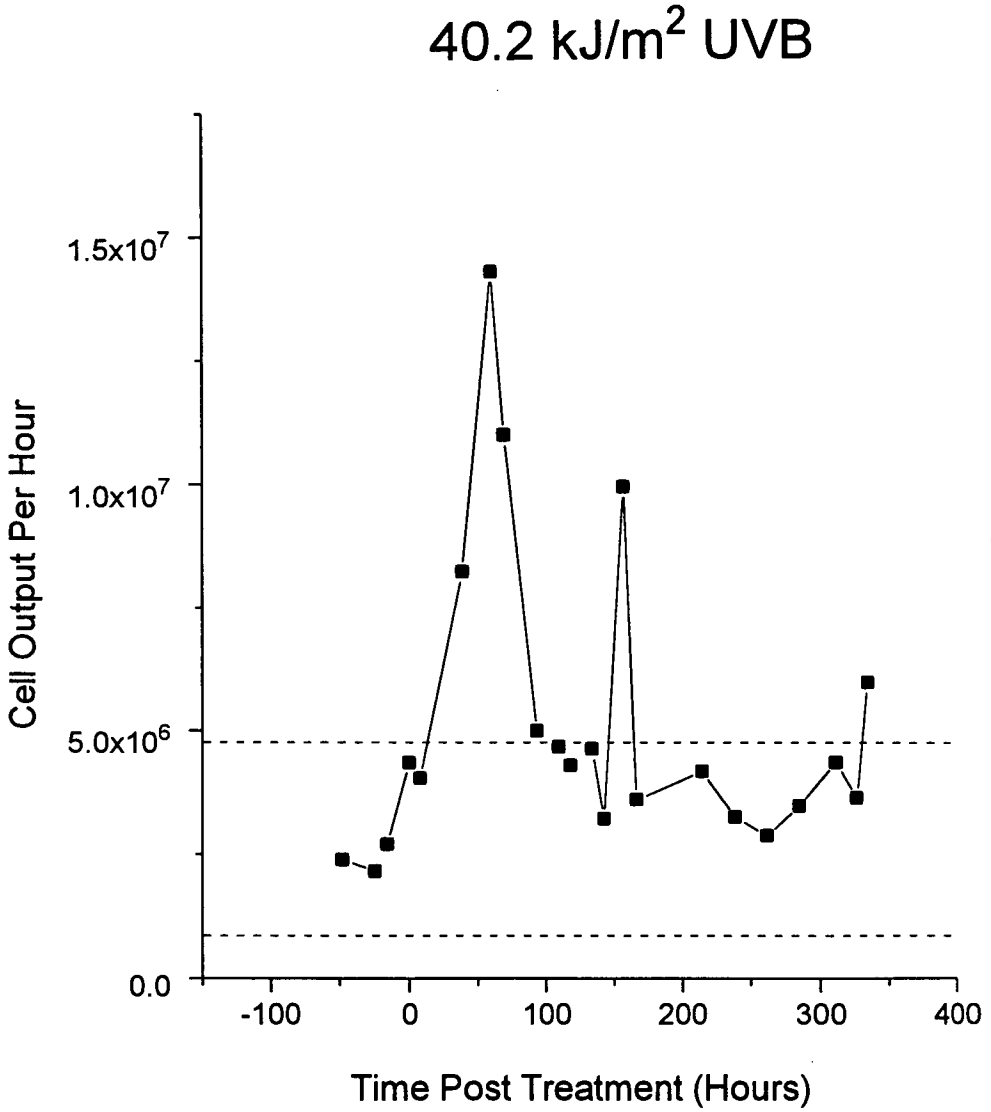



**Figure 3.3.4.2** Total cell output in lymph draining skin treated with 40.2 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

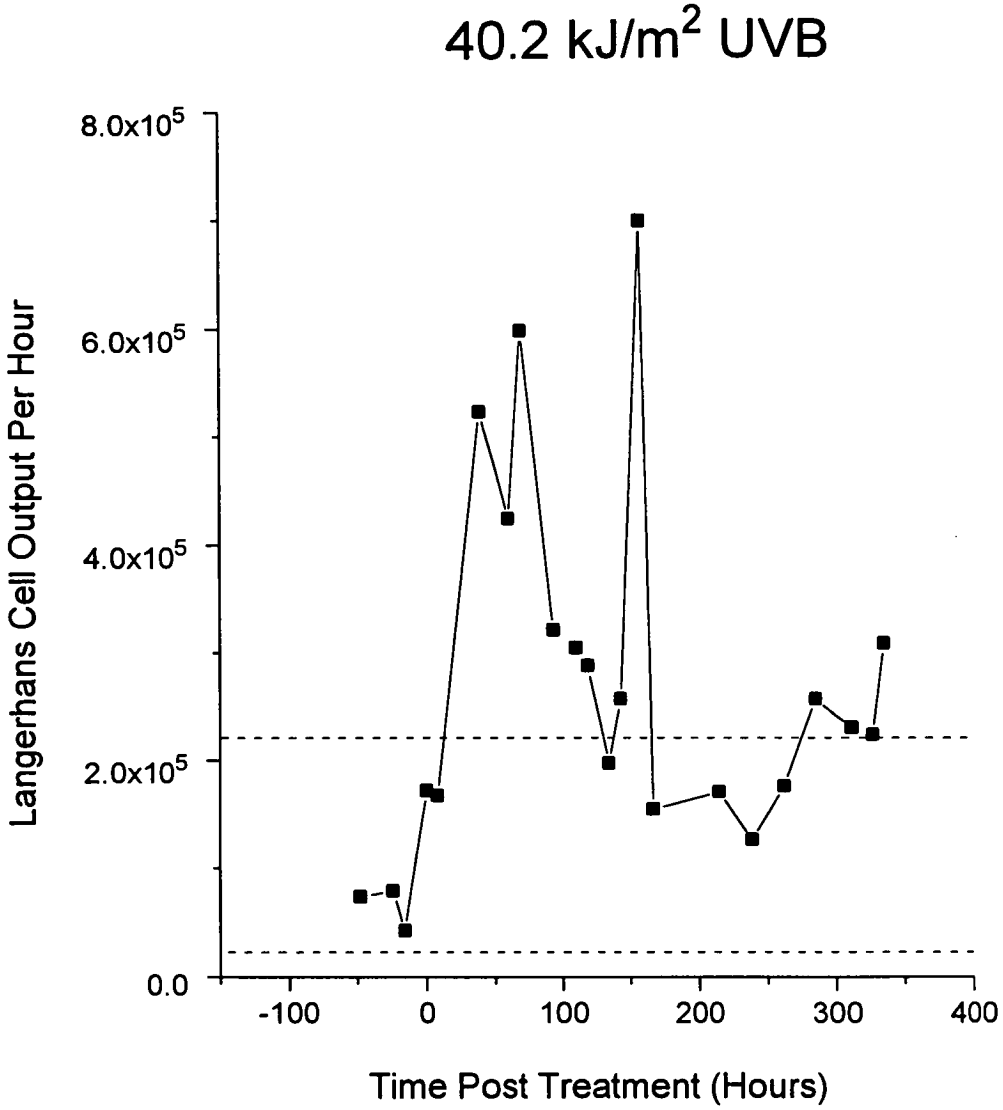



**Figure 3.3.4.3** Numbers of Langerhans cells in lymph draining skin treated with 40.2 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

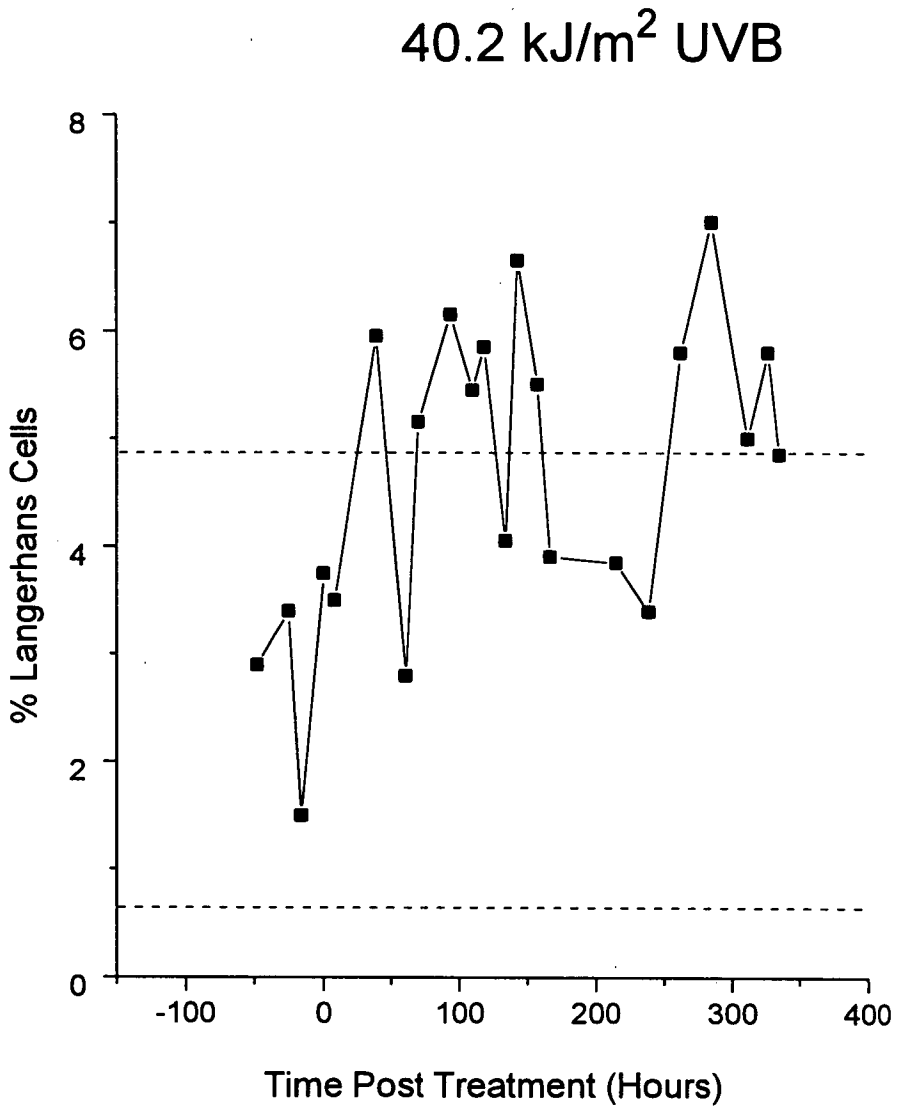



**Figure 3.3.4.4** Percentage of LC in lymph draining skin treated with 40.2 kJ/$m^2$ UVB treated skin. Dashed lines indicate the normal range for this single representative experiment.

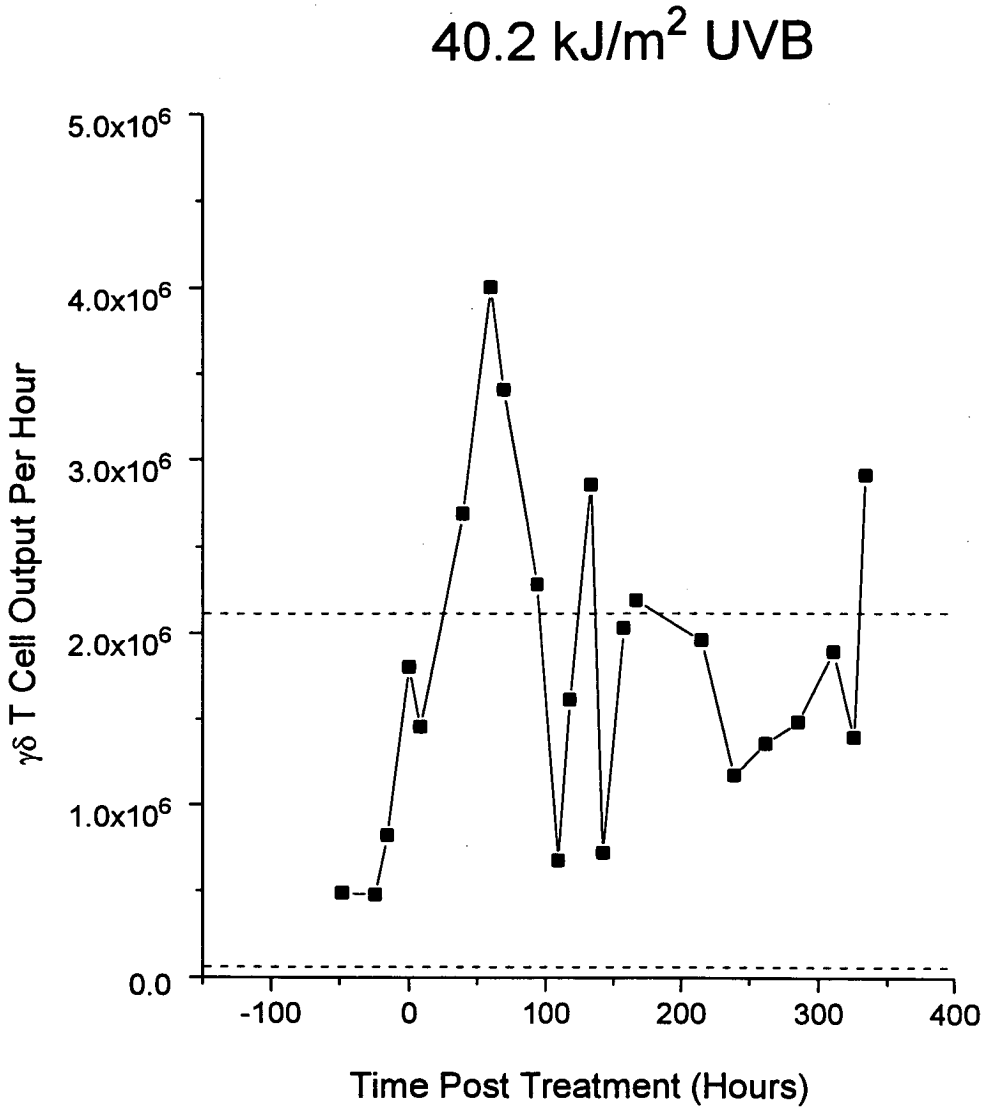



**Figure 3.3.4.5** Numbers of T19 $^{+}\gamma\delta$ T cells in lymph draining skin treated with 40.2 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

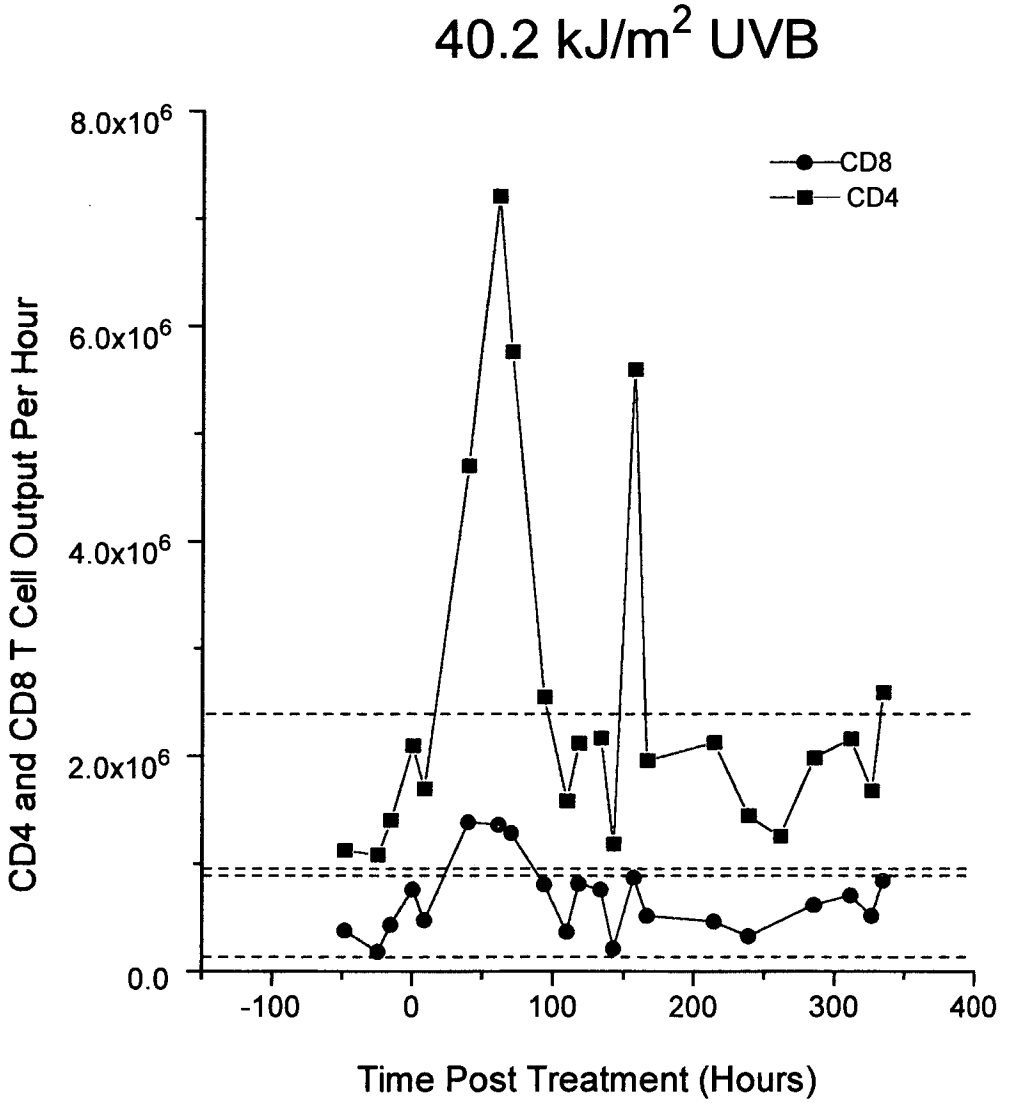



**Figure 3.3.4.6** Numbers of CD4 and CD8 T cells in lymph draining skin treated with 40.2 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

output (130%), whereas the increase in $CD8^+$ was of much lower than the increase in cell output (40%).

**Table 3.4 Summary of Effects after Exposure to 40.2 kJ/m² UVB**

| Parameter | Initial Increase (hours) | Peak increase (hours) | Magnitude of Peak Increase | Period of sustained increases (hours) |
|---|---|---|---|---|
| Lymph output | 40 | 60 & 70 | 1.3 fold | 40-70, 286-311 |
| Total cell output | 40 | 60 | 4.9 | 40-70 |
| LC | 40 | 157 | 7 fold | 40-157 |
| % LC | 40 | 286 | 2.4 fold | 70-118,142-157, 262-328 |
| $T19^+$ γδ T cells | | 60 | 4.5 fold | 40-94 |
| $CD4^+$ T cells | 40 | 60 | 6.4 fold | 40-94 |
| $CD8^+$ T cells | 40 | 40 | 1.9 fold | 40-70 |

### 3.3.4.4 General

Three sheep were exposed to this dose. One experiment lasted 85 hours post-treatment yet showed sustained increases for all parameters (data not shown). Of the two experiments showing the full time course, both showed lymph flow to increase above the normal range. One however, consisted of only a transient increase (81 hours post-treatment) and the other showed a sustained increases up to 70 hours after treatment. A sustained increase was seen again after 157 hours and lasted for approximately 100 hours (See Fig. 3.3.4.1). Cell output showed a sustained increases for all sheep between 81-96 hours (data not shown), 32-85 hours (data not shown) and 40-70 hours (see Fig. 3.3.4.2). All sheep showed substantial increase in the numbers of LC migrating which was reflected in increased proportion of LC in all cases. In both experiments of sustained duration, an initial peak was followed by return to normal and then a second peak. In one experiment the second peak was of

greater magnitude than in the first. In the other experiment the reverse was seen. $T19^+$ γδ T cells both showed increases above the normal range. However, these occurred when cell output increased and increases in % $T19^+$ γδ T cells were not seen in the lymph. $CD4^+$ and $CD8^+$ T cell migration were both observed to increase above the normal level but once again this reflected increases in total cell output rather than dramatic increase in these specific populations.

## *3.3.5 Changes in Lymph Flow, Cell Kinetics and Composition after Exposure of Skin to 80.4 kJ/m² UVB*

### 3.3.5.1 Effect on Total Cell Output and Lymph Flow

Lymph flow showed an increase above the normal range ten hours after treatment. A transient return to within the normal range was then observed, followed by three further points of sustained lymph flow between 34 and 58 hours post-treatment (Fig. 3.3.5.1). Two further transient increases above normal were seen at 130 and 275 hours. Lymph flow generally remained in the high end of the normal range after treatment. Cell output also showed a general increase after treatment, however, the majority of these points were above the normal range. The cell output peaked at 89 hours post-treatment (Fig. 3.3.5.2). Those points not lying above the normal range, were found at the high end of the normal range, apart from the points at 58 and 251-267 hours post-treatment, which were towards the lower end of the normal range.

### 3.3.5.2 Effect on LC Migration

LC numbers showed a dramatic increase above that of the normal range peaking at 34 hours with an 8.75 fold increase above the mean. The numbers of LC remained above normal, apart from a return to normal at 58 hours until hours post-treatment (Fig. 3.3.5.3). After this the LC numbers were seen to lie either above or slightly beneath the normal range. A decrease below the normal range was seen at 251 and 267 hours. This was followed by a further sustained increase in LC numbers above normal between 290 and 315 hours followed once again by fluctuations above and within the normal range. The percentage LC migrating generally reflected the initial changes seen in LC migration. A large increase was observed above the normal range followed by a return to with the normal range after 179 hours (Fig. 3.3.5.4). The % LC in the lymph peaked at 34 hours post-treatment, reflecting the peak seen in LC

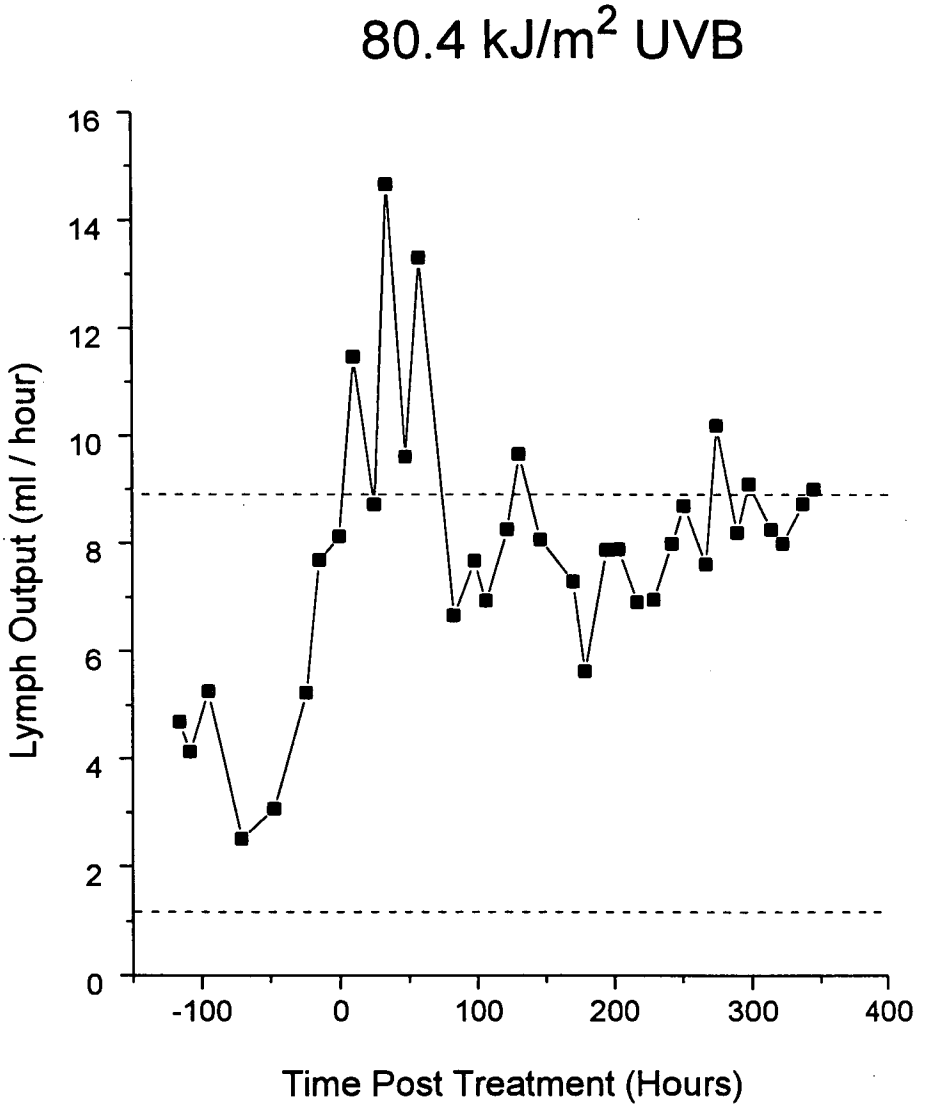



**Figure 3.3.5.1** The rate of lymph flow from skin treated with 80.4 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment

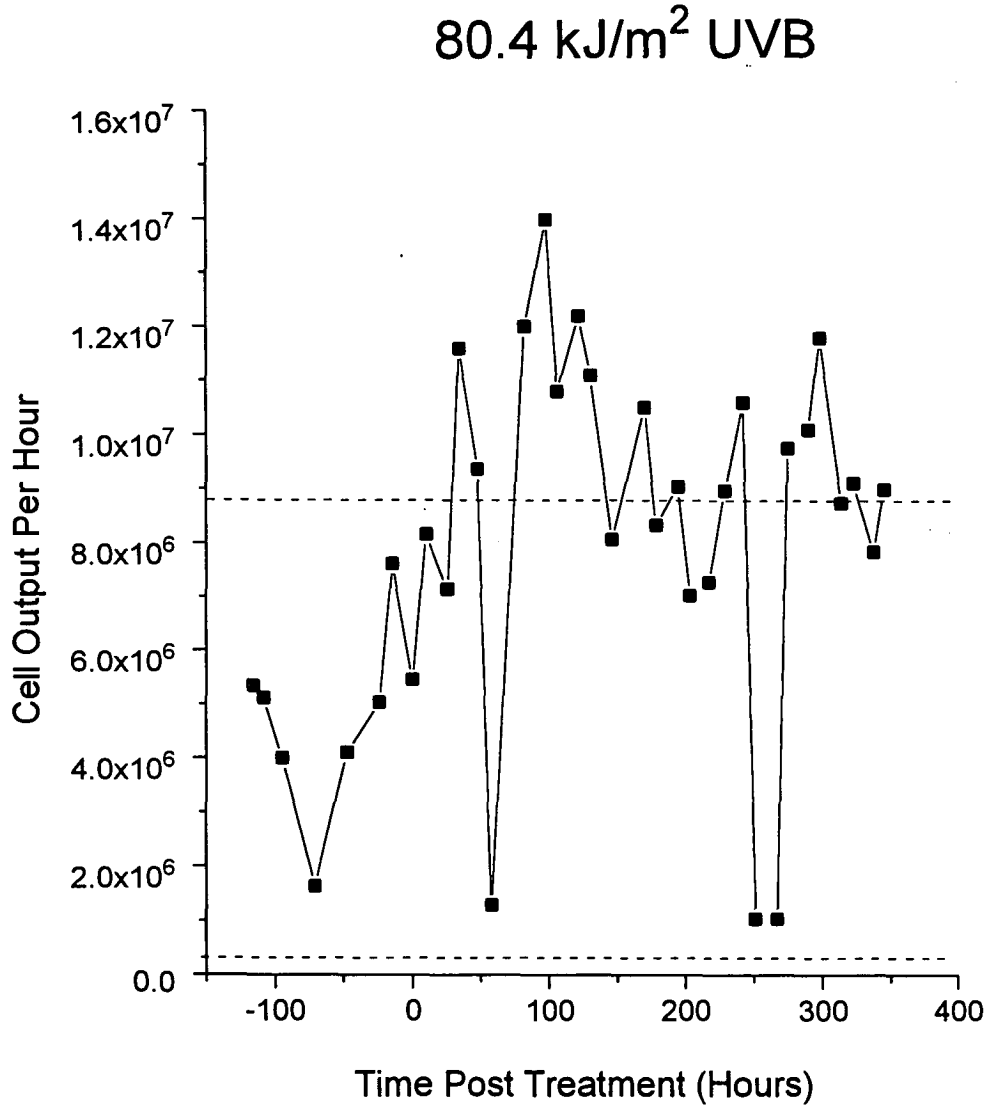



**Figure 3.3.5.2** Total cell output in lymph draining skin treated with 80.4 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

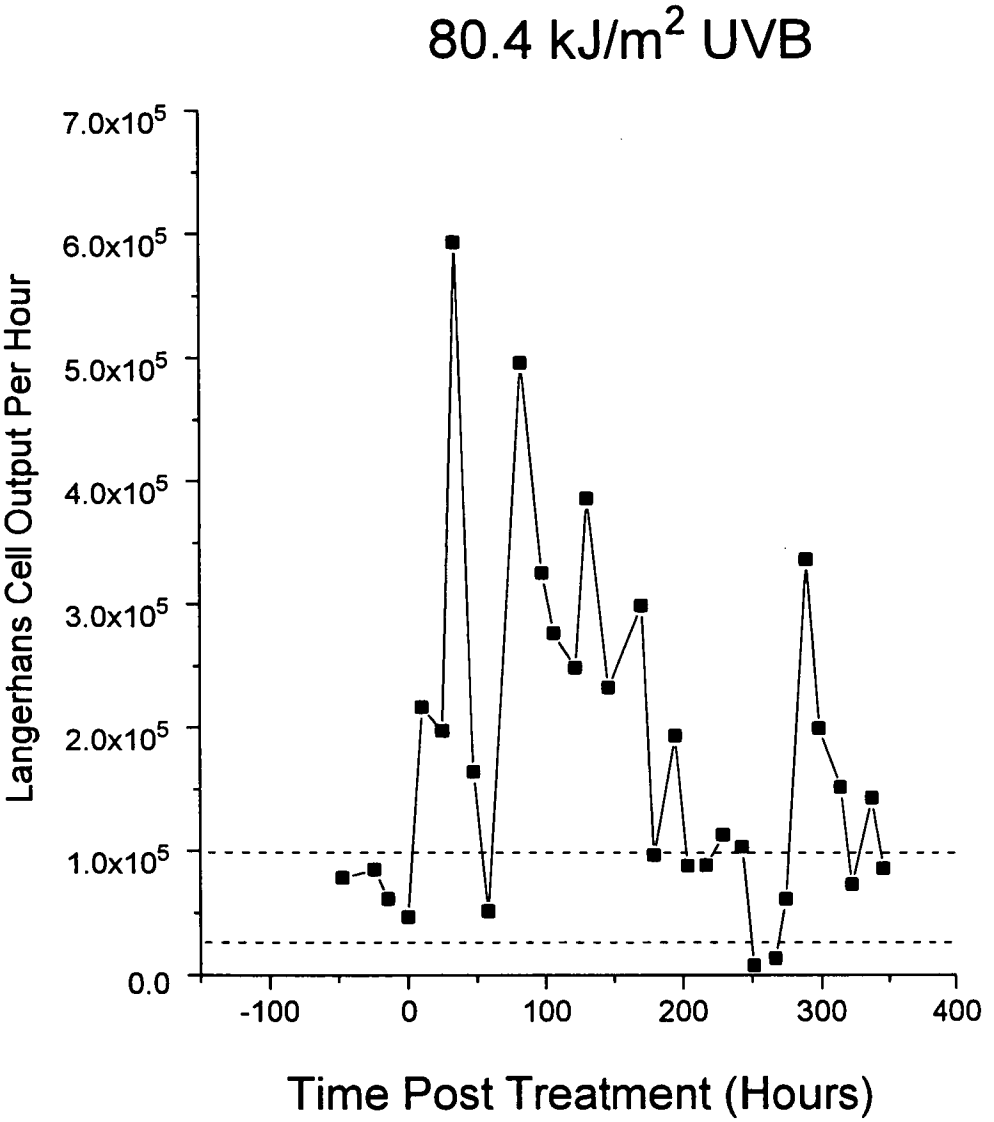



**Figure 3.3.5.3** Numbers of Langerhans cells in lymph draining skin treated with 80.4 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

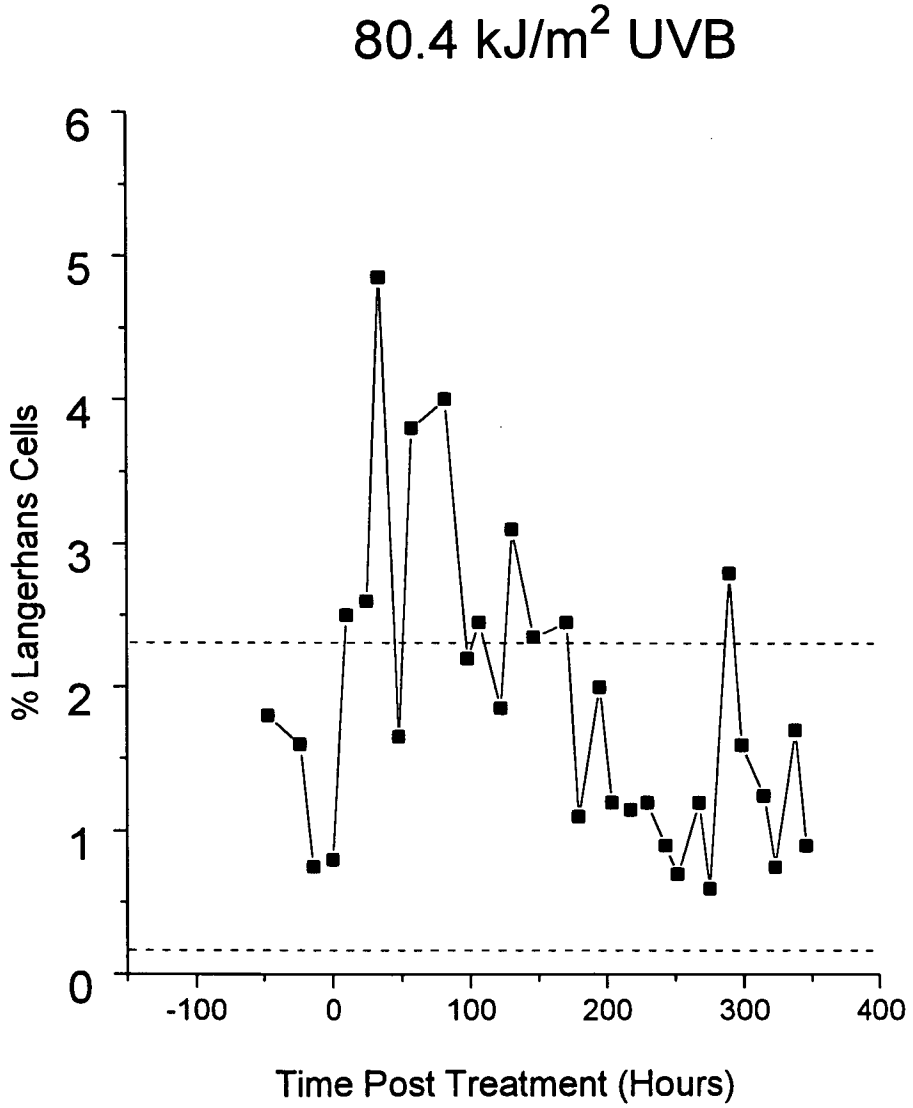



**Figure 3.3.5.4** Percentage of LC in lymph draining skin treated with 80.4 kJ/m$^2$ UVB treated skin. Dashed lines indicate the normal range for this single representative experiment.

numbers at this time. One further increase above normal was noted at 290 hours post-treatment.

### 3.3.5.3. Effect on T Cell Migration

$T19^+ \gamma\delta$ cell numbers increased at 34 hours with a further increase, which was only just slightly outside the normal range, occurring between 83 and 98 hours post-treatment (Fig. 3.3.5.5). Unlike other cell populations, no early sustained increases were seen. Furthermore, unlike other cell populations increases in $T19^+ \gamma\delta$ cell migration peaked after 217 hours and largely remained above normal with some following exceptions. The points at 251 and 267 were below the normal range and 338 hours was within the normal range. The increase of $T19^+ \gamma\delta$ cell migration peaked at 299 hours with a 3.3 fold increase above the mean. $CD4^+$ T cell output pattern followed that seen by the total cell output (Fig. 3.3.5.2) but were increased above the normal range for four sustained periods between 11-48 and 83-131 and 170-243 and 275-346 hours post-treatment (Fig. 3.3.5.6). $CD4^+$ T cell numbers peaked at 83 hours with a 3.7 fold increase in $CD4^+$ migration above the mean levels of pre-treatment points. $CD8^+$ cells followed similar migration patterns but, due to the fact that only two background points were able to be processed for this antibody means no valid normal range could be generated so results must be interpreted with care (Fig. 3.3.5.6). However $CD8^+$ cell migration peaked at 314 hours with and 6.2 fold increase above the mean of the two background points (Fig. 3.3.5.6).

### 3.3.2.4 General

This high dose caused microscopic blistering and damage of the epidermis and was only repeated once, but due to high background variation this data was discarded. So no real trends can be established, however the changes that did occur in this single experiment, especially that of LC migration were significant in magnitude and perhaps the late increases in $T19^+ \gamma\delta$ T cell population are individually interesting. A summary of the observed changes after this single individual experiment is shown in Table 3.5.

## 3.3.6 Summary of Trends Observed in Pseudoafferent Lymph after Exposure of Sheep Skin to UV light

Treatment of the flank skin of sheep with known dose of UVB was observed to produce inconsistencies in the parameters studied. Treatment of different sheep with the same dose was shown to have variable effect on the extent and magnitude of changes taking place. Table 3.6 summarises the

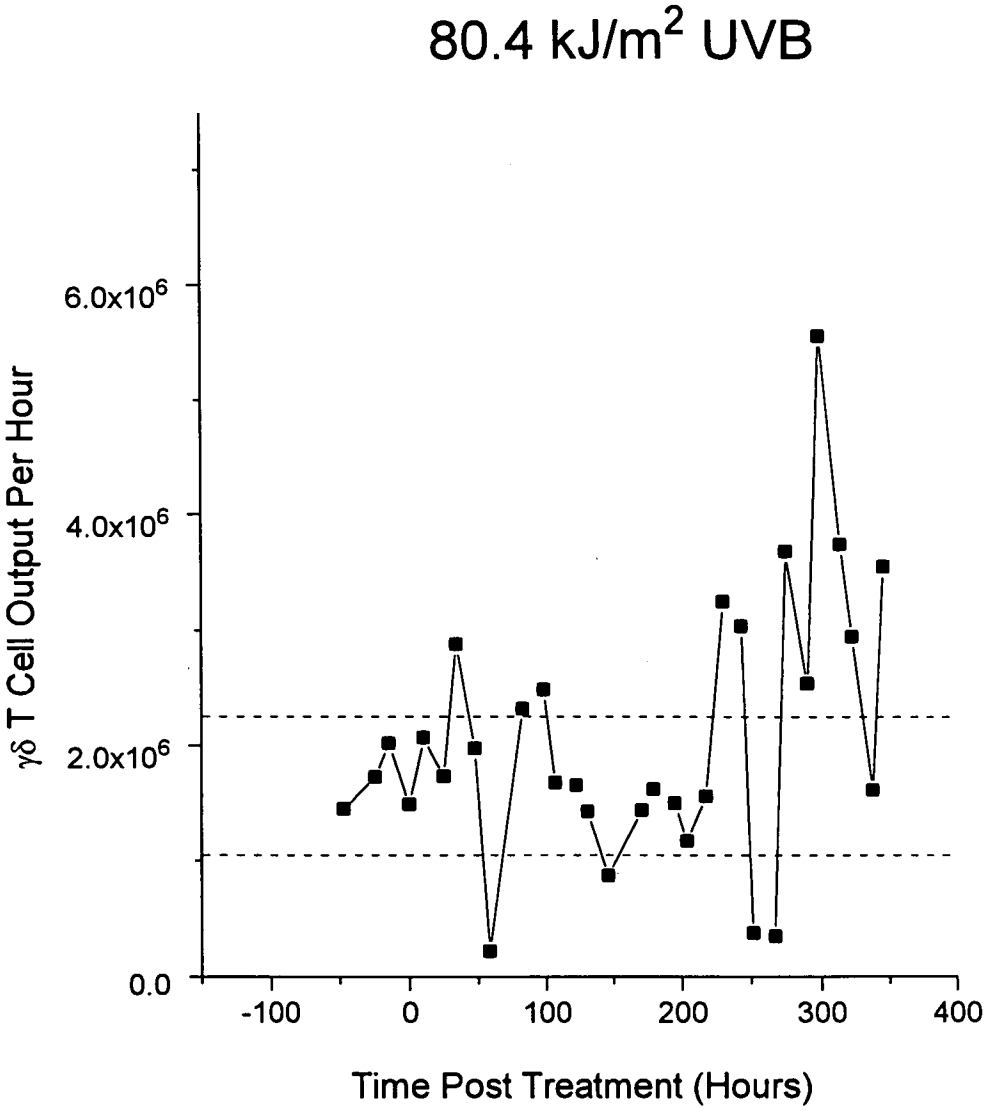



**Figure 3.3.5.5** Numbers of T19 $^{+}\gamma\delta$ T cells in lymph draining skin treated with 80.4 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

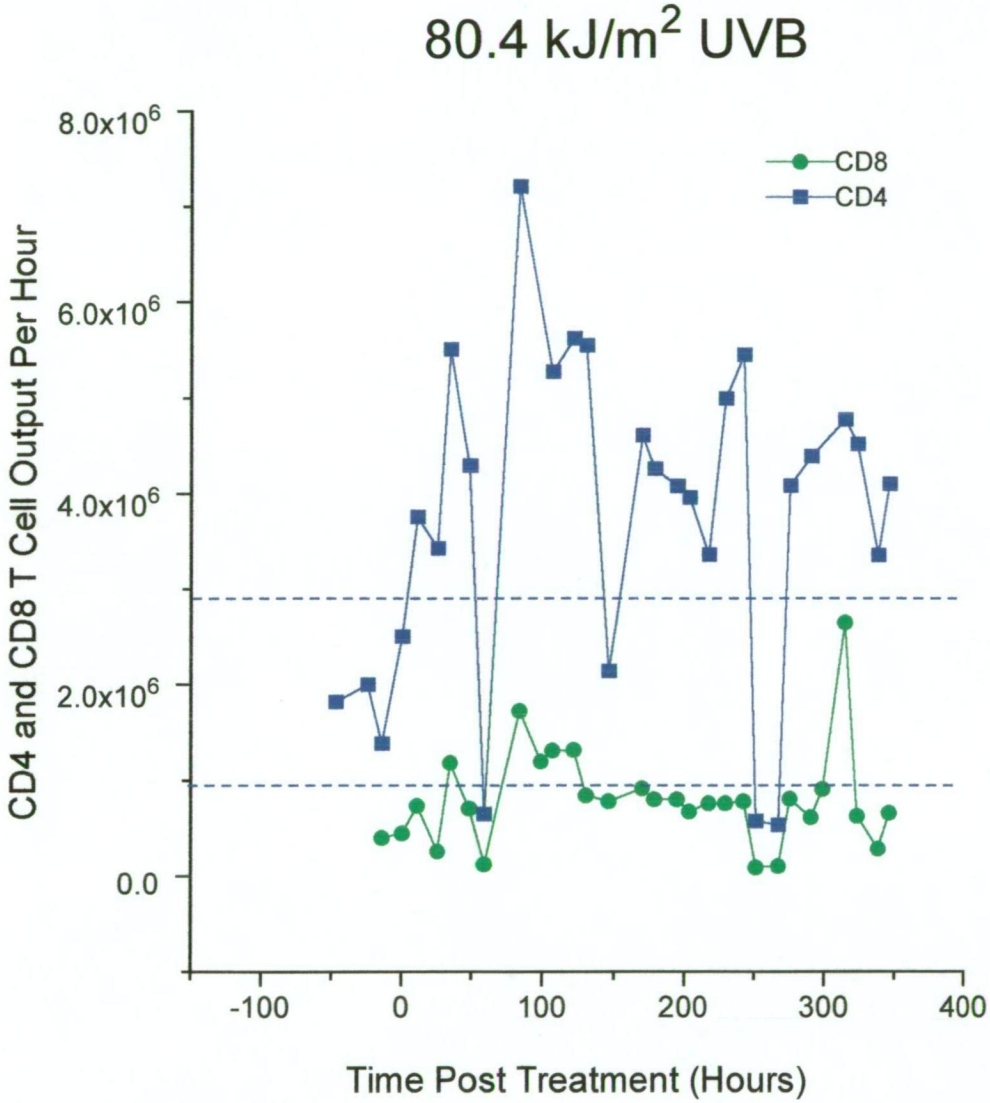



**Figure 3.3.5.6** Numbers of CD4 and CD8 T cells in lymph draining skin treated with 80.4 kJ/m$^2$ UVB. Dashed lines indicate the normal range for this single representative experiment.

numbers of sheep showing sustained changes for each parameter and for each dose.

Lymph flow showed very few sustained increases for sheep treated with 20.1 kJ/m² and below. Lymph flow was generally seen to be elevated for sustained periods after treatment with two highest doses. Sustained increases in LC numbers were observed in the majority of sheep for all doses. The magnitude of these changes was seen to increase with increasing UVB dose (Fig. 3.6). Sustained increases in the proportion of LC migrating in the lymph when compared to other cell populations, were also seen for all sheep treated with doses greater than 8.1 kJ/m². Sustained increases in $T19^+$ γδ T cells were only observed in the majority of sheep treated with the two highest doses. Total cell output was increased all sheep treated with the two highest doses, as were $CD4^+$ and $CD8^+$ T cell numbers. Some sustained decreases below the normal range for cellular output, $CD4^+$ and $CD8^+$ and $T19^+$ γδ T cells were observed for various doses, it must be noted that these were unusual and tended to occur towards the end of the monitoring period. These changes were more pronounced in the $CD8^+$ and $T19^+$ γδ T cells.

**Table 3.5 Summary of Effects after Exposure to 80.4 kJ/m² UVB**

| Parameter | Initial Increase (hours) | Peak increase (hours) | Magnitude of Peak Increase | Period of sustained increases (hours) |
|---|---|---|---|---|
| Lymph output | 11 | 34 | 2.75 fold | 34-58 |
| Total cell output | 34 | 89 | 3.1 | 34-48, 83-130, 230-243,275-323 |
| LC | 11 | 34 | 8.15 | 11-48, 83-195, 229-242, 290-314 |
| % LC | 11 | 34 | 3.91 | 11-34, 58-83, 130-170 |
| $T19^+$ γδ T cells | | 299 | 3.7 | 83-98, 217-340 |
| $CD4^+$ T cells | 11 | 83 | 3.7 | 11-48, 83-131, 170-243, 275-340 |

**Table 3.6 Summary of the effects of increasing doses of UVB on multiple experiments**

| UVB dose ($kJ/m^2$) | Lymph Output Number of sheep showing sustained increases / Total number of sheep used | Cell Output Number of sheep showing sustained increases / Total number of sheep used | LC Numbers Number of sheep showing sustained increases / Total number of sheep used | LC percentage Number of sheep showing sustained increases / Total number of sheep used | $T19^+$ γδ Tcells Number of sheep showing sustained increases / Total number of sheep used | $CD4^+$ Tcells Number of sheep showing sustained increases / Total number of sheep used | $CD8^+$ Tcells Number of sheep showing sustained increases / Total number of sheep used |
|---|---|---|---|---|---|---|---|
| 2.7 | 1/4 | 1/4 | 3/4 | 2/4 | 1/4 | 1/4 | 1/4 |
| 8.1 | 1/4 | 1/4 | 4/4 | 4/4 | 1/4 | 1/4 | 1/4 |
| 20.1 | 1/3 | 2/3 | 3/3 | 3/3 | 1/3 | 2/3 | 0/3 |
| 40.2 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| 80.4 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 |

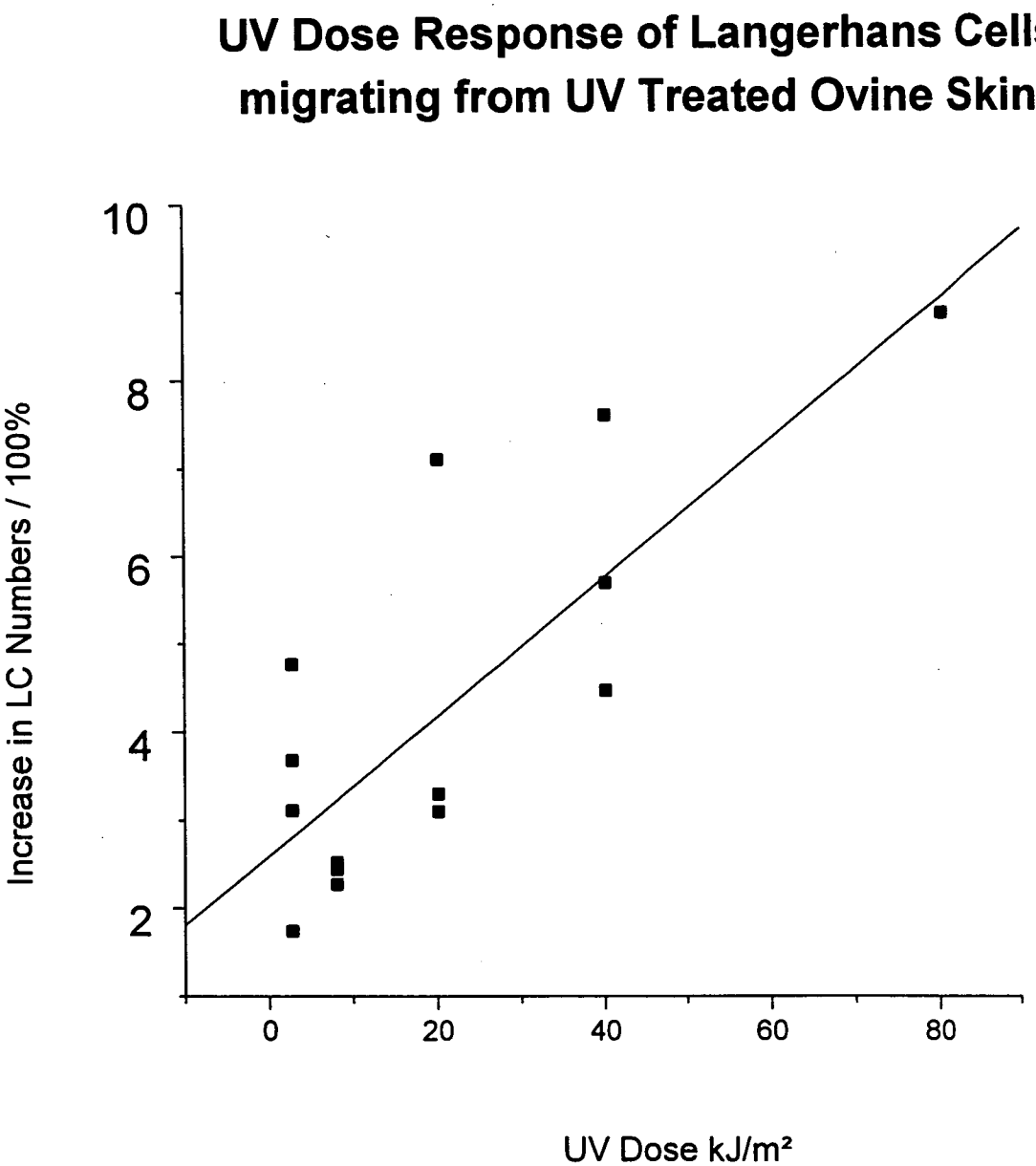



**Figure 3.6** The dose dependence of LC migration from skin treated with increasing doses of UV light. Each point represents the maximum sustained increase in LC migration for each individual sheep.

## 3.4 DISCUSSION

The study presented in this chapter looked at the time course of the migration of Langerhans cells and other cell populations from sheep skin after treatment with increasing doses of UV light. It demonstrated that the kinetics of cell migration are altered by exposure of the skin to UV light. These changes in migration patterns, especially of antigen presenting cells, have downstream ramifications including an alteration of immune responses occurring within the lymph node and therefore, in the whole organism.

The minimum dose used in these studies (2.7 kJ/m$^2$) was suberythemal, with 8.1 kJ/m$^2$ being the lowest dose which caused erythema. 2.7 kJ/m$^2$ is equivalent to 8-9 MED in Caucasian humans (Cooper *et al.*, 1992), suggesting that sheep appear relatively resistant to the inflammatory effects of UVB. In comparison, Negroes who have an MED 33 times greater than their Caucasian counter parts, are relatively resistant to the inflammatory effects of UVB (Olson *et al.*, 1973). Given that sheep skin lacks obvious pigmentation, an alternate mechanism for the resistance to the inflammatory effects of UVB must exist. While small amounts of wool remain after close clipping and the undulating nature of sheep skin may contribute slightly to this resistance, it is unlikely this will have a significant effect. One noticeable difference between human and sheep skin is the secretion of lanolin and other wax esters from ovine sebaceous glands. Whether this contributes to the apparent erythemal resistance of sheep is not, as yet known.

### *3.4.1 The Migration of LC after UV Exposure*

After exposure of sheep skin to UVB, increases in the number of migrating LC (CD1w1$^+$ dendritic cells) are observed in psuedoafferent lymphatic vessels draining the irradiated area of skin. The increase in the migration of viable LC is in accord with previous results obtained from studies in mice, where UV irradiation has been shown to result in an increase in dendritic cell numbers in the lymph node (Moodycliffe *et al.*, 1992). Also observed in the mouse lymph node after irradiation is the presence of MHC II$^+$ thymine dimer$^+$ cells, demonstrating that the cells have migrated from the skin after UV exposure. The increased migration of LC observed in the sheep model at least partially explains LC depletion from skin, which has been shown to occur in a variety of species, including human (Fan *et al.*, 1959, Zelickson and Mottaz, 1970, Aberer *et al.*, 1981, Cooper *et al.*, 1992), murine (Aberer *et*

*al.*, 1981, Obata and Tagami, 1985), guinea pig (Lim *et al.*, 1983, Hanua *et al.*, 1985) and sheep (Lyne and Chase, 1966).

The increased migration of LC was shown to have a linear relationship with UVB dose (Fig. 3.6) as indicated by the regression coefficient of 0.8. However, previous studies have found that the relationship between LC depletion and UVB dose is a log-linear one (Obata and Tagami, 1985, Noonan *et al.*, 1984). A similar relationship between UV dose and LC migration may have been obtained in this study by expanding the dose range to include higher and lower doses of UVB.

While exposure to all doses used in this study caused an increase in LC numbers in pseudoafferent lymph 8.1 kJ/m² proved to be the lowest dose to consistently cause significant increases in the numbers of LC migrating. This change was caused by an increased proportion of LC in the lymph in comparison to minimal changes for other cell types and not just general increases in cell migration (Table 3.2). While it is difficult to compare the magnitude of LC migration to that seen in other models, one study performed showed a four fold increase in DC numbers in the DLN of mice after exposure to 1.44 kJ/m² UVB (Moodycliffe *et al.*, 1992). This dose is nearly half that of the lowest dose used in this study, 2.7 kJ/m², suggesting that sheep may be more resistant to the migratory and hence depletory effects observed in other experimental systems. This may be partially due to the relative erythemal resistance of sheep already mentioned. Increasing the dose above 8.1 kJ/m² leads to increases in other cell types, as well as lymph and cellular outputs, reflecting the increased inflammatory insult to the skin. It should be noted, however that minor variations in the magnitude of increased LC numbers between individual sheep exposed to the same dose did occur. This probably reflects the outbred nature of the model.

Although this study has shown an increase in the numbers of LC migrating from UV exposed skin, the magnitude is moderate when compared to the numbers of migrating LC seen after treatment of sheep skin with the chemical carcinogen DMBA (Dandie *et al.*, 1994). Carcinogens such as DMBA, like UVB exposure, result in a depletion of LC from the epidermis (Woods *et al.*, 1996). While the sustained rate of increased LC migration observed after UV exposure may account for some of these differences, it is likely that there are other, non-mutually exclusive possibilities for the loss of Langerhans cells from the epidermis after UV exposure. These include both the loss of expression of the CD1 marker used to detect LC and LC cell death occurring *in situ*. LC

depletion from the skin after UV exposure was proposed by Aberer *et al.*, (1981) to be due to the loss of LC specific markers such as ATPase and MHC II. Obata and Tagami (1985) found that this was due to cytotoxicity, with only a minority of LC showing loss of MHC II expression. Tang and Udey (1992) also found that the previously observed down-regulation of ICAM-1 on LC (Tang and Udey, 1991) was due to cytotoxicity. Neither CD1 or MHC II have been observed to be down-regulated by *in vitro* UV exposure of epidermal cells (Czernlewski *et al.*, 1984). This, in conjunction with the increased numbers of viable LC ($CD1w1^+$ DC) observed to migrate from the skin in this study, suggests that loss of expression by LC is unlikely.

In contrast, there is substantial evidence for LC death in the skin after exposure to UVB. LC undergoing changes, characteristic of apoptosis are observed in mouse skin after UVB exposure (Obata and Tagami, 1985). Ultrastructural evidence of LC death and its resulting debris have also been reported to occur in the skin (Mommas *et al.*, 1993). The elimination of dead and dying cells by macrophages (Duvall *et al.*, 1985) occurring in the skin, may account for the lack of increased cell death observed in lymph during flow cytometric analysis in this study. However, 48-96 hours after UVB exposure of sheep skin an increased number of hard white clots were repeatedly observed in the cannula. These clots could have formed from dead cells or debris flushed from the skin by increased lymph flow. Further, DC with UV specific DNA damage has been found in the DLN of mice after UVB exposure of the skin (Sontag *et al.*, 1995). While the increased migration of viable LC was observed in this study, the presence UV induced DNA damage within these cells cannot be ruled out. The use of thymine dimers in the ovine system may make it possible to distinguish whether the increased numbers of LC observed migrating from the skin are a resident population (thymidine dimer positive) or a population of recent emigrants (thymine dimer negative). Initial studies to clarify this by looking for the presence of thymine $dimer^+$ dendritic cells in psuedoafferent lymph after UV exposure of the skin are currently being undertaken.

The monoclonal antibody used in these studies to identify LC (SBU-T6), recently has been reclassified as CD1w1 (Nassens *et al.*, 1997) and was previously thought to be a pan CD1 marker (Dutia and Hopkins, 1991). It is therefore possible it was detecting not only LC but the sheep equivalent of dermal DC, which express CD1b in humans (Richters *et al.*, 1995). Richters *et al.*, (1996) argues that the number of LC migrating from skin, is reduced after

UV exposure. However, examination of their studies which utilised skin explants, reveal that, while the proportion of $CD1a^+$ DC (LC) was decreased, these cells still greatly outnumber $CD1b^+$ and $CD14^+$ cells. Therefore the majority of ovine CD1w1+ DC observed in this study are still likely to be LC. The continuing development of ovine specific reagents, may enable LC to be distinguished from dermal DC cell in the future, although this will probably involve multi-parameter flow cytometric analysis. Further, the use of *in vitro* systems, such as explants, eliminates input from other areas such as the circulatory and nervous systems. It can be strongly argued that the sheep model, which studies cells collected *ex vivo* from wholly intact skin after UV exposure, is much more likely to represent the "normal" response of LC to this physical insult.

The SBU-T6 mAb used in this study, as well as possibly reacting with dermal DC has been shown to react with some ovine tissue macrophages (Dutia and Hopkins *et al.*, 1991), as well as ovine macrophages produced by culturing PBMC (Gorrell *et al.*, 1992). Macrophages are one of the cell types shown to infiltrate the skin after UV exposure (Cooper *et al.*, 1992) and migrate from skin explants after UV exposure (Richters *et al.*, 1996). Further cells with a macrophage-like phenotype are seen in the DLN of mice after UV exposure (Moodycliffe *et al.*, 1992). It may therefore be possible that the mAb used may be reacting with migrating macrophages. Evidence is presented in chapter 4 showing that this it is unlikely, although it cannot be eliminated entirely. Nonetheless, macrophages remain the major candidate other than UV altered LC, for down-regulation of antigen specific immune function detected after UV light exposure of the skin.

Other studies examining LC migration from sheep skin have been performed in this department, and it is interesting to compare the timing, magnitude and duration of these studies using skin exposure to chemicals with data obtained in this current study investigation UV light exposure of the skin. The contact sensitiser TNCB has been shown to cause increased LC migration in the sheep model, as have the complete chemical carcinogens DMBA (Dandie *et al.*, 1992, Dandie *et al.*, 1994) and BP as well as the tumour promoter, TPA (Ragg *et al.*, 1994). All of these chemicals have been shown to deplete LC from the epidermis of mice (Halliday *et al.*, 1987b, Muller *et al.*, 1985, Woods *et al.*, 1993, Woods *et al.*, 1996). In the sheep model, LC migration was shown to be increased immediately after application of TNCB (Dandie *et al.*, 1992, Dandie *et al.*, 1994) , BP, TPA (Ragg *et al.*, 1994) or

DMBA (Dandie *et al.*, 1992, Dandie *et al.*, 1994). Similarly UV irradiation of the skin caused increased migration, usually observed to begin within the first 24 hours. The magnitude of this initial increase in LC migration after cutaneous exposure to BP, TPA and DMBA, was approximately 5-10 fold, suggesting antigen induced migration as it was of similar timing and magnitude to that induced by TNCB (Dandie *et al.*, 1992, Dandie *et al.*, 1994). The magnitude of LC migration in this study, to the highest doses of UVB was also of a similar size.

Increased LC migration following UVB exposure of skin is likely to be a response to inflammatory stimuli proposed by Ibrahim *et al*, (1995) causing DC to migrate, rather than a response to UV induced antigens. It is likely to involve UV specific alterations such as alterations in the local cytokine milieu in the skin (reviewed by Granstein, 1996 and Takishima and Bergstresser, 1996), *cis*-UCA production (Moodycliffe *et al.*, 1992, Kurimoto and Streilein, 1992) or DNA damage (LeVee *et al.*, 1988). The migration of LC in response to UVB appears to be sustained for a much longer period than that of any chemical exposure of the skin.

### 3.4.2 *Lymph Output and Total Cell Output after UV Exposure of the Skin*

The treatment of skin with UV light causes vascular changes, including vasodilatation, which in turn leads to fluid accumulation in the skin which has been shown to occur for at least 24 hours after UV exposure (Gilchrest *et al.*, 1981). Exposure of the skin to large doses of UV causes oedema or blistering. However, only microscopic blistering was evident in the skin of sheep treated with the highest dose used in this study, 80.4 kJ/m$^2$ (unpublished observations). Despite the lack of obvious erythema, transient increases in lymph flow was observed in 3/5 sheep treated with 2.7 kJ/m$^2$ suggesting that erythema may have been present to some extent and that measurement more precise than visual estimation of erythema are required. However for this dose sustained increases above the normal range were only observed in one sheep. Transient increases were common in the first 48 hours after exposure to all doses of UVB assessed and sustained increases in lymph flow are seen for all sheep treated with doses higher than 20.1 kJ/m$^2$. This possibly reflects increased fluid accumulation at the inflammatory site. The increased rate of lymph flow observed after UV exposure of the skin may account for some of the

increases in cellular output as increased fluid flow may have had a flushing effect.

Increased cellularity of the lymph was observed after the flank skin was exposed to UV light in the majority of sheep treated with 20.1 kJ/m² UVB and above. It should be noted that, sustained increases were observed only in one out of four sheep after treatment with the 8.1 kJ/m² UVB. The increased cellularity of the lymph is due to a combination of two factors, the migration of resident cells from the skin and/or the migration of infiltrating cells. The latter is in agreement with Cooper *et al.*, (1993), who found that exposure of BALB/c mice to 3.12 kJ/m² caused the epidermis to be extensively infiltrated with neutrophils, macrophages and monocytic cells. Gilchrest *et al.*, (1981) also found some lymphocytes infiltrate the skin after UV exposure. It should be noted that the origin of the infiltrating macrophage\monocyte cells is a subject of debate and may originate from within the skin (Meunier *et al.*, 1995). Increased inflammatory cell infiltrate of sheep skin due to ecto-parasite infection, is also associated with increased cell output from cannulated pseudoafferent lymphatic vessels, the magnitude being similar (2-4 fold) to that observed after UVB exposure of the skin (Egan *et al.*, 1996.)

However for those sheep treated with UV light that did not show an increased number of cells in the lymph it may mean that the UV dose was too low to stimulate cells to be recruited into the skin or alternatively these cells and\or resident cells did not emigrate to the DLN in significant numbers. Increased cellularity was not due to the increased migration of LC, as this cell type was still relatively minor in number, even when significant increases in LC numbers were observed.

### 3.4.3 *Migration of T cell Subsets after UV Exposure of the Skin*

There is very little data on the migration of lymphocytes into or out of the skin after UV exposure. However, it has been shown that a slight lymphocytic influx occurs in human skin 24-72 hours after exposure with UV (Gilchrest *et al.*, 1981). The data shown in this current study indicates that treatment of the skin with UV causes a general increase in the migration of lymphocytes to the draining lymph node, which included $CD4^+$ T cells and, to a lesser extent, $T19^+$ $\gamma\delta$ T cells and $CD8^+$ T cells. Lymphocytes in this study appear to be less sensitive to these changes than LC which are the most sensitive to the effects of UVB light.

$CD4^+$ positive lymphocytes comprised the largest individual population of cells in the lymph after UVB. $CD4^+$ cells consistently made up the majority of T cells in the lymph, followed by $T19^+$ γδ T cells and $CD8^+$ T cells. The proportion of CD4 to CD8 T cells approximately reflected the ratio previously found in psuedoafferent lymph of 3.5 to 1 (Mackay *et al.,* 1988), albeit the numbers of $T19^+$ γδ T cells appeared to vary between sheep some being close to the numbers of $CD4^+$ T cells, while others were similar in number to the $CD8^+$ T cells. $T19^-$ γδ T cells were not assessed as anti TCR1 antibodies were not used in this study, although the number of these cells is small (Mackay and Hein, 1989, Hein and Mackay, 1991). Accordingly it was concluded that UV exposure of the skin caused an increase in the migration of T cells and CD4 T cells were increased in all sheep showing increased cellular output. It should be noted that T cell sub-populations, especially $CD8^+$ T cells, did not appear to be as sensitive to the migratory effects of UV light as that of LC, perhaps indicating that LC are much more susceptible to the effects of UV light. It also illustrates the function of DC, which readily migrate in response to inflammation and cell damage (Ibrahim *et al.*, 1995).

It is known that inflammatory endothelium preferentially extracts T cells of the memory phenotype (Mackay *et al.*, 1992). These cells traffic through the inflamed tissue back to the draining lymph node (Mackay *et al.*, 1990). E-selectin is up-regulated on endothelial cells after exposure to 2 MED UVB after 6 hours, peaking at 24 hours (Norris *et al.*, 1991). This correlated with increased accumulation of inflammatory cells. Human studies show E-selectin preferentially attracts a sub-population of skin homing memory T cells expressing the cutaneous lymphocyte associated antigen (CLA) (Picker *et al.*, 1993). While an equivalent antigen is yet to be determined in sheep, the observed increases in migrating lymphatic $CD4^+$ T cells, $CD8^+$ T cells and $T19^+$ γδ T cells may be memory cells that have responded to changes in the vascular endothelium by trafficking through the UV inflamed skin.

Further evidence for the attraction of memory T cells into ovine skin comes from another model of a cutaneous inflammation using *Lucila cuprina*, an ectoparasite (Egan *et al.*, 1996), where the authors found that the increased cellularity of the lymph was primarily due to the migration of $CD4^+$ T cells and γδ T cells both of a memory phenotype. The same cell types also predominate in the inflammatory lesion. It should be noted that this model did not cause significant increases in the migration of LC, which the authors suggest was due

to the production of different cytokines in comparison to those produced in the skin after UV light exposure.

Likewise, infiltration of $CD4^+$ T cells and $CD8^+$ T cells has been observed to occur in ovine skin using *Trypansoma congolese* (Mwangi *et al.*, 1990), and was paralleled by increases in these specific cell populations in bovine pseudoafferent lymph studies (Flynn *et al.*, 1994). No infiltration of γδ T cells was observed and these were few in lymph draining the infected site. This indicates that specific cell types observed to infiltrate the skin after a specific inflammatory stimulus, subsequently results in similar cell migration to the DLN. Extrapolating this to the present study, $CD4^+$ T cells, $CD8^+$ T cells and $T19^+$ γδ T cells all show an increased migratory response to UV light, reflecting the phenotype of cells infiltrating the skin. In support of this increased numbers of these T cell subsets have been observed in the dermis of chronically UV exposed sheep skin (Gorrell *et al.*, 1995).

$T19^+$ γδ T cells are proposed to represent mature γδ T cells (Mackay 1989). Gorrell *et al*, (1995) and Hein and Mackay (1991) have both shown that the immature γδ T cells ($T19^-$ γδ T cells) preferentially home to epithelia in sheep, and thus are more likely to represent the equivalent population to the DETC seen in mouse skin. Depletion of resident γδ T cells in response to UV light in mouse skin has been demonstrated (Aberer *et al.*, 1986 and Alcalay *et al.*, 1989). However, the fate of these cells is unknown as γδ T cells (including DETC), are not found within the DLN with UV specific damage (Sontag *et al.*, 1995). This suggests that they either migrate from the epidermis and are retained in the dermis; or undergo apoptotic cell death prior to reaching the node; or are not susceptible to UV induced damage.

A separate analysis of large, possibly dendritic, $T19^+$ γδ T cells has been investigated in conjunction with the study presented in this chapter (Dandie *et al.*, in preparation). This revealed that while migration of dendritic $T19^+$ γδ T cells is increased after UV exposure, this event was not dose related. DETC have been proposed as mediators of some of the suppressive effects of UV light especially in rodent models where a network of DETC is observed (Okamoto and Kripke, 1987). However, the human equivalent DETC population is not normally present in the epidermis (Bergstresser *et al.*, 1993). Furthermore, DETC have not been demonstrated in either the epidermal or dermal layers in the sheep in normal skin or chronically UV exposed skin (Gorrell *et al.*, 1995). In the current study only a small proportion of $T19^+$ γδ T

cells are dendritic. These cells may be large granular lymphocytes, rather than true DC.

In sheep skin $CD8^+$ T cells outnumber $T19^+$ γδ T cells (Gorrell *et al.*, 1995), yet in lymph draining normal and UV treated skin $CD8^+$ T cells were only rarely observed to outnumber $T19^+$ γδ T cells despite increases in their migration. Gorrell *et al.*, (1995), also found that $CD8^+$ T cells were less likely to be found next to superficial vessels were $T19^+$ γδ T cells predominate and suggested that $T19^+$ γδ T cells represent a more transient population within ovine skin. $CD8^+$ T cells are the most numerous T cell sub-population in ovine skin, yet they were the sub-population least sensitive to UV light, in terms of observed migration patterns. The migration of resident T cell populations, such as the epidermal $CD8^+$ T cells after UV exposure could be masked by the influx and migration of T cells responding to the inflammatory stimulus. The use of thymine dimers would appear to be the most useful technique for elucidating what proportion of $CD4^+$ T cells, $CD8^+$ T cells and $T19^+$ γδ T cells migrating were resident in the skin at the time of irradiation.

Gorrell *et al.*, (1995) also observed that in sheep skin chronic exposure to UV light resulted in an increased number of $CD4^+$ T cells in the epidermis, while in the dermis T lymphocytes of all subtypes were increased, but especially $CD4^+$ T cells with the positioning of these cells near blood vessels indicating immigration rather than replication. Therefore as well as $CD4^+$ T cells migrating to the DLN which has been demonstrated in this chapter, after chronic exposure to UV light they and other subtypes return to the skin.

## 3.5 SUMMARY

The migration kinetics of Langerhans cells and other cell populations has been investigated after UV exposure of the flank skin in sheep. These studies show that, in the ovine model UVB doses of 8.1 $kJ/m^2$ and greater cause increased LC migration. Consistent increases in general cell output were observed at higher doses and this reflected the migration of lymphocytes, including $CD4^+$ T cells, $T19^+$ γδ T cells, and to a lesser extent $CD8^+$ positive T cells. It was found that these cell types were much less sensitive to UV irradiation than LC which are resident in the skin. Evidence presented in this study supports the idea that LC migration to the draining lymph node is increased after UV exposure of the skin, in a dose dependant manner. This conclusion partially explains the depletion of LC from the epidermis which has been previously demonstrated in other models.

# CHAPTER 4: PHENOTYPE OF AFFERENT LYMPHATIC DC AND MACROPHAGES

## 4.1 INTRODUCTION

Exposure of skin to UVB draws inflammatory cells into the skin as well as affecting the resident DC. These inflammatory cells include neutrophils (Logan and Wilhelm, 1963, Hawk *et al.*, 1988, Cooper *et al.*, 1992), lymphocytes (Gilchrest *et al.*, 1981) and macrophages (Cooper *et al,* 1992). The induction of tolerance by epicutaneous immunisation 2-14 days after UV exposure, coincides with the timing of the influx of macrophages into the epidermis (Cooper *et al.*, 1985b). Separation of DC and macrophages is difficult given that they share many markers, perhaps reflecting their close relationship (Peters *et al.*, 1996). This problem is exacerbated in the ovine system where a more limited range of reagents against leucocyte antigens are currently available. In this chapter experiments will be described which have used a number of the relevant mAb's which are available to specifically identify any macrophages, which may be present in pseudoafferent lymph draining normal or UV exposed skin. Macrophages obtained from alveolar lavage fluid have been used as a readily identifiable population of ovine macrophages for the purpose of mAb testing and comparison in flow cytometric studies.

The exposure of the skin to UVB while drawing other cell populations into the skin appears to crucially affect the ability of LC to induce Th1 responses. The alteration of MHC II, adhesion molecules and co-stimulatory molecules on UV irradiated LC has been a topic of considerable debate. A crucial group of co-stimulatory molecules are the B7 family, CTLA-4 ligands. These have been previously noted in ovine pseudoafferent lymph DC (Ragg *et al.*, 1997). This chapter will further investigate the affect of UV exposure on the expression of CTLA-4 ligands on migrating ovine afferent lymph DC.

The use in this chapter of the more modern FACSCAN™ (BD) and ELITE™ (Coulter) flow cytometers in comparison to the EPICS V flow cytometer used in chapter 3. These cytometers allow precise gating and have capabilities for greater multi-parameter analysis, permitting the phenotype of cells to be studied in greater detail, thus enabling a more thorough search for macrophages in pseudoafferent lymph and a more accurate analysis of the

expression of B7 molecules on migrating LC, before and after cutaneous UV exposure.

## 4.2 MATERIALS AND METHODS

### *4.2.1 Experimental Plan*

#### 4.2.1.1 Isolation of Pseudoafferent Lymph DC and Alveolar Macrophages

The study of migrating DC was performed using cells from the ovine pseudoafferent lymphatic model as described previously (Sections 2.2-2.5), while the isolation of isolation of alveolar macrophages (AM) is described in Section 2.10.

#### 4.2.1.2 Detection of Macrophages

Large cells consisting of either macrophages from alveolar lavage fluid or DC from pseudoafferent lymph, were identified by forward and 90° scatter profiles (see Fig. 4.1). These cells were gated and analysed separately to ensure the populations of DC and AM were relatively pure. The levels of MHC I and MHC II expression were measured using mAbs SBU-I (41.19) and SBU-II (49.1). Antibodies against various cell markers, were also used, including SBU-T6 (20.27) which is an anti-CD1w1 mAb (Naessens *et al.*, 1997), which reacting with ovine LC in the skin and with migrating LC in pseudoafferent lymph (Dandie *et al.*, 1992). To identify $CD4^+$ T cells, $CD8^+$ T cells and $T19^+$ $\gamma\delta$ T cells the mAbs SBU-T4(44.38+44.97), SBU-T8 (38.65) and SBU-T19 (19.19) were used respectively. OM1, OM2 and OM3, which have been shown to stain macrophages and macrophage sub-populations but not pseudoafferent lymphatic DC, were used in an attempt to identify macrophages (Pepin *et al.*, 1992). OM1 has been recently reclassified as a marker of CD11c (Nassens *et al.*, 1997). The mAb CC-G33, which was raised against bovine CD14 (Sopp *et al.*, 1996), cross reacts with ovine CD14 (reviewed by Nassens *et al.*, 1997) and was used as a specific marker of macrophages. Directly conjugated isotype controls for IgG1 and IgGa (BD), were used to assess non-specific binding. For lavage fluid samples 25,000 cells and for lymph samples 10,000 cells were analysed for each phenotypic marker. The immunofluorescent data from each sample were stored as list mode files using the EPICS ELITE flow cytometer (Coulter) and were analysed using Elite software (Coulter). The staining patterns of the various mAb were used to compare the profiles of the two distinct cell types, alveolar macrophages and pseudoafferent lymph DC, to

determine if macrophages could be identified in pseudoafferent lymph under normal conditions, or after UV induced inflammation of the skin.

#### 4.2.1.3 CTLA-4 Ligand Expression by DC

CTLA-4 is a molecule expressed on T Cells which has been shown to be a receptor for the B7 (Lindsey *et* al., 1991) family of co-stimulators. Previous use of the CTLA-4 marker against ovine DC showed that it reacted with 68 +/- 7% of ovine DC (Ragg *et al.*, 1997). Although the CTLA-4 fusion protein is a construct made from the human CTLA-4 receptor this molecule has been shown to be conserved across species (>70% homology) (Lindston *et al.*, 1993). MCD40 was used as the control for the CTLA-4 construct protein. Two colour immunofluorescence was used to detect the expression of CTLA-4 ligands on LC ($CD1w1^+$ DC). The second layer used to detect the mAbs in this case contained phycoerytherin (PE) conjugated anti-mouse $F(ab')_2$ fragment (Silenus) to detect CD1w1 expression and FITC conjugated anti human $F(ab')_2$ (Silenus) to detect CTLA-4 ligand expression. These samples were processed using the FACSCAN flow cytometer (Becton-Dickenson), generously made available by the Red Cross Blood Bank, (Hobart) . Once pre-treatment levels were stabilised, the region of flank skin to be irradiated was shorn and exposed to UVB light (see Section 2.4) and monitoring continued. Doses of 2.7 $kJ/m^2$, 8.1 $kJ/m^2$ and 20.1 $kJ/m^2$ UVB were administered to shorn flank skin (Section 2.4). Care was taken to ensure that the conditions for each staining were kept as similar as possible. Samples were stored in FACS FIX (see Section 2.5), until the time course was finished. Samples were then analysed on the same day using the same cytometer settings to reduce possible variation.

### *4.2.2 Presentation of Data*

For each phenotypic marker an example of the staining profile of AM and psuedoafferent lymphatic DC draining normal skin, is depicted in a histogram showing log FITC staining for each cell population. The findings are summarised in Table 4.1 showing the mean percentage of positive cells in both the DC and AM populations and the mean fluorescence intensity for each marker.

The CLTA-4\SBU-T6 staining's are presented as intensity of staining of LC ($CD1w1^+$ DC) with the CTLA-4 fusion protein. This is shown as a time course after UV exposure. Because of the variability observed two representative graphs are used to illustrate the trends observed for each dose.

## 4.3 RESULTS

### *4.3.1 Phenotype of Afferent Lymphatic DC and Alveolar Macrophages*

#### 4.3.1.1 Flow Cytometric Characteristics of Afferent Lymph DC and Alveolar Macrophages

It can be seen from Fig. 4.1a that macrophages (large granular cells) appear to make up approximately 67% of cells isolated from lavage fluid. Small-lymphocyte like cells compose between 7-22% of the cells isolated from lavage fluids. The remaining cells are likely to be dead cells or debri and were exlcluded from the anlysis. Cells with the characteristic FSC vs SSC profile of DC are a minor cell population of pseudoafferent lymph as illustrated by Fig. 4.1b where they comprised 11.6% of the total afferent lymph cells. To assess the phenotype of dendritic cells and macrophages these specific cell populations were gated and separately analysed as "macrophages" or "DC".

#### 4.3.1.2 Controls

The use of isotype controls containing two directly conjugated isotypes, IgG1 (FITC) and IgG2a (PE) revealed that very few cells were positive. It should be noted that macrophages appeared to have a higher intensity of staining with IgG1 (Fig. 4.2a) than did DC (Fig. 4.2b). Controls containing the second layer, anti-mouse FITC conjugated $F(ab')_2$ fragments (Silenus), only showed a higher fluorescence intensity than those of the isotype controls. Therefore this control was used to set which cells could be regarded as "positive" or "negative" for both macrophages and DC , and is represented by the red lines in the Figs. 4.3-4.11.

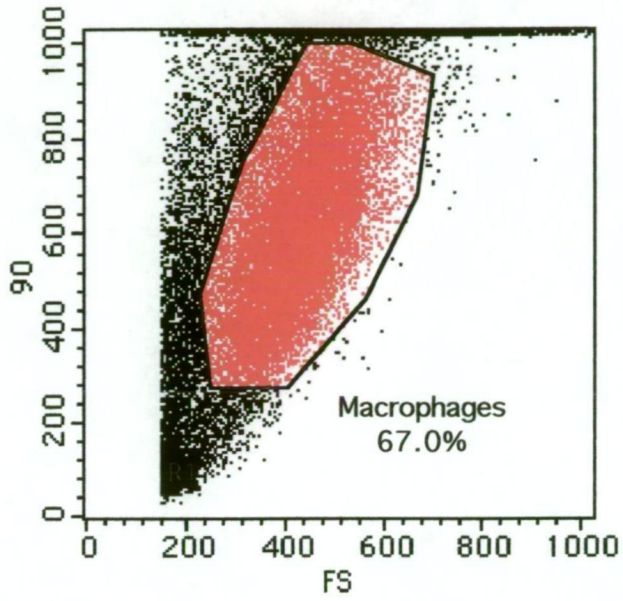
#### 4.3.1.3 Major Histocompatability Complex

Macrophages and DC expressed MHC I, with 98.3% (Fig. 4.3a) and 96.4% (Fig. 4.3b) respectively being positive. The level of expression was similar, with positive cells for both populations generally found within the third decade (log $10^2$ and log $10^3$) of immunofluorescence.

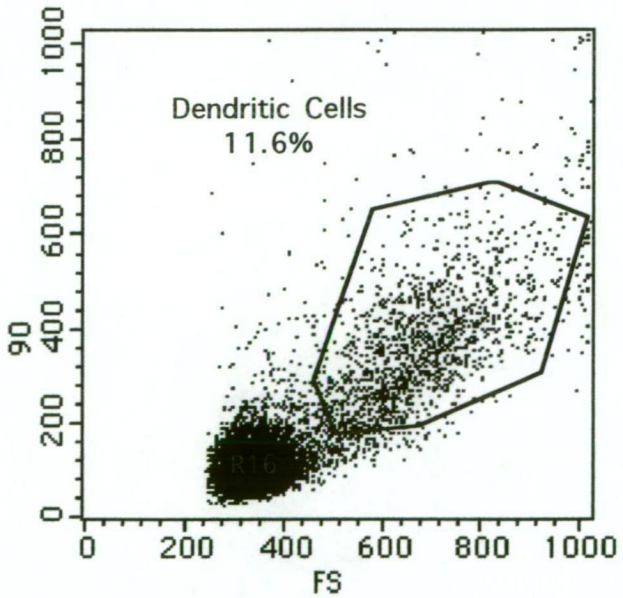
Both, macrophages and DC were also found to express MHC II to varying degrees, with 91% (Fig. 4.4a) and 98.4% (Fig. 4.4b) respectively being MHC II positive. However, it appeared that a minority of cells lying within the macrophage gate macrophages were negative. Further, the levels of expression of MHC II by AM and DC were quite distinct with macrophages

# Figure 4.1

(a) Gating of Alveolar Macrophages

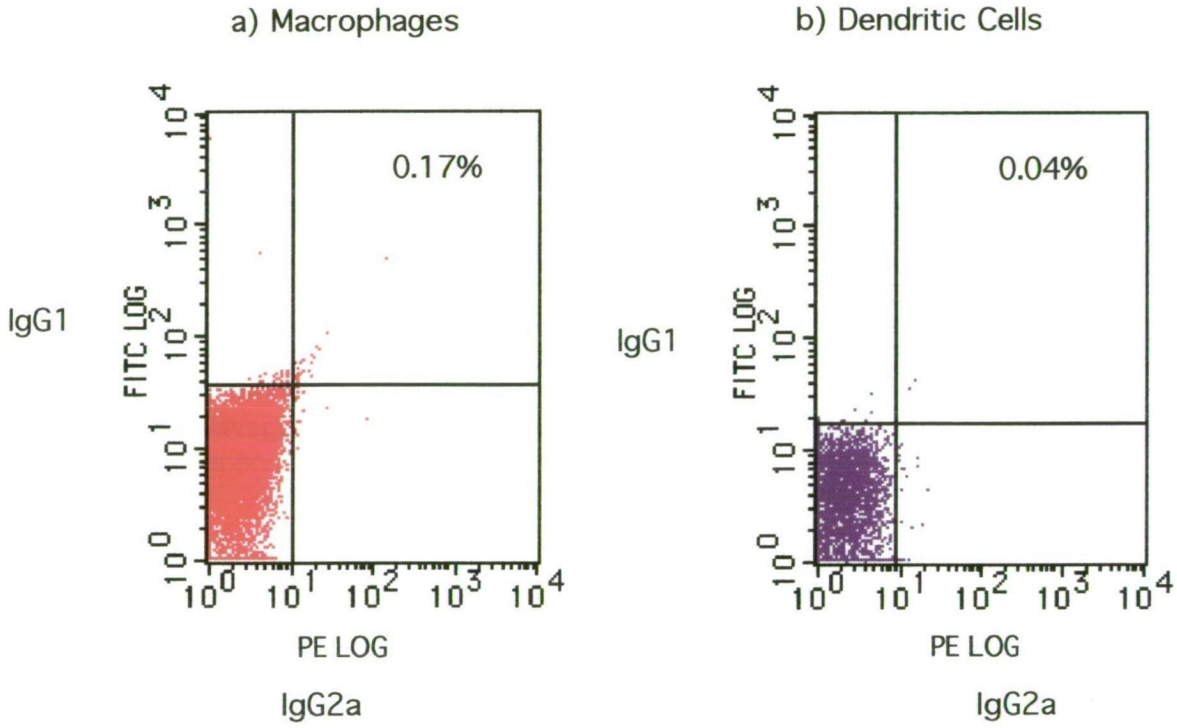



(b) Gating of Pseudoafferent Lymph Dendritic cells




Dot plot representation of flow cytometric analysis of forward scatter (FSC) and side scatter (90) of cells comprising (a) alveolar lavage fluid and (b) psuedoafferent lymph, showing the gating used for analysis purposes and the % of cells lying within the gate for this representative example

## Figure 4.2 Isotype Controls

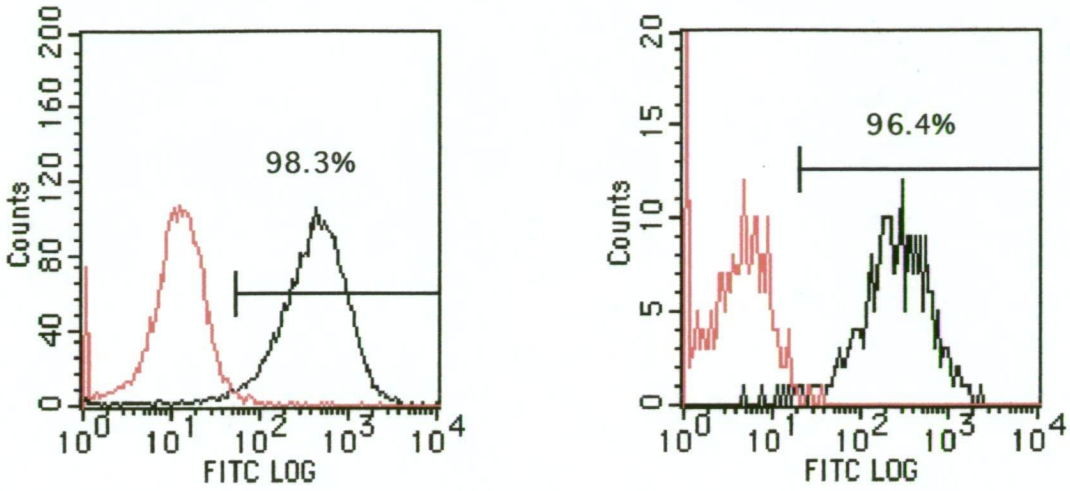



Dot plot representation of flow cytometric analysis of directly conjugated IgG1(FITC LOG) and IgG2a (PE LOG) isotype controls showing the percentage of double postive cells in the upper right quadrant. This analysis was performed on gated macrophages (a) from alveolar lavage fluid(red) and gated dendritic cells (b) from pseudoafferent lymph (purple)

## Figure 4.3 MHC I

a) Macrophages

b) Dendritic Cells

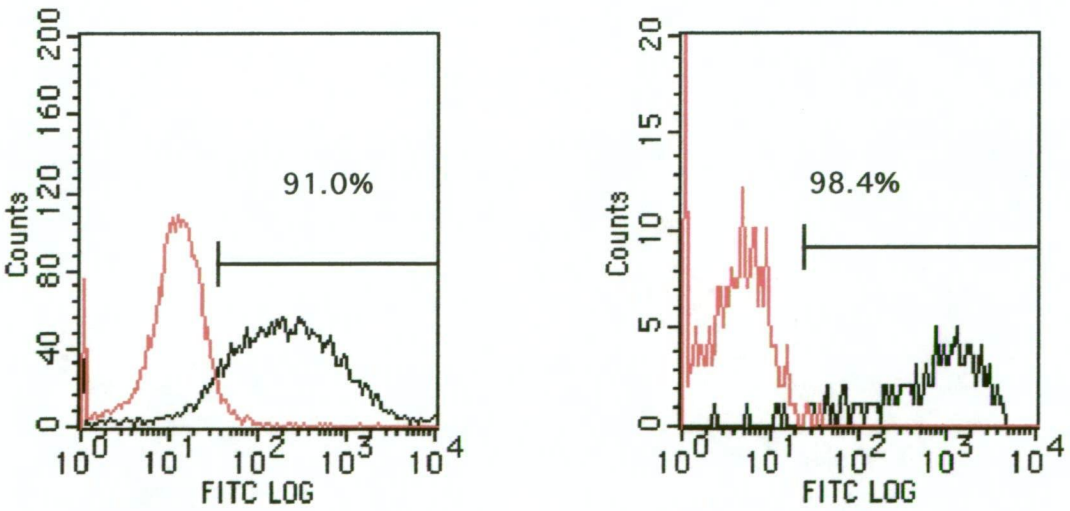



Flow cytometric staining profile of the anti-MHC I monoclonal antibody (SBU I - 41.19) showing the percentage of postive cells from gated macrophages from alveolar lavage fluid (a) and gated dendritic cells from pseudoafferent lymph (b) in comparison to cells from control samples (red).

## Figure 4.4 MHC II

a) Macrophages

b) Dendritic Cells




Flow cytometric staining profile of the anti-MHC II monoclonal antibody (SBU II - 49.1) showing the percentage of postive cells from gated macrophages from alveolar lavage fluid (a) and dendritic cells from pseudoafferent lymph (b) in comparison to cells from control samples (red).

having a continuum from negative through weakly positive to quite intensely positive. The immunofluorescent levels of the majority of positive macrophages lay within the third decade, while the majority of DC lay within the upper third and fourth decades.

#### 4.3.1.4 T Cell Markers

Staining with the SBU-T6 mAb revealed that, in general, AM were negative for this marker (Fig. 4.5a), whereas a majority of DC, 61.4% in the example shown (Fig. 4.5b) were positive. Fig. 4.6a shows that macrophages were negative for CD4 whereas a minority of cells lying within the DC gate were positive, in this case, 7.7% (Fig. 4.6b). The expression of this T cell marker was quite variable on DC, but its expression was consistently observed. The fluorescence intensity of CD4 staining was relatively low lying within the upper second decade. Macrophages were observed to be CD8 negative (Fig. 4.7a). However a small number of pseudoafferent lymph DC were positive (Fig. 4.7b). The percentage of CD8 positive cells were consistently smaller than that of CD4 positive cells and the fluorescence intensity of these cells was very low. Very few $T19^{+}$ $\gamma\delta$ T cells were found in either the macrophage gate (0.1%) or within the DC gate, (2.0%) (Fig. 4.8).

#### 4.3.1.5 Macrophage Markers

Macrophages were found to express CD11c (OM1) (Fig. 4.9a) as were the majority of DC (Fig. 4.9b), 89.7% and 65.1% respectively. However, approximately one third of DC did not express this marker. Similarly OM2 was expressed on 94.3% of cells within the macrophage gate (Fig. 4.10a), whereas only a minority of cells within the DC gate, 35.8%, expressed this marker (Fig. 4.10b). OM3 was not expressed significantly by either AM or DC; and only 1.5% and 0.9% of AM and DC, respectively were weakly positive (Fig. 4.11). CD14 was expressed by 87.7% of macrophages (Fig. 4.12a) but only by 0.9% of cells lying within the DC cell gate (Fig. 4.12b).

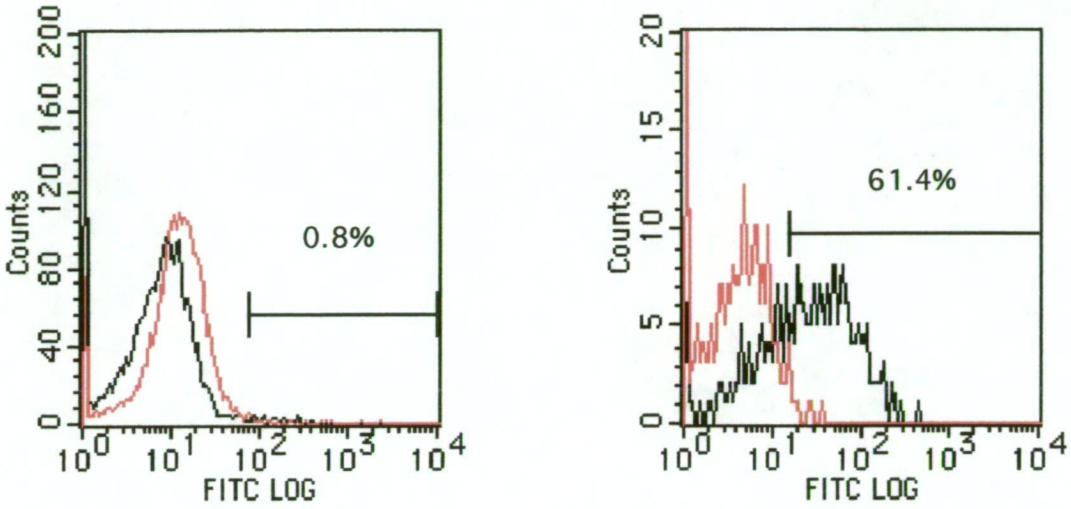
#### 4.3.1.6 Summary

Table 4.1 summarises the percentage of cells observed to express the various markers examined. While macrophages express MHC II, the intensity of expression was lower than that of DC. It was also consistently found that a minor population of DC expressed the T cell marker, CD4. Two other differences were the expression of CD1w1 by the majority of dendritic cells and not macrophages and the expression of CD14 by macrophages and not DC.

## Figure 4.5 CD1w1

a) Macrophages

b) Dendritic Cells

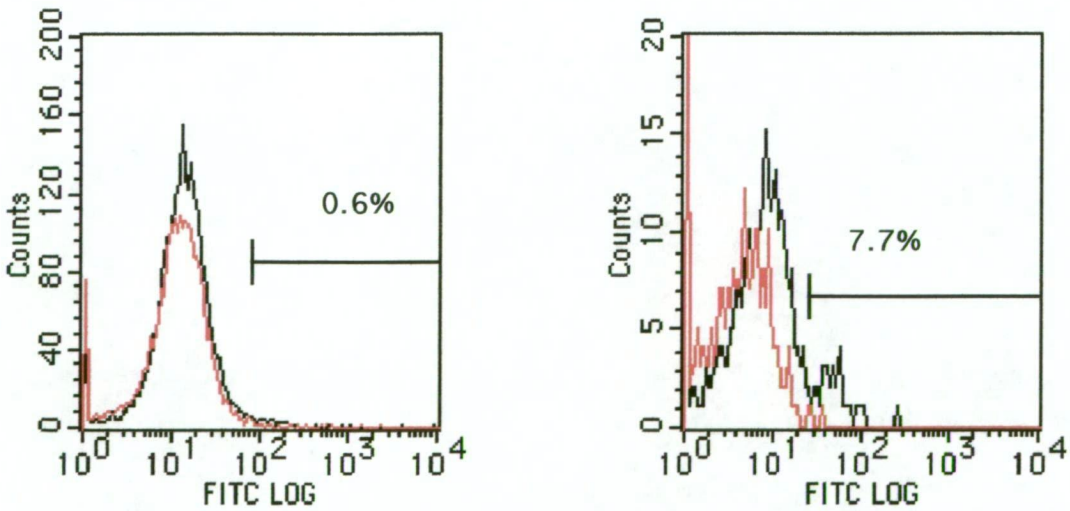



Flow cytometric staining profile of the anti-CD1w1 monoclonal antibody (SBU T6 - 20.27) showing the percentage of postive cells from gated macrophages from alveolar lavage fluid (a) and gated dendritic cells from pseudoafferent lymph (b) in comparison to cells from control samples (red).

## Figure 4.6 CD4

a) Macrophages

b) Dendritic Cells

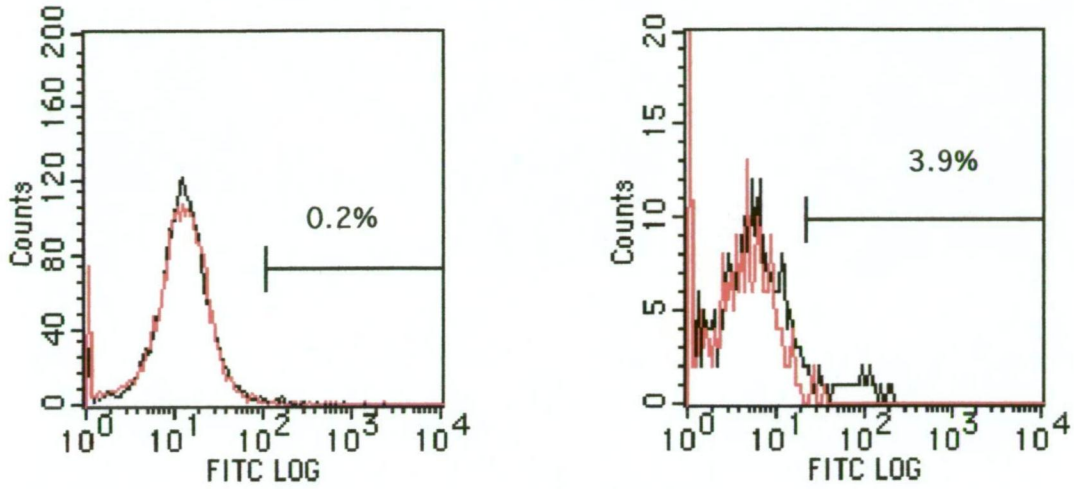



Flow cytometric staining profile of the anti-CD4 monoclonal antibodies (SBU T4 -44.38+44.97) showing the percentage of postive cells from gated macrophages from alveolar lavage fluid (a) and gated dendritic cells from pseudoafferent lymph (b) in comparison to cells from control samples (red).

## Figure 4.7 CD8

a) Macrophages

b) Dendritic Cells

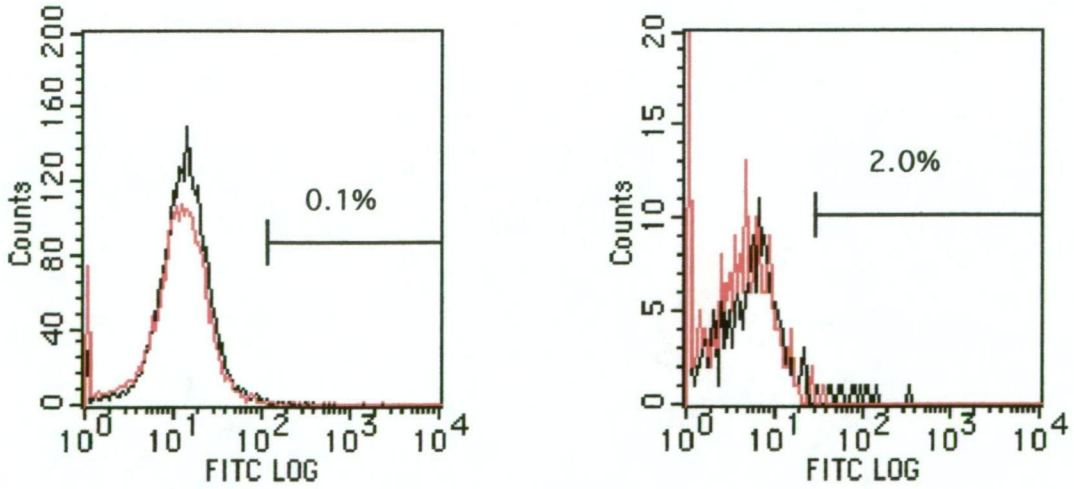



Flow cytometric staining profile of the anti-CD8 monoclonal antibody (SBU T8 - 38.65) showing the percentage of postive cells from gated macrophages from alveolar lavage fluid (a) and gated dendritic cells from pseudoafferent lymph (b) in comparison to cells from control samples (red).

## Figure 4.8 T19 γδ T cells

a) Macrophages

b) Dendritic Cells

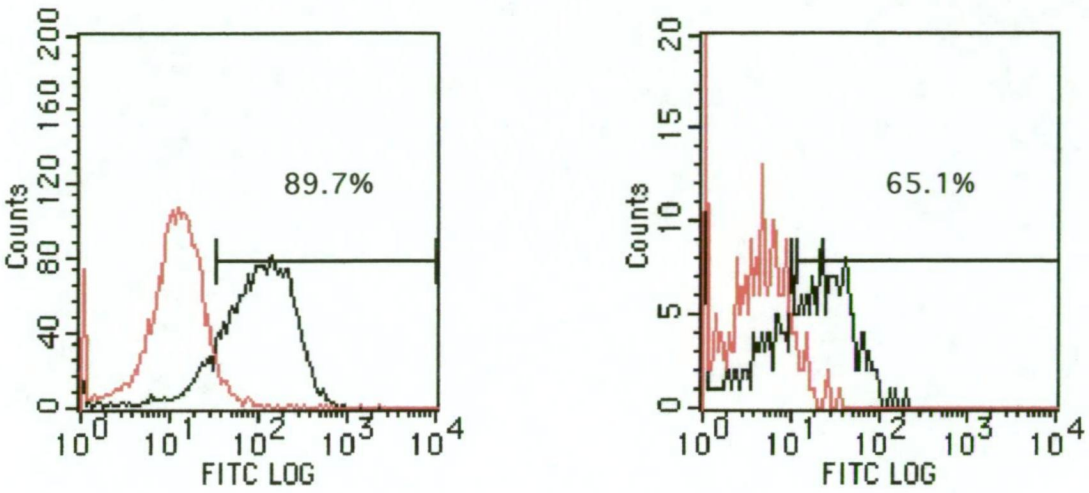



Flow cytometric staining profile of the anti-T19 monoclonal antibody (SBU T19 - 19.19) showing the percentage of postive cells from gated macrophages from alveolar lavage fluid (a) and gated dendritic cells from pseudoafferent lymph (b) in comparison to cells from control samples (red).

## Figure 4.9 CD11c

a) Macrophages

b) Dendritic Cells

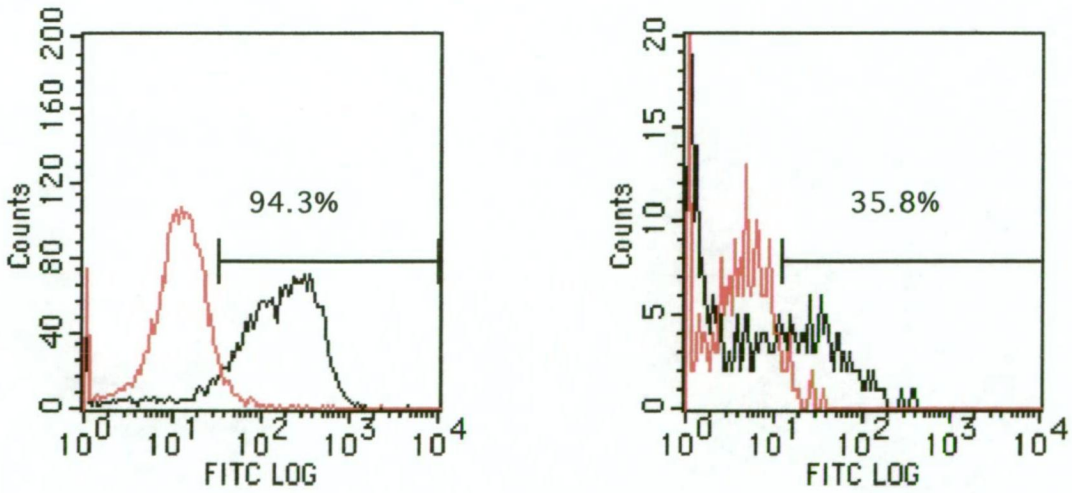



Flow cytometric staining profile of the anti-CD11c monoclonal antibody (OM1) showing the percentage of postive cells from gated macrophages from alveolar lavage fluid (a) and gated dendritic cells from pseudoafferent lymph (b) in comparison to cells from control samples (red).

## Figure 4.10 OM2

a) Macrophages

b) Dendritic Cells

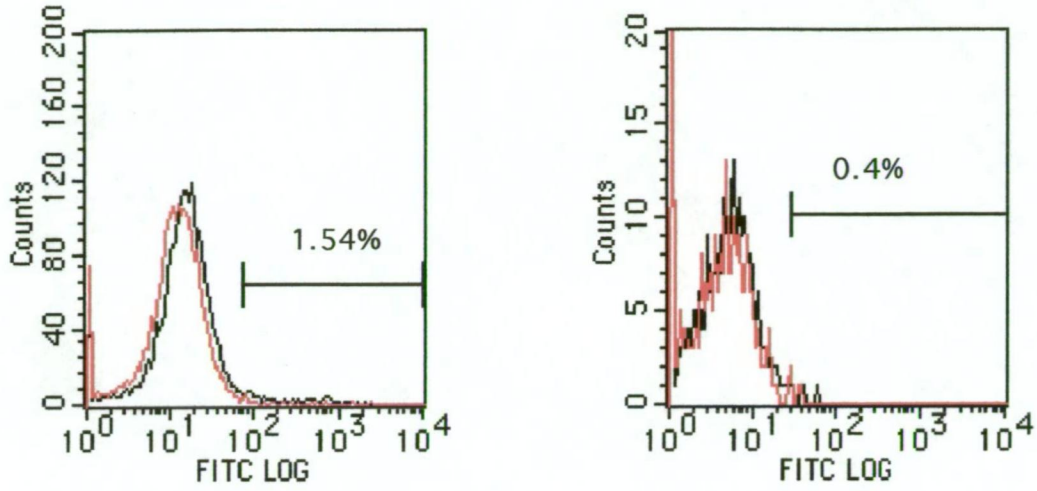



Flow cytometric staining profile of the anti-OM2 monoclonal antibody showing the percentage of postive cells from gated macrophages from alveolar lavage fluid (a) and gated dendritic cells from pseudoafferent lymph (b) in comparison to cells from control samples (red).

## Figure 4.11 OM3

a) Macrophages

b) Dendritic Cells

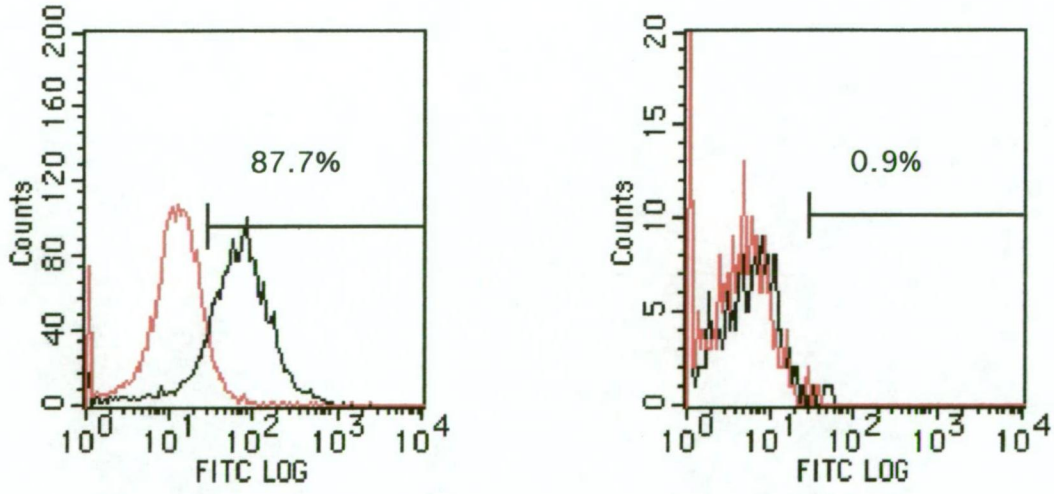



Flow cytometric staining profile of the anti-OM3 monoclonal antibody showing the percentage of postive cells from gated macrophages from alveolar lavage fluid (a) and gated dendritic cells from pseudoafferent lymph (b) in comparison to cells from control samples (red).

## Figure 4.12 CD14

a) Macrophages

b) Dendritic Cells




Flow cytometric staining profile of the anti-CD14 monoclonal antibody (CC-G33) showing the percentage of postive cells from gated macrophages from alveolar lavage fluid (a) and gated dendritic cells from pseudoafferent lymph (b) in comparison to cells from control samples (red).

The crucial finding was that the CD14 surface molecule was not expressed by pseudoafferent lymph DC. These findings provided a critical base to assess macrophages migrating after skin exposure to UVB.

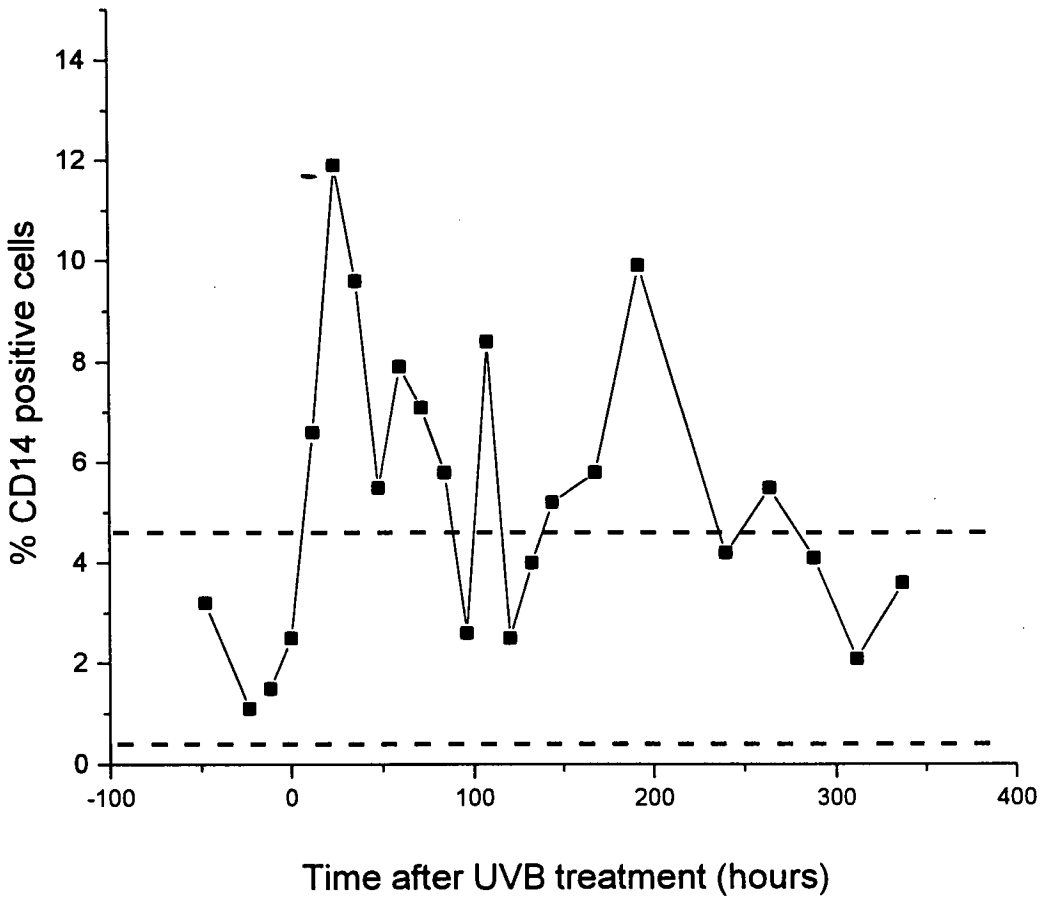
**Table 4.1 Expression of Phenotypic Markers by Alveolar Macrophages and Psuedoafferent lymphatic Dendritic Cells**

| Marker | Pseudoafferent Dendritic Cells | | Alveolar Macrophages | |
|---|---|---|---|---|
| | % Positive cells (Standard Deviation) | Mean channel of Fluorescence | % Positive Cells (Standard Deviation) | Mean channel of Fluorescence |
| MHC I | 99.3 (0.4) | 21.3 | 97.1 ( 3.7) | 39.1 |
| MHC II | 95.7 (3.9) | 82.4 | 71.4 (27.5) | 20.1 |
| CD1w1 | 58.7 (10.9) | 4.5 | 2.4 (1.5) | 7.0 |
| CD4 | 29.1 (10.4) | 3.1 | 3.1 (1.5) | 9.6 |
| CD8 | 6.8 (2.8) | 3.9 | 1.8 (1.1) | 5.8 |
| T19 γδ T cells | 2.4 (0.7) | 4.2 | 1.6 (1.3) | 5.5 |
| OM1 | 60.8 (8.2) | 3.9 | 91.6 (3.5) | 12.9 |
| OM2 | 27.7 (8.6) | 3.8 | 94.6 (1.3) | 21.3 |
| OM3 | 0.9 (1.0) | 3.8 | 3.9 (1.6) | 8.2 |
| CD14 | 2.9 (1.6) | 2.4 | 88.4 (7.2) | 10.7 |

### 4.3.2 Migration of $CD14^+$ DC after UV Exposure

The flank skin of three cannulated sheep was exposed to 20.1 kJ/m² UVB and the proportion of migrating $CD14^+$ cells occurring within the DC gate was monitored to determine if macrophages were being induced to migrate. From Fig. 4.13 can be seen that there was a sustained increase in the percentage of large granular cells expressing the CD14 molecule. This peaked 24 hours after exposure, with an approximate 4 fold increase above the mean pre-UVB exposure levels. The proportion of CD14 cells within the DC gate remained elevated until 96 hours were they returned to within the normal range. This change was transient and was immediately followed by another increase above the normal range which was followed by subsequent decrease between 120 and 132 hours post treatment. Following this a gradual increase in the proportion of CD14 positive DC occurred, peaking at 192 hours, after which the percentage of CD14 returned to the normal range apart from a transient increase above the normal range at 264 hours.

**Figure 4.13 Migration of CD14+ cells after Exposure of Flank Skin to 20.1kJ/m² UVB**




Dotted lines indicate the normal range for this single representative experiment.

Increases in the percentage of $CD14^+$ DC were seen for two other sheep both peaking within the first 24 hours. The up tempo migration $CD14^+$ DC was observed to last for 168 and 216 hours for these two replicate experiments Thus it appears that after UV exposure of the skin $CD14^+$ cells with the FSC and SSC characteristics of both DC and macrophages were induced to migrate to the draining lymphatics.

## *4.3.3 Expression of B7 molecules by Afferent lymph DC after UV exposure of the skin*

### 4.3.3.1 Expression of CTLA-4 Ligands by Pseudoafferent DC

Fig. 4.14 illustrates how the expression of CTLA-4 ligands by LC ($CD1w1^+$ DC) was assessed. The conjugate only control was used so negative cells could be excluded from the analysis (Fig. 4.14a). The construct MCD40 (Fig. 4.14b) was used as an isotype control for the CTLA-4 construct (Fig. 4.14c) and cells lying within this region were excluded by logical gating from the histogram analysis of the levels of CTLA-4 ligand expression (Fig. 4.14d).

### 4.3.3.2 Expression of CTLA-4 Ligands by Pseudoafferent DC after Skin Exposure to 2.7 kJ/m² UVB

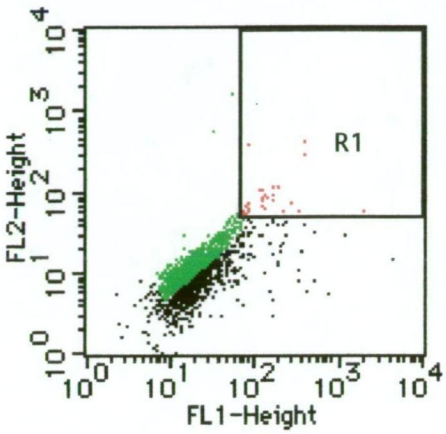
There was a variable response in the level of B7 expression on $CD1w1^+$ DC observed after UV exposure of skin to 2.7 kJ/m². It can be seen from Fig. 4.15 that the fluorescence intensity did not deviate from the normal range after exposure of the skin to 2.7 kJ/m². However, Fig. 4.16 shows that in a different experimental subject the intensity of fluorescence, was first observed to increase 22 hours after treatment but subsequently returned to within normal levels. Between 48 and 72 hours the intensity of fluorescence was again observed to be above the normal range. After this the level of B7 expression by DC returned to normal levels apart from subsequent increases above the normal range at 176 and 298 hours. It should be noted that the increases in B7 expression were not very dramatic for the one experimental subject which showed increases above the normal range. So typically it was found that only small increases, if any, in the levels of B7 expression were observed after exposure of sheep flank skin to 2.7 kJ/m².

### 4.3.3.3 Expression of CTLA-4 Ligands by Pseudoafferent DC after Skin Exposure to 8.1 kJ/m² UVB

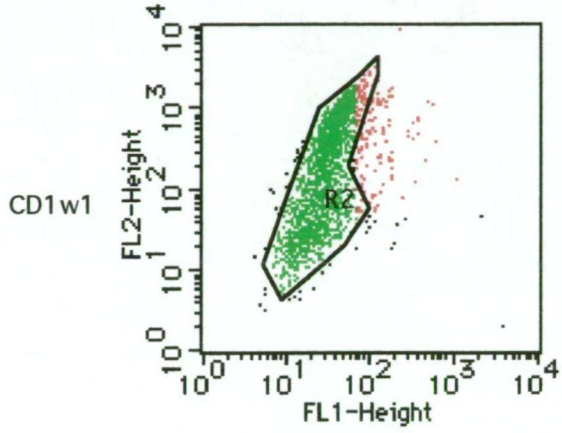
When sheep skin was exposed to higher doses of UVB, similar results were noted. Figs. 4.17 and Fig. 4.18 illustrate that after treatment of the skin

# FIGURE 4.14 ANALYSIS OF CTLA-4 LIGANDS ON MIGRATING LC (CD1w1+ DC)

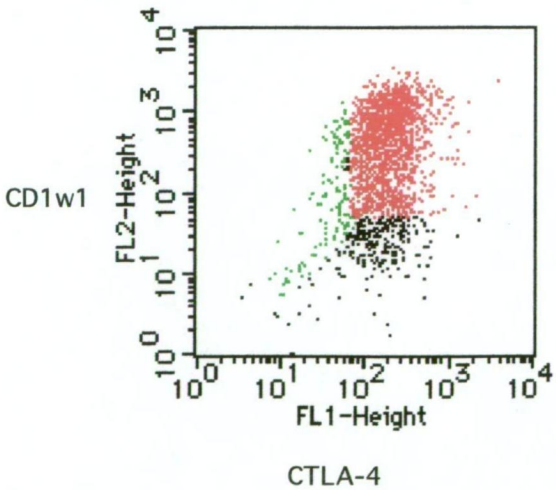
a) Second layer only




b) MCD40 + SBU-T6




c) CTLA-4/Ig + SBU-T6




d) gated CTLA-4+ and CD1w1+ DC using the logical gate, G3 = R1 AND NOT R2

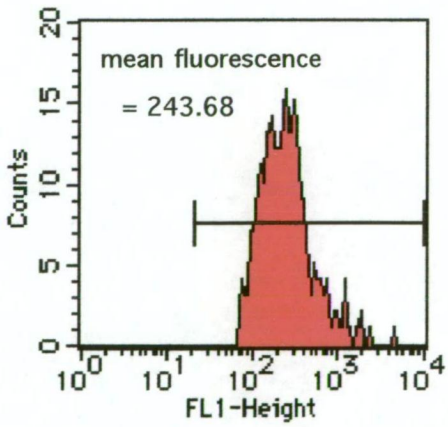
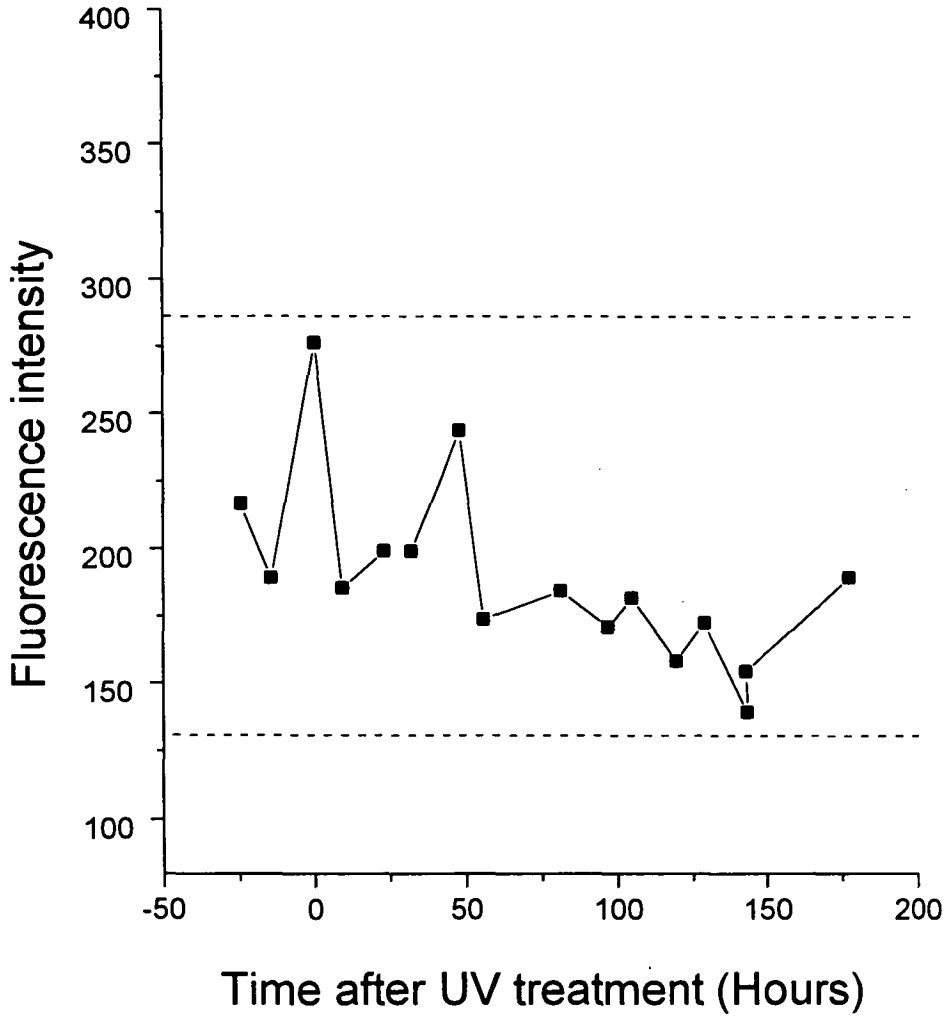


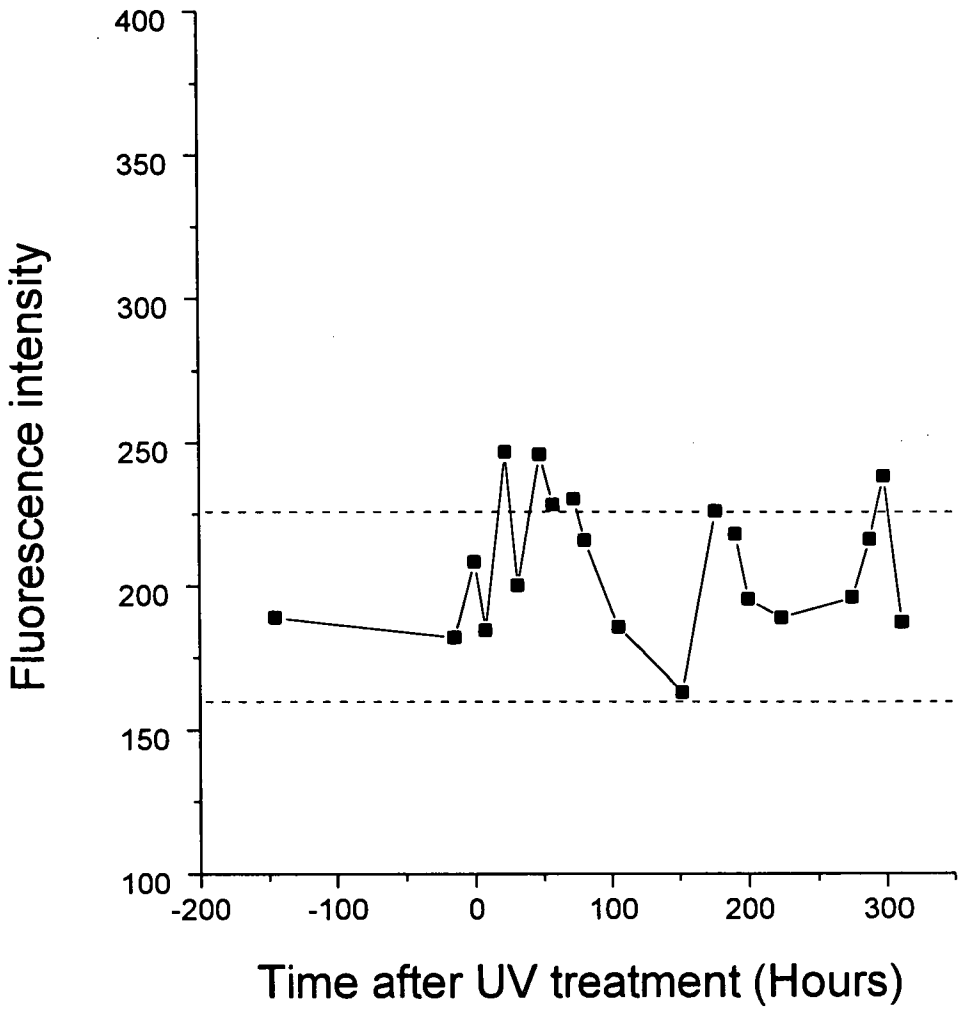

Figure 4.14a, b & c are dot plot representations of dendritic cells labelled with (a) directly conjugated FITC and PE second layer $F(ab')_2$ only, (b) with the MCD40 control construct and the SBU-T6 mAb and (c) with the CTLA-4 construct and SBU-T6. Regions are shown in colors (R1 = Red and R2 =Green. Figure 4.14d is histogram gated on region G3 showing intensity of CTLA-4 staining on CD1w1+ dendritic cells

## Figure 4.15 B7 expression after 2.7 kJ/m$^2$ UVB




Fluorescence intensity of CTLA-4 ligand expression on migrating CD1w1$^+$ DC after exposure of the skin to 2.7 kJ/m$^2$ UVB. Dashed lines represent the normal range for this representative experiment.

## Figure 4.16 B7 expression after 2.7 kJ/m²




Fluorescence intensity of CTLA-4 ligand expression on migrating CD1w1$^{+}$ DC after exposure of the skin to 2.7 kJ/m² UVB. Dotted lines represent the normal range for this representative experiment.

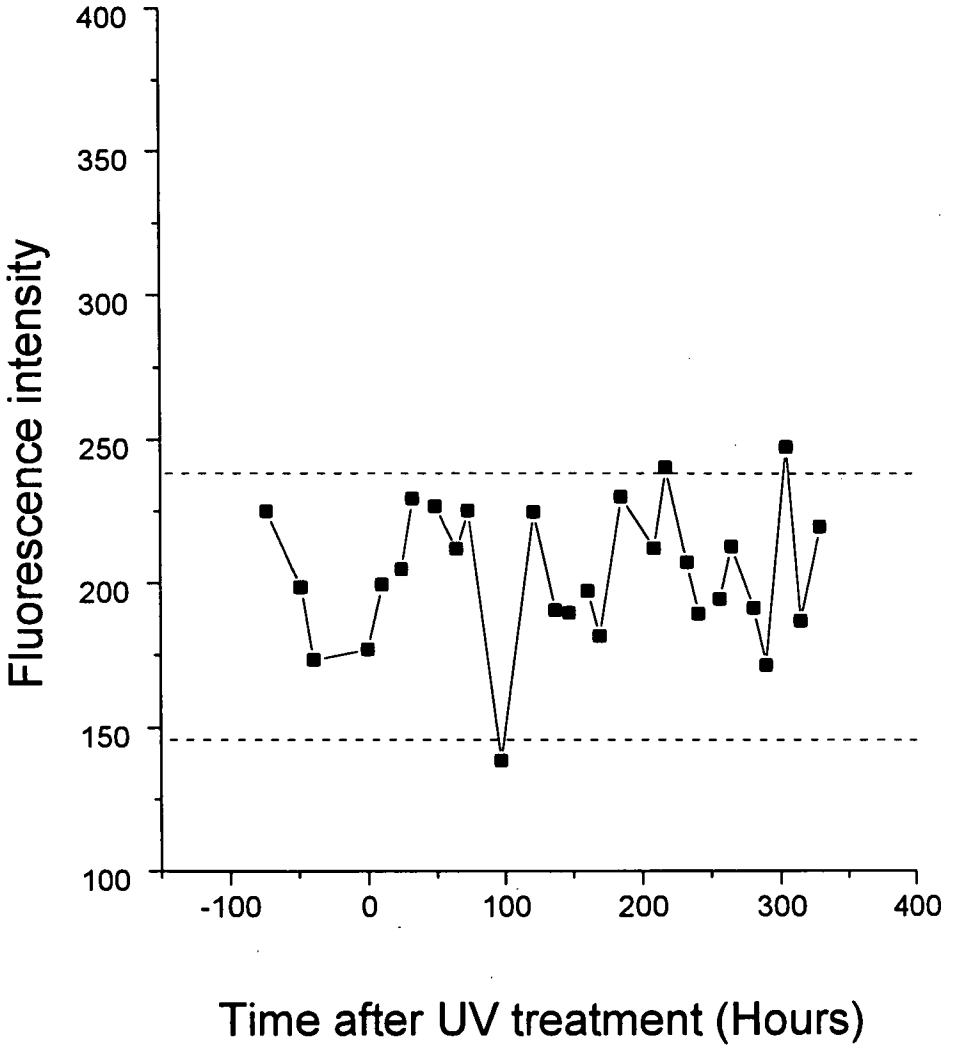
with 8.1 kJ/m² UVB, the intensity of expression of CTLA-4 ligands were substantially unchanged. The exceptions were two transient increases just above the normal range at 218 and 305 hours and a transient decrease below the normal range at 96 hours after UV exposure (Fig. 4.17). It should be noted that the pre-treatment level of B7 expression were quite variable, between experimental subjects and ranged from a mean fluorescence channel of 120-220 (Fig. 4.18).

#### 4.3.3.4 Expression of CTLA-4 Ligands by Pseudoafferent DC after Skin Exposure to 20.1 kJ/m² UVB

Exposure of sheep skin to 20.1 kJ/m² had variable effects on the expression of CTLA-4 ligands. This is illustrated by Fig. 4.19 and Fig. 4.20. Fig. 4.19 shows that a sustained decrease in the intensity of CTLA-4 ligand expression occurred within the first 24 hours after UV exposure. While a transient return to within the normal range, the substantial decrease in the expression of CTLA-4 ligands was sustained throughout the duration of the monitoring period. In contrast, data from another experimental subject (Fig. 4.20) shows a sustained increase in the expression of CTLA-4 ligands, initially between 80-120 hours after exposure and then between 240-312 hours following exposure.

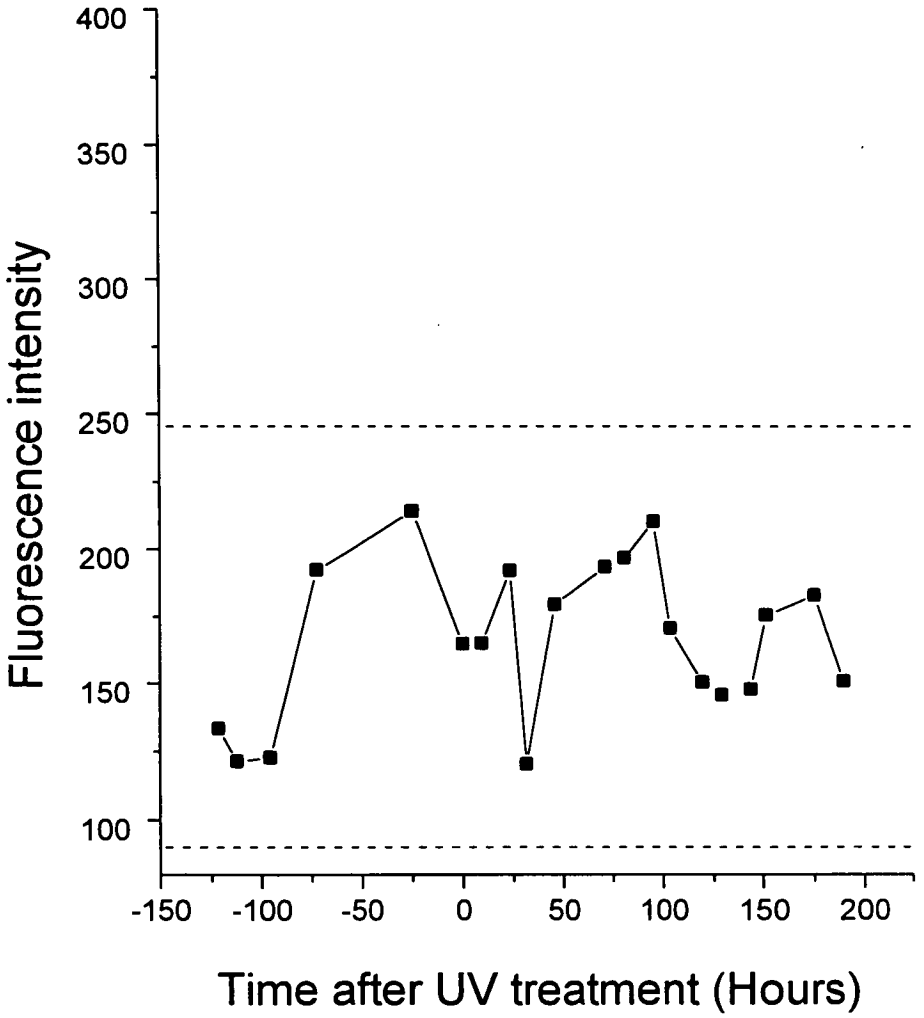
Exposure of two further sheep to this dose revealed no decreases in the levels of intensity of CTLA-4 expression but increases in the intensity of CTLA-4 ligand expression were observed at 48 hours and between 96-120 hours respectively. Therefore, levels of B7 expression on migrating DC after exposure of the skin to 20.1 kJ/m² were generally observed to be elevated in expression in the majority of experimental subjects (3/4).

## Figure 4.17 B7 expression after 8.1 kJ/m$^2$ UVB



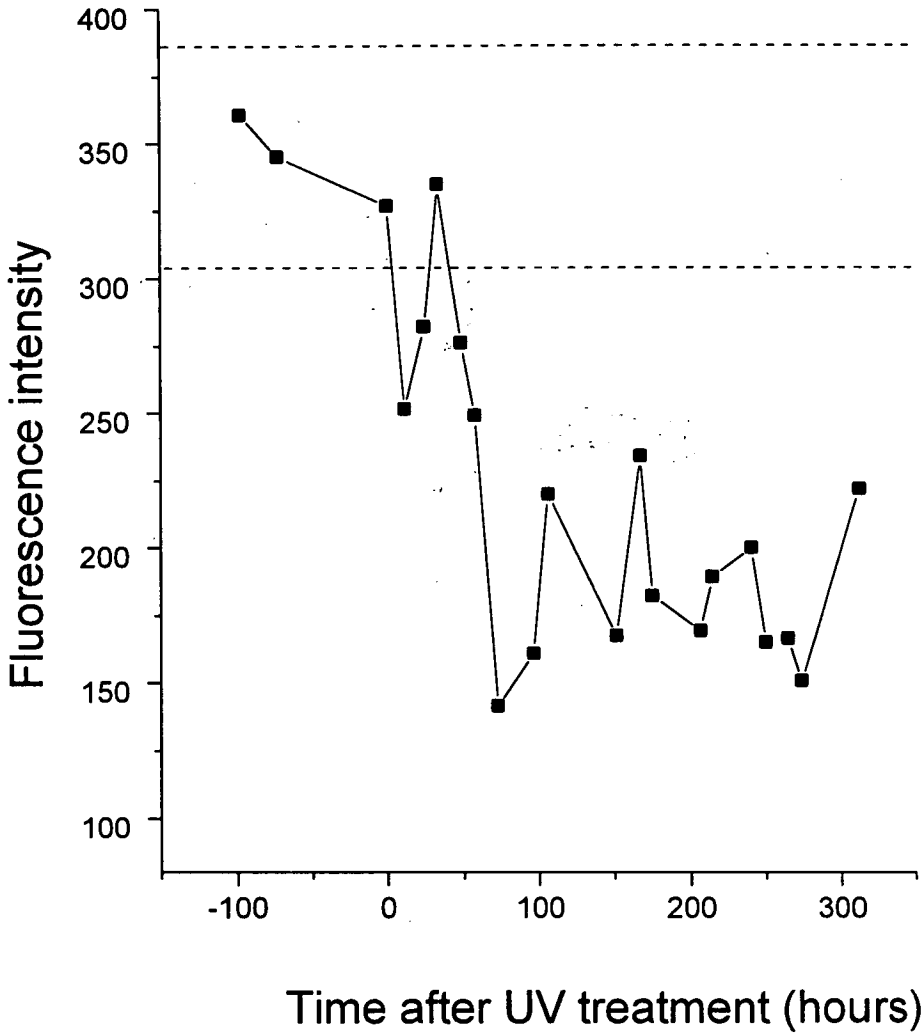

Fluorescence intensity of CTLA-4 ligand expression on migrating CD1w1$^+$ DC after exposure of the skin to 8.1 kJ/m$^2$ UVB. Dashed lines represent the normal range for this representative experiment.

## Figure 4.18 B7 expression after 8.1 kJ/m$^2$ UVB



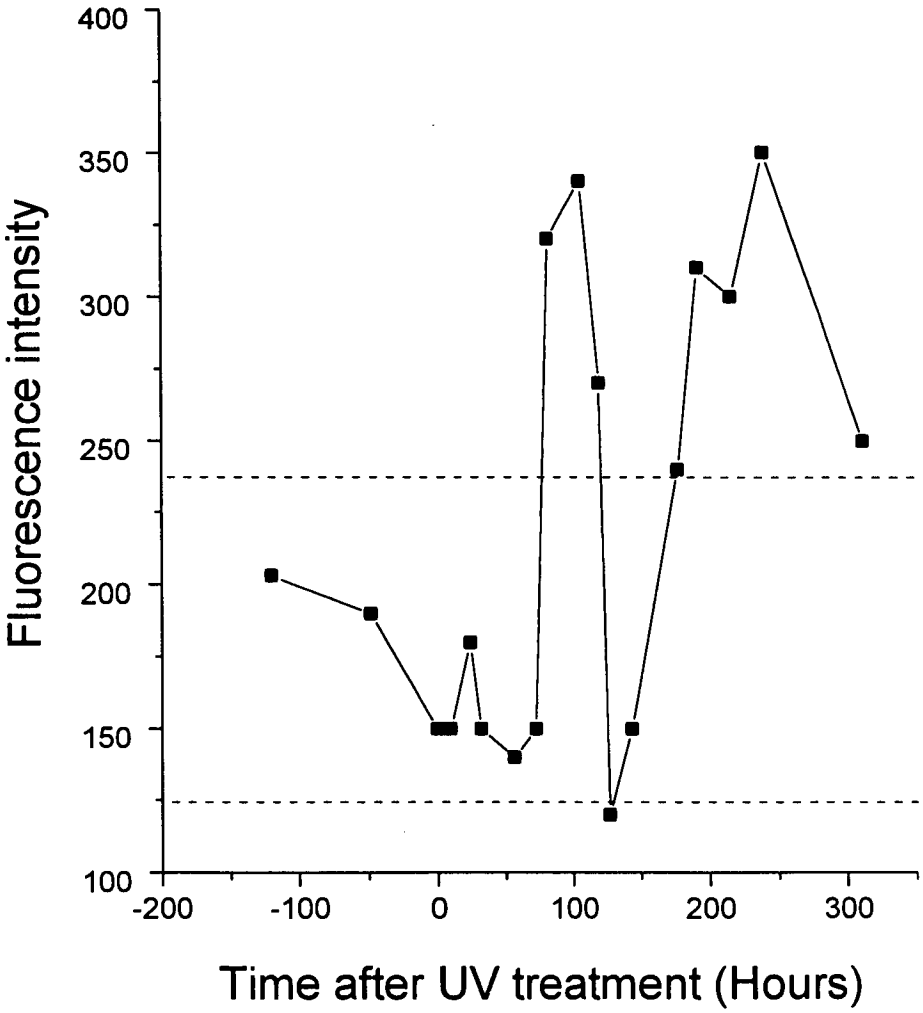

Fluorescence intensity of CTLA-4 ligand expression on migrating CD1w1$^+$ DC after exposure of the skin to 8.1 kJ/m$^2$ UVB. Dashed lines represent the normal range for this representative experiment.

## Figure 4.19 B7 expression after 20.1 kJ/m² UVB




Fluorescence intensity of CTLA-4 ligand expression on migrating CD1w1$^{+}$ DC after exposure of the skin to 20.1 kJ/m² UVB. Dashed lines represent the normal range for this representative experiment.

## Figure 4.20 B7 expression after 20.1 kJ/m$^2$ UVB




Fluorescence intensity of CTLA-4 ligand expression on migrating CD1w1$^+$ DC after exposure of the skin to 20.1 kJ/m$^2$ UVB. Dashed lines represent the normal range for this representative experiment.

## 4.4 DISCUSSION

The previous chapter used the EPICS V, flow cytometer to assess the migration of various cell types, including DC in pseudoafferent lymph. The use of the FACSCAN and ELITE flow cytometers allows a more accurate analysis to be performed, as these cytometers are capable of more precise gating of individual cell populations. This, combined with the multi-parameter capacity of FACSCAN and ELITE cytometers, allows a more detailed analysis of individual cell phenotypes. The two objectives of experiments reported in this chapter were to see if a population of migrating macrophages could be detected in pseudoafferent lymph after UV exposure and secondly, to see if the exposure of skin to UVB altered the expression of B7 molecules on migrating DC.

### *4.4.1 Macrophage Migration Induced by UV Exposure of the Skin*

The expression of MHC I by both AM and DC was expected, and therefore used mainly as a positive control. While the majority of AM expressed MHC II, the intensity of expression was highly variable but generally much lower than that of pseudoafferent DC. The high levels of MHC II expression on DC shown here is in agreement with previous reports of high levels of MHC II in sheep afferent lymph DC (Budjuso *et al.*, 1989).

The lack of CD1w1 expression by alveolar macrophages was unexpected as some ovine tissue macrophages (Dutia *et al.*, 1991) and macrophages generated from culture of ovine PBMC (Gorrell *et al.*, 1992) have been shown to express this marker. This suggests that the expression of this molecule by macrophages may be dependent on the tissue of origin and that perhaps the microenvironment of the lung is not conducive to its expression. This lack of expression by macrophages supports the use of the SBU-T6 monoclonal antibody to specifically identify migrating LC in this study.

A T cell marker found to be expressed by a sub-population of DC in pseudoafferent lymph is CD4, which has been shown to be expressed by LC in human skin (Wood *et al.*, 1983). However, the expression of CD4 by ovine DC is less well characterised but has been noted on 50% of dendritic "macrophages" in pseudoafferent lymph (Mackay *et al.*, 1988b). In contrast to this finding the expression of CD4 has since been shown not to occur on ovine macrophages from tissue, blood and culture experiments (Gorrell *et al.*, 1988a,

Gorrell *et al*, 1988b, Gorrell *et al.*, 1992), suggesting that Mackay *et al.*, (1988), were in fact observing CD4 expression on DC, possibly migrating LC.

CD8 expression by the DC population was restricted to a minor group of cells in the DC fraction. AM were not found to express this marker. While DC have been shown to express CD8 in the thymus and spleen of mice, this has not been previously demonstrated in lymph. While it is possible this antigen is expressed on DC, it is more likely this result represents contaminating CD8 positive lymphocytes attached to DC as they are examined. The lack of expression of the T19 marker of mature γδ T cells (Mackay *et al.*, 1989) by both DC and AM was not surprising. The lack of expression by migrating DC supports the previous findings of Gorrell (1995) and Hein and Mackay, 1991), that it is unlikely that sheep possess the equivalent of the mouse DETC.

This study showed that OM1, which was recently classified as equivalent to CD11c (Gupta *et al.*, 1995), reacted with a majority of cells lying within the DC gate. The reactivity of OM1 with afferent lymph DC was noted previously by Gupta *et al.*, (1995). Gupta's study led to the characterisation of OM1 as an antibody directed against ovine CD11c. Panfilis *et al.*, (1989) found the expression of CD11c on resting human LC, which agrees with the findings from DC from other species (Steinman *et al.*, 1991). The reason for the lack of expression of OM1 on LC reported by Pepin *et al.*, (1992) is unknown. It may be due to differences in the strain of sheep used or methods employed to detect CD11c expression. However, it confirms the results of both Pepin *et al.*, (1992) and by Gupta *et al.*, (1995) which show that, CD11c is expressed on the majority of alveolar macrophages. The percentage of DC expressing either CD1w1 or OM1 suggests that some overlap in the expression of these molecules occurs. Future studies using two colour immunofluorescence may reveal co-expression of these molecules on migrating ovine DC.

OM2 was shown to react with a smaller portion of the DC fraction whereas nearly all AM were found to be OM2 positive. The reactivity with AM is in agreement with Pepin *et al.*, (1992). However, OM3 was not observed to stain either alveolar macrophages or DC. This contrasts with Pepin's study were OM3 was observed to react with "nearly all" ovine AM. This lack of expression by sheep AM in the current study may reflect differences in the age and sheep strain used, which in Pepin's study were 3 month old Préalpes du Sud lambs .

One major aim of this part of the current study was to identify a marker which could be used to distinguish migrating macrophages from DC. This was

viewed as being of particular importance as after UV exposure macrophages are noted to enter the human epidermis (Cooper *et al.*, 1985, Cooper *et al.*, 1992), and are increased in the lymph node of mice (Muller *et al.*, 1994) after UV exposure. These cells are thought to play a down-regulatory role in the subsequent immune responses occurring in the skin (Baadsgaard *et al.*, 1988, Baadsgaard 1990, Kang *et al.*, 1994). This migration of macrophages appears to also occur in the ovine system, as very few $CD14^+$ cells were detected in ovine lymph under normal circumstances, but a dramatic increase in the percentage of $CD14^+$ large granular cells appeared after exposure of the skin to 20.1 $kJ/m^2$ UVB.

The migration of macrophages was observed to be maximal 24 hours after UVB exposure, with increased migration occurring for up to two weeks after irradiation. This early increase in the percentage of $CD14^+$ macrophages migrating from UV exposed skin is in accordance with the findings of Richters *et al.*, (1996), who observed macrophage migration from skin explants to be increased 24 and 48 hours after UV exposure. However, as the maximal migration of macrophages into the skin occurs 48-72 hours after exposure (Baadsgaard *et al.*, 1987), the initial efflux of macrophages from the skin may represent the migration of dermal macrophages. The increased macrophage migration at later time points may be composed of macrophages migrating from the epidermis. Vink *et al.*, (1995) demonstrated that cells with UV damage were found in the dermis, thus providing evidence that macrophages in the dermis might be directly affected by UV light exposure leading to their migration. However, the migrating macrophages appear to be only a minor population when compared with the proportion of LC in the pseudoafferent lymph. This agrees with evidence from the mouse model (Muller *et al.*, 1994) and human skin explant studies (Richters *et al.*, 1995), where the number of cells expressing characteristic macrophage markers is increased but is still a relatively minor population in comparison those expressing DC markers such as CD1 or MHC II

It might be argued that the expression of CD14 by alveolar cells may not necessarily be related to the expression of CD14 by macrophages in the lymph after UV exposure. However, UV induced epidermal macrophages have been proposed to originate from the spleen (Jun *et al.*, 1989) and the dermis (Meiuner *et al.*, 1995), while normally characterised by their expression of CD11b (Cooper *et al.*, 1993), they have been shown to co-express CD14 in both spleen (Buckley *et al.*, 1991) and skin (Cooper *et al.*, 1986, Sonthiemer *et*

*al.*, 1989), whereas LC do not express CD14 (Davis *et al.*, 1988). This strongly suggests that the $CD14^+$ cells detected in the ovine lymph were the same UV induced macrophages proposed by Cooper *et al.*, (1993), and were migrating from skin to the draining lymph node via the afferent lymphatics. This agrees with current theory that inflammatory macrophages migrate from inflammatory sites to the draining lymph node *in vivo* (Belligan *et al.*, 1996).

The phenotypic characteristics of the infiltrating macrophages also include the expression of CD11b (Cooper *et al.*, 1993), which has also been shown to be expressed by some LC in human skin (Panfilis *et al.*, 1989). However, Duraiswamy *et al.*, (1994) by combining the expression of MHC II, CD11b and Ly6c (monocyte/endothelial antigen) have been able to distinguish between macrophages and LC. While macrophages were found to express MHC II, CD11b and are $Ly6c^+$ LC were found to be weakly $CD11b^+$ and $Ly6c^-$. The dual expression of CD11b and CD14 by migrating ovine macrophages after UV light would perhaps be a more definitive study, than looking at the expression of a single marker. However, it was not possible during the duration of this investigation to obtain access to a mAb specific to CD11b, albeit a number of these are available (Naessens *et al.*, 1997). The expression of non-specific esterase is also used to characterise macrophages. But this is expressed by freshly isolated ovine LC (M Sandeman, personal communication) and so ruled out its use as a histochemical marker of macrophages to compliment the flow cytometric studies.

While previous studies have shown that the migration of cells with macrophage characteristics occurs after UV exposure of the skin (Cooper *et al.*, 1993, Muller *et al.*, 1994, Richters *et al.*, 1995), due to the limitations of these models, a time course over an extended period has not been able to be performed. The study presented in this chapter extends the previous studies in other species and shows that that the migration of macrophages occurs for an extended time following UV exposure of the skin. This event perhaps contributes to the alteration of the cutaneous immune response for extended periods, due to the infiltrating macrophages ability to both induce tolerance *in vivo* (Hammerberg *et al.*, 1994) and produce the cytokine IL-10 (Kang *et al.*, 1994). However, it should be noted that while the percentage of $CD14^+$ DC increased, LC ($CD1w1^+$ DC) were still the major migrating dendritic cell population.

### 4.4.2 *Expression of CTLA-4 Ligands by Migrating LC after UV exposure of the Skin*

The study presented in this section utilised the multi-parameter capabilities of the FACSCAN flow cytometer (Becton-Dickenson) to examine the expression of CTLA-4 ligands on migrating LC ($CD1w1^+$ DC). The CTLA-4 ligands include all members of the B7 family of co-stimulatory molecules. The B7 family of co-stimulators includes two molecules expressed on mature LC, B7-1 and B7-2 (Symington *et al.*, 1993), which are crucial for T cell stimulation (reviewed by Lenschow *et al.*, 1996). In this study exposure to 2.7 $kJ/m^2$ and 8.1 $kJ/m^2$ did not cause sustained decreases in B7 expression. Although, the occasional transient decrease was observed in some subjects such changes were not observed in repeat experiments. Similarly, some sustained increases were observed after exposure to 2.7 $kJ/m^2$ but not in other experiments. While three sheep clearly showed increases in B7 expression after exposure of the skin to 20.1 $kJ/m^2$, a clear decrease in B7 expression by migrating LC was observed in one sheep. While care was taken to ensure that cytometer settings and staining conditions were kept constant, these apparent inconsistencies may have been due to the outbred nature of the model. Nonetheless these studies suggest B7 expression appears not to be down-regulated for either the 2.7 $kJ/m^2$ or 8.1 $kJ/m^2$ doses and was up-regulated for the majority of sheep exposed to the 20.1 $kJ/m^2$ dose.

The maturation stage of DC influences the expression of B7 on DC, such that, in human skin CTLA-4 ligands are expressed only weakly or not at all by LC freshly isolated from the skin (Symington *et al.*, 1993, Weiss *et al.*, 1995). After fresh LC are cultured for 48 hours the expression of B7-1 and B7-2 is dramatically up-regulated (Weiss *et al.*, 1995). Culturing is proposed to represent the maturation of LC, to lymph node DC (Romani *et al.*, 1989, Teunissen *et al.*, 1990) and the increased expression of B7-1 and B7-2 parallels this theory. The current study examined LC freshly isolated from afferent lymph which appear to express an intermediate level of B7 molecule in comparison with cultured human LC (Weiss *et al.*, 1995). This intermediate expression may reflect an intermediate stage of maturation associated with ovine LC migrating from the skin or may reflect a species difference. Weiss *et al.*, (1995) also found that the up-regulation of B7-1 and B7-2 was inhibited when LC were irradiated *in vitro*, prior to culture. This lead Weiss *et al.*, (1995) to propose that the loss of expression of B7 molecules on UV irradiated LC may be responsible for the lack of stimulation and induction of anergy in Th1 cells.

A more recent study by Laihia and Jansen, (1997) has shown that *in vivo* exposure of human skin to erythemal levels of UV light resulted in increased levels of B7-1 and B7-2 expression on LC within the skin, which was maximum between 12-24 hours after exposure. The levels of expression returned to normal at 48 hours before being elevated again at 72 hours. This in agreement with the present study, which also showed some up-regulation especially after exposure to the 20.1 $kJ/m^2$ dose, although generally this occurred after the time points observed by Laihia and Jansen, (1997). The delay in the increased levels of B7 expression may represent the time taken for LC to migrate from the epidermis to the dermis via the draining lymphatics.

The expression of B7 molecules is up-regulated by cytokines such as IFN$\gamma$,IL-4 and the CD40/CD40L pathway (reviewed by Lenschow *et al.*, 1996). However, no evidence for the up-regulation of either cytokine has been observed within the skin after UV irradiation (Takashima and Bergstresser, 1996). The involvement of CD40 or its ligand in the cutaneous UV immune response at this time is unknown. Conversely IL-10 has been shown to specifically down-regulate the expression of B7-2 on the surface of peripheral blood DC, resulting in the inhibition of allogeneic T cell proliferation (Buelens *et al.*, 1995). However as the CTLA-4 immunoglobulin fusion protein detects both B7-1 and B7-2 specific changes in either co-stimulatory molecules cannot be detected in the current study. In contrast to the changes reported by Buelens *et al.*, (1995), IL-10 has also been shown to inhibit both B7-1 and B7-2 up-regulation on peritoneal macrophages (Willems *et al.*, 1994). The effects of IL-10 on migrating LC have not yet been studied, so whether this effect is restricted to B7-2 on this cell type is unknown. Sustained down-regulation was apparent in only one of the experimental subjects used in the present study. However, if the migrating LC had been cultured for extended periods, a lack of up-regulation may have been observed, which in turn may result in the reduction of T cell proliferation in response to antigens presented by LC.

While the expression or lack of expression of B7-1 or B7-2 on migrating LC may be important to the resulting immune response, equally important is the expression of the counter receptors CD28 or CTLA-4 on T cells. The interaction of B7 molecules with CD28 appears to costimulate T cells and prevent the induction of anergy and apoptosis. Conversely the interaction with CTLA-4 appears to cause down-regulation of the immune response either by competing with CD28 for its ligands or by the induction of apoptosis (reviewed by Lenschow *et al.*, 1996). While it was observed in a previous chapter that T

cell migration from UV exposed skin was altered, the lack of reagents specific for the CD28 or CTLA-4 receptors in the ovine system means that any alterations in the expression of these molecules on migrating T cells is unknown.

## *4.5 Summary*

This chapter describes a series of experiments designed to investigate the expression of a variety of surface molecules on migrating ovine psuedoafferent lymph DC after UV exposure of the skin. The study included an investigation of the expression of the B7 family of co-stimulators on migrating LC and the identification of migrating macrophages from UV exposed skin. The expression of CD14 by alveolar macrophages and not DC, was used to distinguish macrophages migrating in pseudoafferent lymph. A sub-population of large granular cells that were $CD14^+$ showed substantially increased migration after UV exposure of the skin. While the migration was maximal at 24 hours it was sustained for extended periods after exposure, thereby possibly identifying migrating macrophages which have been shown to infiltrate the epidermis after UV exposure in other species. The expression of B7 molecules on LC migrating from the skin was found to generally remain within or above normal levels after exposure to 2.7, 8.1 and 20.1 $kJ/m^2$ UVB. The timing and extent of these changes varied between experimental subjects and in one case a substantial sustained decrease in the expression of B7 was observed. These findings agree with and extend recent findings that B7 expression appears to increased on human LC residing in the skin after cutaneous UV exposure.

# CHAPTER 5: EFFECTS OF UVB ON THE FUNCTION OF MIGRATING ANTIGEN PRESENTING CELLS

## 5.1 INTRODUCTION

LC initially function as a sentinel in the epidermis; when stimulated to migrate, they carry antigen to the draining lymph node (DLN). In the DLN, LC function changes from antigen uptake to one of antigen presentation. They present antigen to T cells, thereby initiating an immune response (as reviewed by Ibrahim *et al.*, 1995). During this maturation into interdigitating cells, LC undergo dramatic phenotypic changes including up-regulation of MHC I, MHC II and adhesion molecules (Romani *et al.*, 1989, Teunissen *et al.*, 1990), as well as the appearance of the important co-stimulatory molecules B7-1 and B7-2 (Yokozeki *et al.*, 1996). LC become functionally unable to phagocytose (Sousa *et al.*, 1993) or process soluble antigens (Steinman *et al.*, 1991). These changes reflect the maturation of LC from a sentinel, to antigen presenting cell (Ibrahim *et al.*, 1995). It is this transformation which enables LCs to become "the most potent stimulators of naive T cells that have been identified" (Udey, 1997). LCs are critical for the initiation of anti-tumour responses in the cutaneous environment (Muller and Halliday, 1991). Furthermore, LCs have been shown to present tumour associated antigens in both the initiation and elicitation phases of the anti-tumour response (Grabbe *et al.*, 1991, Grabbe *et al.*, 1992).

Following UV exposure of the skin, the role of LC in tumour responses becomes crucial. Mutations which occur in the DNA of exposed cells, together with the altered microenvironment of the skin and the suppression of the immune system have been well documented. These factors undoubtedly contribute to the generation of tumours within the skin. Evidence presented in Chapter 3 demonstrated that following UV exposure, the number of LC ($CD1w1^+$ DC) migrating from exposed skin increased. This poses the question, whether these cells migrating from the skin function normally. That is, are the LC able to process and present tumour associated antigens that may arise at this time. This chapter describes experiments, which assessed the functional capacity of DC post UVB exposure, exploiting the unique properties of the sheep pseudoafferent lymphatic model.

## 5.2 MATERIALS AND METHODS

### *5.2.1 Experimental Plan*

#### 5.2.1.1 Assessment of Antigen Presenting Cell Function

Sheep were immunised twice over a 48 hour period with a 2 mg/ml suspension of alum precipitated ovalbumin (OVA) (see section 2.7.1). This immunisation protocol was repeated 4 weeks later and serum antibody levels were assessed by haemagglutination two weeks after this (see section 2.6). If it was found that titres were low sheep were re-immunised and titres re-assessed. Bilateral pre-femoral lymphatic cannulation was carried out on lymphadenectomised immune sheep (section 2.2). A post-operative recovery period of 5-7 days was allowed for stabilisation of lymphatic output. Sterile collections of pseudoafferent lymph then commenced (see section 2.3.2) as did collection of blood for use in a proliferation assay. This assay assessed the ability of DC to process and present OVA to responding cells contained in the PBMC fraction. Briefly, DC were enriched using discontinuous metrizamide gradient (14.5% + 15% FCS) centrifugation. Similarly, PBMC were enriched by centrifugation over a discontinuous histopaque gradient (density 1.077). The DC were pulsed with OVA then treated with mitomycin C, which inhibited DNA synthesis and thus cell division. The DC were then co-cultured with autologous PBMC for 5 days and pulsed with [$^3$H] thymidine over the last 16 hours, as a measure of proliferation. Control wells were used to assess the proliferation of DC alone and PBMC alone. This procedure was adapted from the method of Bujdoso et al., (1989) by Ragg et al., (1995) and is presented in greater detail in Section 2.7

Once a sufficient number of background points had been used to establish the normal range of stimulation indices, the sheep were treated with 8.1 kJ/m$^2$ UVB. The dose (8.1 kJ/m$^2$ UVB) was used on the basis of the previous finding that exposure of ovine skin to this dose caused consistent increases in LC migration with minimal increases in other cell populations and minimal erythemal changes. This minimised the overwhelming inflammatory effects that would be evident with higher doses. These experiments were repeated three times to ascertain if the observed trends were consistent.

#### 5.2.1.2 Assessment of PBMC function

Full body UVB exposure has also been shown to result in suppression of lymphocyte function (Morison *et al.*, 1979). Where possible, contralateral sheep flanks were used as controls in order to distinguish any systemic effects of UVB. However, due to shortage of successful bilateral cannulations, it was decided also to test lymphocyte function with mitogen stimulation, using phytohaemagglutin (PHA). PBMC remaining from the antigen presentation assays were cultured in triplicate for three days in dilutions of PHA. The cells were pulsed with [$^3$H] thymidine during the last 16 hours of culture and uptake of the radioactive label was used as a measure of proliferation.

### *5.2.2 Presentation of data*

#### 5.2.2.1 Antigen Presenting Assay

The ability of DC to present antigen to PBMC is presented as a stimulation index (SI), calculated at a ratio of 1 DC :16 PBMC. The method for calculation of the SI is detailed below.

$$SI = \frac{\text{Mean CPM OVA pulsed DC : PBMC co-cultures}}{\text{Mean CPM CONTROL DC : PBMC co-cultures}}$$

The mean of at least four pre-treatment points +/- two standard deviations from this mean were used as the normal range ( >90% confidence limits Zar, 1984) SI. The limits of this normal range were shown as dotted lines (see Fig. 5.2). SI outside these lines are considered to be significantly different. The post treatment samples, are shown as the mean SI +/- the error accumulated by the division of CPM of quadruplicate samples of PBMC cultured with antigen pulsed DC by mean CPM values of quadruplicate samples of PBMC cultured with unpulsed DC. The data presented is from a representative experiment, illustrating the trends observed. SI generally represented a 5-8 fold greater stimulation of T cells by the OVA pulsed DC than that of control DC.

#### 5.2.2.2 PHA cultures

Data obtained from triplicate cultures as CPM were averaged for each concentration of PHA. The above statistical method was used to calculate the normal range of CPM expected for a given PHA concentration prior to treatment with UVB. Similarly, the data is from another single representative experiment, illustrating the trends observed.

## 5.3 RESULTS

### *5.3.1 Effects of UVB Exposure of Flank Skin on DC Function*

The exposure of ovine flank skin to UVB was observed to result in the changes previously outlined in Chapter 3. These include transient but substantial increases in lymph flow during the first 24-48 hours post exposure, increases in the number of migrating DC and the presence of hard white clots within the cannula. A reduced number of DC were also found to survive the mitomycin C step in the antigen presentation assay after UVB irradiation. Therefore, occasionally insufficient DC were obtained to allow the assay to performed for each lymph collection.

### *5.3.2 Exposure of flank skin to 8.1 kJ/m²*

#### 5.3.2.1 Assessment of DC Function

The exposure of the flank skin to a dose of 8.1 kJ/m² UVB has previously been shown to result in the increased numbers of DC migrating from the skin. These experiments assess the functional ability of UVB induced migrating DC to process and present OVA to T cells within the PBMC fraction. From Fig. 5.1 the optimal ratio of DC to responder cells was 1:16.

Monitoring the ability of DC to induce responder cell proliferation at this ratio revealed that the functional capacity of DC was reduced (Fig. 5.2). As detailed in this representative experiment, DC function was impaired within 48 hours of UVB irradiation. While the mean pre-UV exposure SI was 4.98, the loss of antigen presenting cell activity between 60-168 hours after exposure led to a substantially decreased SI (range 1.65 - 0.98). Following this period of time, the SI returned to a level within the normal range. It remained within the normal range until the final two collection points at 336 and 480 hours, which were significantly below normal.

#### 5.3.2.3 Assessment of Responder Cell Function

The optimal concentration of PHA required to stimulate proliferation was found to be 12.5 μg/ml (Fig. 5.3) This concentration was therefore used to asses the ability of PBMC to proliferate after the flank skin was exposed to 8.1 kJ/m² UVB. The proliferation of the responder cells was decreased between 24 and 72 hours after UVB exposure of the flank skin (Fig. 5.4). The reduction in

## Figure 5.1 Functional Ability of Antigen Pulsed DC vs non Pulsed DC to induce proliferation of PBMC

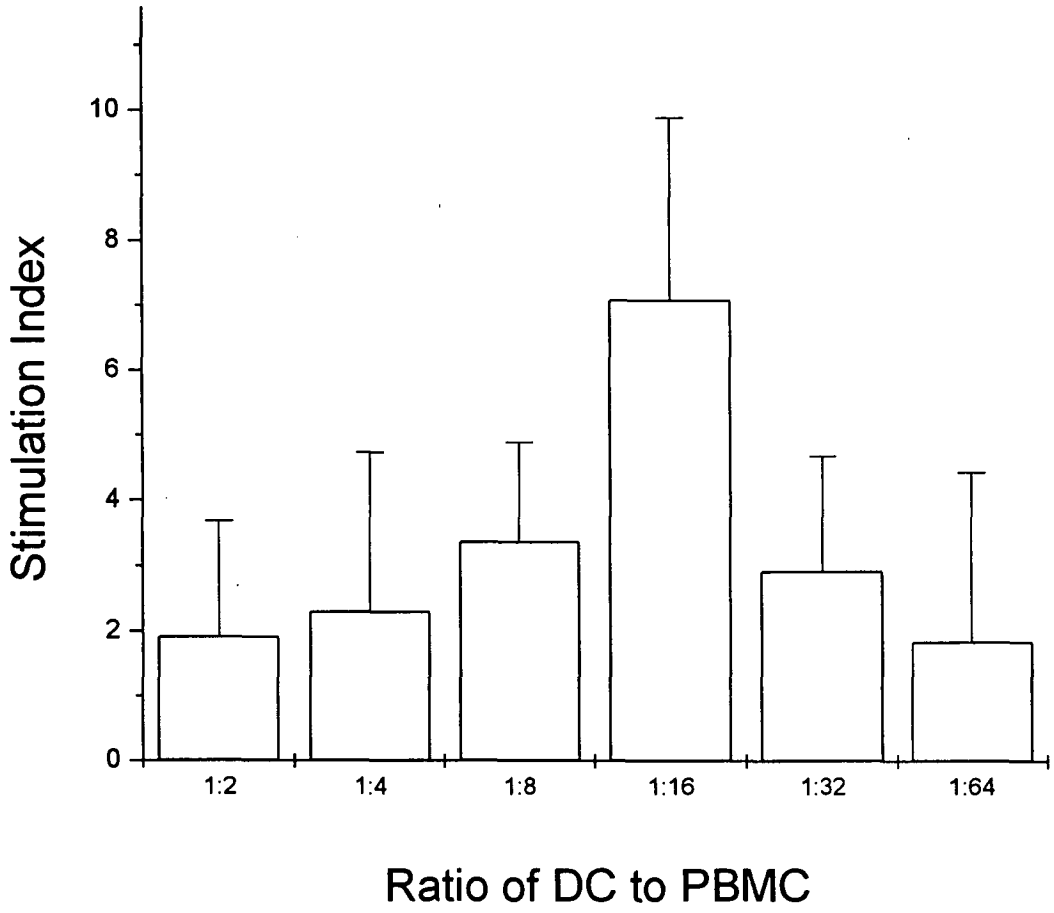
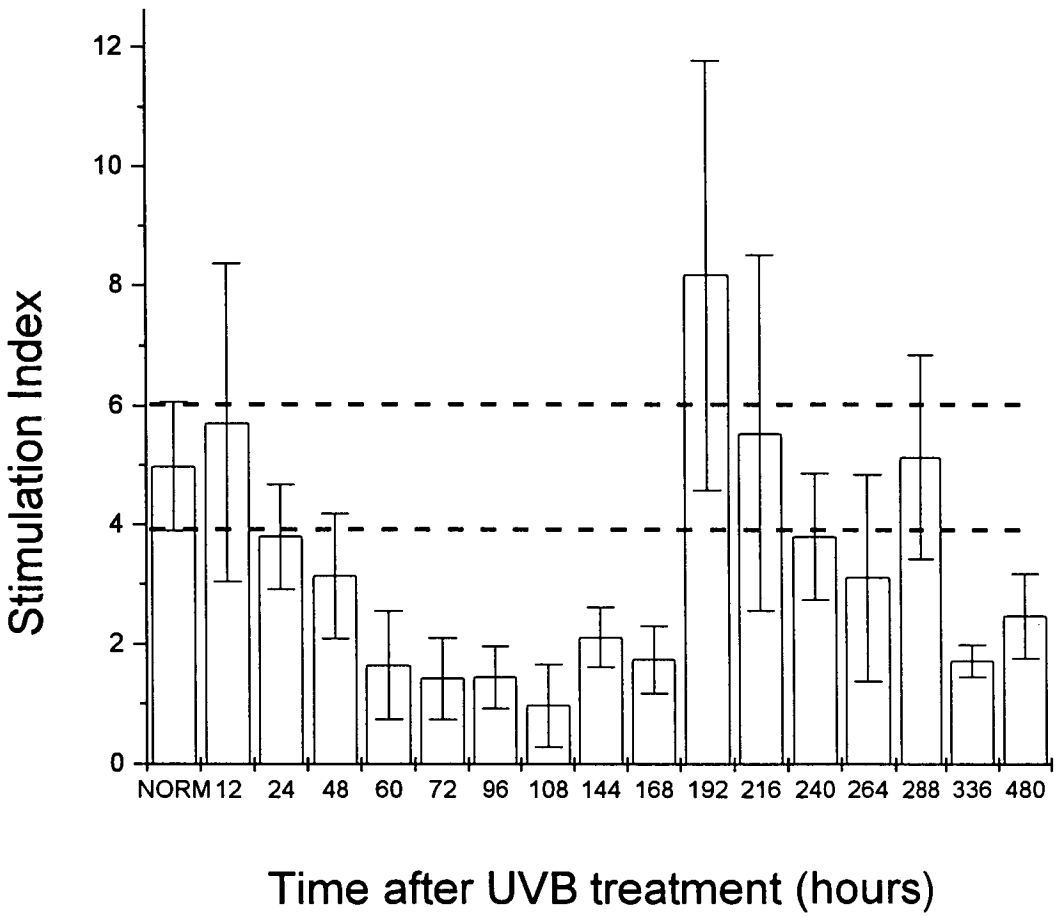


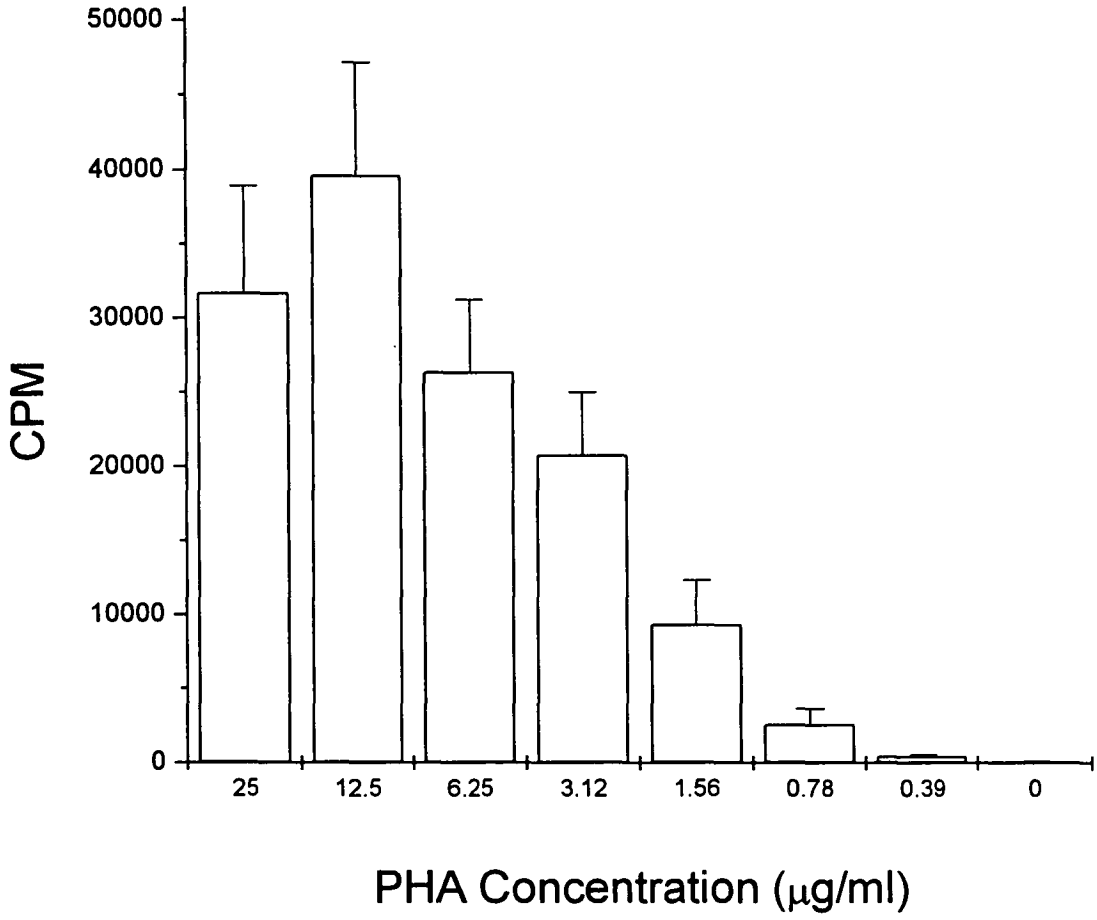

Figure shows the stimulation indicies after culturing$10^5$ PBMC with doubling dilutions of DC for 5 days. Stimulation indicies are calculated by dividing PBMC proliferation incubated with OVA pulsed DC by PBMC proliferation after incubation with non-pulsed DC. Proliferation was measured by thymidine uptake during the last 16 hours of culture.

**Figure 5.2 Functional Ability of Migrating Antigen Pulsed DC to Stimulate the Proliferation of PBMC after Exposure of Flank Skin to 8.1 kJ/m² UVB**



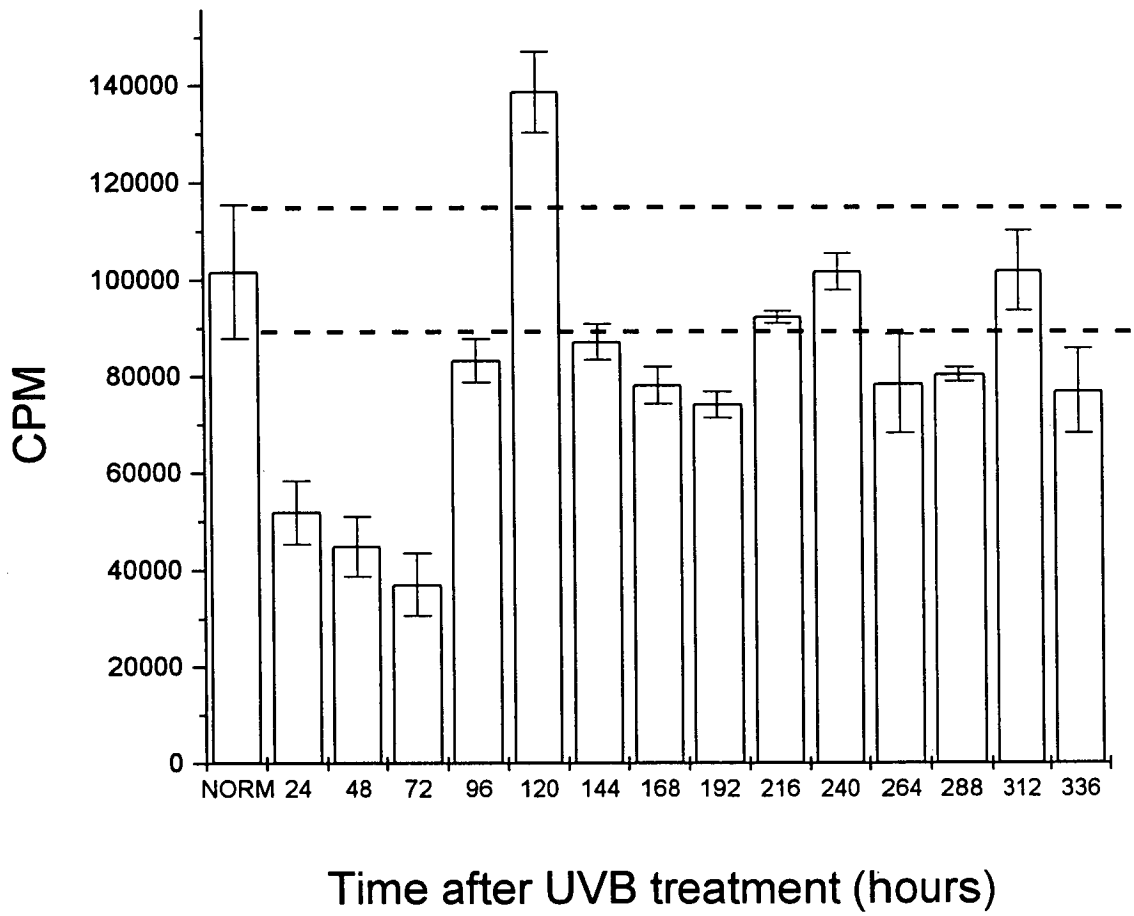

Stimulation index (Y axis) represents the ability of pulsed DC divided non-pulsed DC to stimulate PBMC proliferation. Norm represents the the mean stimulation index (+/- 2 standard deviations) of four pre-treatment points, the normal range of which is indicated by dotted lines Error bars for other time points represent the cumulative error accrued from the division of quadruplicate samples of pulsed/unplused DC. Maximal Control CPM was 1160 for this experiment.

## Figure 5.3 PBMC PHA Induced Proliferation




Proliferation of $10^5$ PBMC after 3 days culture in various concentrations of phytohaemagglutin (PHA). Proliferation was measured by thymidine uptake over the last 16 hours of culture

## Figure 5.4 PHA Induced Proliferation of PBMC after Exposure of Flank Skin to 8.1kJ/m² UVB



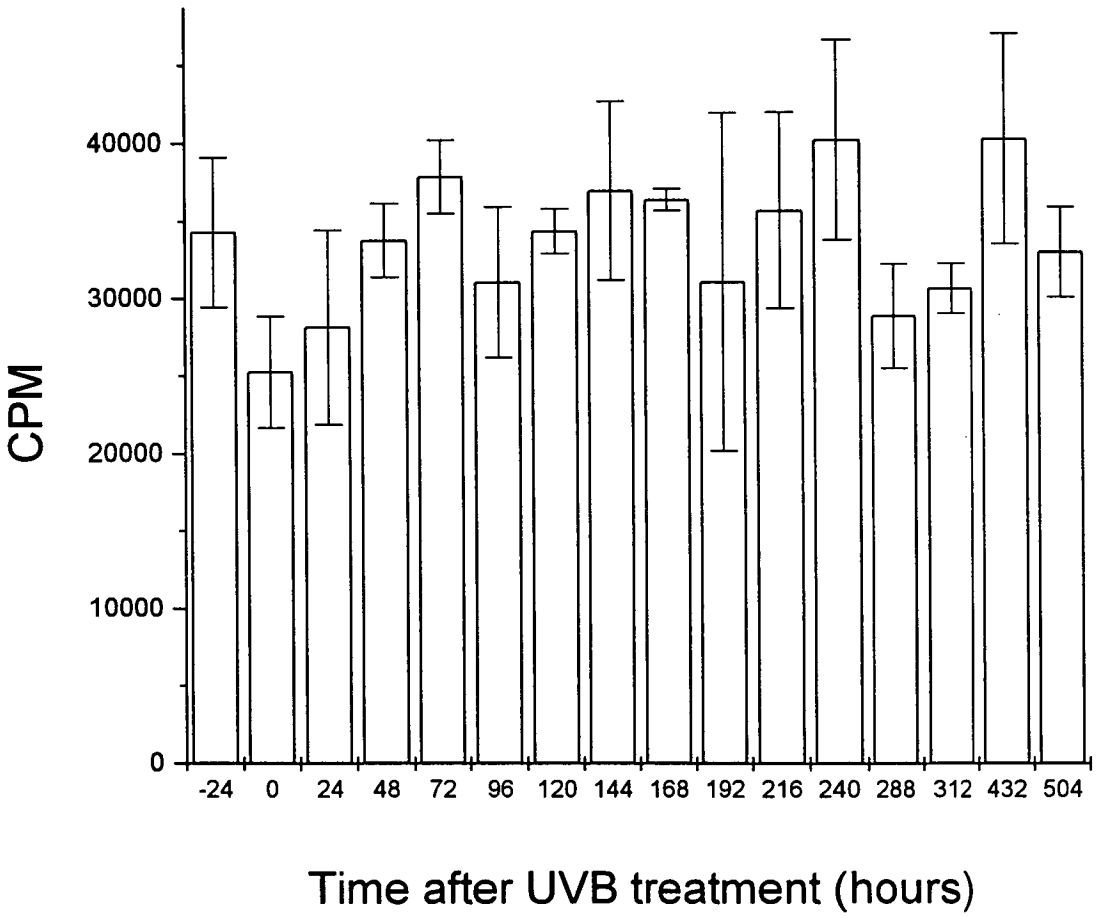

PBMC proliferation induced by 72 hours culture in 12.5μg/ml PHA. Normal sample (Norm )represents the mean CPM +/- 2 standard deviations of at least four pre-treatment points used to set the normal range (dotted lines). Error bars for other time points represent the calculated standard deviation. Maximal Control CPM was 2132 for this experiment.

proliferation was maximal at 72 hours and represented a 63% reduction in CPM in comparison to mean pre-treatment levels. Two repeat experiments were performed and showed a similar loss of mitogen induced proliferation between 24 and 72 hours post irradiation. One experimental subject was sham UV irradiated (Fig. 5.5) in parallel to the experiment shown in Fig 5.4 and no loss of PHA induced responder cell proliferation was observed in this control experiment.

#### 5.3.2.2 General

Two repeat experiments were performed examining the APC function of migrating DC after exposure of ovine flank skin to 8.1 kJ/m$^2$. These subsequent experiments showed a similar loss of functional ability to that shown in Fig 5.2. However the timing of these effects varied between individual experimental subjects. The two repeat experiments showed the initial loss of function occurred at 24 hours after UV exposure and the return to normal function also varied and occurred at 144 and 192 hours, respectively after UV exposure. The shorter duration of the two repeat experiments did not allow the assessment of the later loss of function observed in Fig 5.2. A systemic loss of DC function was observed between 24-72 hours after UV exposure, for one experiment using a bilaterally cannulated sheep. However this sheep was exposed to a slightly lower dose due to the failure of 2 UV lamps. Further bilateral cannulations of sheep which lasted the duration of the experiment were unsuccessful.

## Figure 5.5 PHA Induced Proliferation of PBMC after Sham Exposure of Flank Skin to UVB




Proliferation of $10^5$ PBMC induced by 72 hours culture in 12.5μg PHA. Error bars represent the calculated standard deviation.

## 5.4 DISCUSSION

The ovine model allows direct investigation of the functional ability of DC that under normal circumstances, would reach the DLN after UV exposure. It has been demonstrated that the ability of antigen pulsed DC to stimulate the proliferation of PBMC responder cells was seriously inhibited after exposure of skin to 8.1 kJ/m$^2$. Previous investigations were limited to the study of cells from within UV exposed skin or alternatively the cells found in the DLN in response to UV exposure and a fluorescent contact sensitiser, FITC. The former involves traumatic removal of tissue from the animal and includes cells which may be incapable of reaching the lymph node to present antigen, while the latter has large numbers of contaminating DC, not fluoresceinated and therefore potentially not exposed to UV light. The ovine model allows the investigation of the function of *ex vivo* migrating DC after UV exposure of intact skin. It does not require the application of an external agent (which may add confounding factors to the experiment). These migrating cells are likely to stimulate an immunologic or tolerogenic response to antigens arising in the skin after UVB exposure.

The experiments described in this chapter have examined the ability of DC to cause proliferation of PBMC responder cells in an *in vitro* assay system. A similar assay system was used by Stingl *et al.*, (1980) using *in vitro* irradiation of murine epidermal cells (EC). These cells were pulsed with antigen and then washed. Their ability to stimulate purified T cells was shown to weaken in response to UVB in a dose dependant manner. Similarly the ovine system has shown that the DC migrating from UV exposed skin have a reduced ability to stimulate T Cells. Further, the return of normal function was not observed until 144-192 hours post exposure. Accordingly, the capacity of the skin immune system to deal with infection or tumour cells arising in the skin during this period would be seriously compromised. While this series of experiments concentrated on the function of antigen presenting cells migrating from the skin, previous investigations have concentrated on the function of LC within the skin. For example, it has been shown that LC from normal murine skin were able to stimulate T cells (Green *et al.*, 1980), while Toews *et al.*, (1980) observed that mice exposed to low doses of UV light failed to induce CHS, which was associated with a loss of LC and the generation of suppressor cells. Later experiments have also demonstrated that epidermal cells isolated from UV exposed skin had a reduced capacity to stimulate both allogeneic or

antigen specific T cells *in vitro* (Lynch *et al.*, 1983, Cooper *et al.*, 1985 and El-Ghorr *et al.*, 1994) and this was at least partially due to alteration in the antigen presenting activity of LC. Cruz *et al.*, (1990) found that purified MHC $II^+$ cells which were irradiated and conjugated to hapten *in vitro*, then subsequently injected into mice resulted in the failure of the mice to develop CHS to the specific hapten. This was due to induced tolerance, as a subsequent application of the same hapten failed to induce CHS.

LC purified from the skin and then UV irradiated *in vitro* retain their ability to stimulate Th2 cells but are unable to stimulate Th1 clones (Simon *et al.*, 1990). Due to UV irradiated LC inducing anergy in the Th1 clones (Simon *et al.*, 1991). The failure of migrating ovine DC in this study to cause proliferation of PBMC after UV exposure of the skin may reflect the failure of the OVA specific Th1 cells to respond to UV altered DC. It should be noted that this antigen has been shown to preferentially induce Th1 responses at high and intermediate doses (reviewed by Constant and Bottomly, 1997). Saijo *et al.*, (1997) found that that lymph node DC from UV irradiated mice induced less T cell proliferation than non-irradiated controls, a result supported by the current study. Further, the cytokine profile of the cells in Sajio's experiments suggest that this was not limited specifically to Th1 cells, but also included Th2 cells. Sajio's *et al.*, (1997) study also illustrates the differences that may be observed between *in vivo* and *in vitro* experiments. Thus the experiments using Th clones such as those performed by Simon *et al.*, (1990) may be an oversimplification of what occurs *in vivo*. While the antigen presentation assay used in this chapter took place under *in vitro* culture conditions, the dendritic cells were irradiated *in vivo* and the responder cells did not solely comprise specific clones, thus being more representative of the heterogenous population of T cells *in vivo*.

Failure to take up antigen due to physical alteration of the LC is another possible reason for this loss of function, as UV light has been previously shown to physically alter LC. DC migrating after UV exposure used in the functional assay, appeared to have normal morphological characteristics under the light microscope. Alterations in LC morphology in the skin have been observed in previous studies (Aberer *et al.*, 1981, Obata and Tagami, 1985), and there is evidence of LC death (Mommas *et al.*, 1993). In addition, modification of the LC cytoskeleton by vinblastine, which alters microtubules, causes similar morphological changes to those induced by UVB exposure and also reduces CHS (Bacci *et al.*, 1996). This suggests that physical damage of LC may play a

role in this loss of function (Bacci *et al.*, 1996), as the characteristic ruffles and projections on the LC membrane are crucial for antigen uptake (Steinman and Swanson, 1995). As the DC used in the assay system are capable of migration from the skin, they would appear to be relatively intact as the integrity of the cytoskeleton is crucial for cell shape, motility and migration (Ingber *et al.*, 1994). Physical damage of the LC may contribute to the apparent loss of function of DC migrating from skin by causing the premature death of these cells during 5 days culture. While DC were initially viable, it has been previously reported by Pretell *et al.*, (1984) that the reduced viability of *in vitro* UV exposed adherent cells, is not apparent until 48 hours after culture. The impairment of APC function may be mediated in part by DNA damage (Vink *et al.*, 1996) and UVB exposure is known to lead to the induction of delayed apoptosis (Godar *et al.*, 1995). This is unlikely to account for the long term alterations in migrating DC function (6-8 days), as this effect on DC viability would appear to be limited to within the first two days after UVB exposure (Godar *et al.*, 1996)

One unexpected result found in the current study was the reduction of mitogen induced proliferation of PBMC for 72 hours after UV exposure of the flank skin. This systemic loss of proliferation was confirmed in one experiment which found reduced stimulation of PBMC in response to antigen pulsed DC migrating from the flank contra-lateral to the exposed flank. Likewise, a reduction in mitogen induced proliferation of PBMC has been observed in humans after whole body irradiation (Morison *et al.*, 1979). The current study indicates that irradiation of a small area of the body (200 $cm^2$) is sufficient to alter the function of circulating cells. This suggests the responder cell population in the antigen presentation assay may have been directly affected by UV irradiation. The ability of APC to stimulate T cell proliferation was reduced for up to 192 hour post treatment, while the reduction in the ability of PBMC to proliferate in response to mitogen lasted only 72 hours post exposure. Thus it appears these are two separate affects of UV exposure. Systemic effects were noted in one experiment on the contra-lateral side which confirmed the systemic effects of UV exposure using the mitogen and supported the finding that the systemic loss of function lasted for between 24-72 hours being identical to that observed in the assay utilising mitogen induced proliferation. This similarity in the observed time courses supports the systemic loss of function being due to the loss of responder cells ability to proliferate. However, a systemic loss of APC activity has been reported after UV irradiation possibly mediated by *cis*-UCA (Noonan *et al.*, 1988) or IL-10 (Ullrich *et al.*, 1994) and it

cannot be ruled out that this also occurred. Another effect of UVB exposure that may have a bearing on the experiments conducted is the phenotype of T cells in the blood, with a reduction in the numbers of $CD4^+$ circulating T cells the most commonly observed effect (reviewed Hersey *et al.*, 1995) although this occurred after whole body exposure to sunlight. It is possible that the loss of CD4 cells from the responder cell population would account for a portion of the loss of function observed. One way to manage this effect in the future, would be to isolate and store responder cells prior to commencement of the experiment, which could then be used after irradiation. This would eliminate any effects that UV exposure has on the responder cell population. There are two distinct possibilities for the systemic loss of function of mononuclear cells, either the direct irradiation of cells passing through the circulatory system in skin is occurring at the time of irradiation or alternatively a cytokine or soluble factor may being produced which systemically alters PBMC function. The latter hypothesis is examined in chapter 6. The failure to detect thymine dimers in PBMC from mice which had their dorsal surface UVB irradiated would seem to reduce the possibility of the former occurring (Sontag *et al.*, 1995).

## 5.5 SUMMARY

The ability of DC induced to migrate from UV exposed ovine skin to take up and present antigen to autologous T cells in culture was examined. After exposure of the skin to 8.1 $kJ/m^2$ (approximately 1 MED UVB) the ability of antigen pulsed DC to stimulate immune responder cells was reduced. This effect was found to last as long as 192 hours post irradiation. While this may have been initially due to the reduced ability of PBMC to proliferate, it is apparent that LC migrating from UV exposed skin are unable to fulfil their role as sentinels of the skin and their ability to induce an effective immune response is seriously impaired. The lowering of immunosurveillance in UV exposed skin and the systemic nature of the observed UV effects may thus facilitate the escape of neoplastic cells that otherwise would be eliminated.

# CHAPTER 6: ALTERATION OF CYTOKINES AND SOLUBLE FACTORS AFTER UV EXPOSURE OF OVINE SKIN

## 6.1 INTRODUCTION

Cells of the skin immune system produce many soluble factors that are required for the growth, activation and differentiation of immunocompetent cells. Keratinocytes are the major producers of many cytokines in the cutaneous micro-environment (Matsue *et al.*, 1992). LC are also a source of cytokine secretion and are the major, if not only source, of the T cell stimulating cytokines IL-1β (Matsue *et al.*, 1992, Enk *et al.*, 1993a) and IL-12 (Kang *et al.*, 1996) in normal epidermis. However, levels of cytokines in normal skin are generally below detectable levels and it requires an exogenous stimulus, such as that provided by UV exposure, to significantly induce the secretion of a vast array of cytokines (Reviewed by Luger *et al.*, 1997). Such changes in the skin's microenvironment undoubtedly affect the LC resident in the skin and may in turn, alter those which subsequently migrate to the DLN. This, in turn would, alter the interaction between migrating LC and T cells, perhaps changing the range of cytokines produced by the T cells. Accordingly, it was an important part of this study to examine alterations in cytokine and soluble factor production in both lymph draining the skin and in DC:PBMC co-cultures, to determine if there were any changes which might result in altered immunity after UV exposure.

The aberrant antigen presenting function of DC migrating from UV exposed skin discussed in chapter 5 may be due to the lack of a co-stimulatory cytokine such as IL-1β. This cytokine is regarded as the typical T cell stimulatory cytokine and has been shown to be essential for the growth of some T cell clones (Weaver and Unanue, 1990). Experiments have since determined that LC are a source of IL-1β in the epidermis (Matsue *et al.*, 1992, Enk and Katz, 1992). Further, LC express the IL-1 converting enzyme (ICE), which is essential for IL-1β secretion (Ariizumi, *et al.*, 1995). The secretion of IL-1β by LC has been demonstrated to be essential for the initiation of primary immune responses occurring in the skin (Enk *et al.*, 1993a). Any reduction in secretion of IL-1β by DC migrating from UV exposed skin may reduce antigen

specific T cell proliferation. Thus an investigation of the quantities of IL-1β in DC:PBMC co-cultures as part of these studies was deemed to be essential.

The loss of antigen presenting function by LC may also be due to presence of inhibitory soluble factors, such as the cytokine IL-10 or the secretion of prostaglandins by cells affected by UV exposure. IL-10 has been shown to be a cytokine crucial to both UV induced immunosuppression (Ferguson *et al.*, 1994, Navarro *et al.*, 1994, Rivas and Ullrich, 1994) and apparent reduction in LC function (Enk *et al.*, 1993b, Peguet-Navarro *et al.*, 1994). As this cytokine is produced both by cells in the skin and systemically after UV irradiation (Rivas and Ullrich, 1992), IL-10 was assayed in DC:PBMC cell co-cultures and lymph draining UV irradiated skin. Prostaglandins such as $PGE_2$ are up-regulated in the skin after UV light exposure (Imokawa and Tejima, 1989) and appear able to inhibit cutaneous immune responses (Chung *et al.*, 1986, Jun *et al.*, 1988). As LC are one source of $PGE_2$ synthesis (Teunissen *et al.*, 1991, Rosenbach *et al.*, 1990), so the amount of $PGE_2$ was assayed in DC:PBMC co-culture supernatants after UV exposure to determine if the level of production was altered.

Another cytokine known to influence LC function is GM-CSF, which is secreted by resting keratinocytes in the skin (Kupper *et al.*, 1986) and most T cell subtypes (reviewed by Mosmann and Sad, 1996). It is an important regulatory factor required for differentiation of LC into potent immunoregulatory cells (Heufler *et al.*, 1988). GM-CSF has also been shown to stimulate LC migration *in vitro* (Rupec *et al.*, 1996). Further, increased GM-CSF production has been noted in the skin after UV irradiation (Schwarz and Luger, 1989). The investigation of the presence of this cytokine in both lymph and DC:PBMC culture supernatants was therefore undertaken as part of this study.

The interaction between a DC and a T cell may induce apoptosis, anergy or activation of T cells capable of responding to that antigen. Further, the activation of T cells may cause cells to become further differentiated into the Th1 or Th2 subtypes. These subtypes lead to the production of different cytokine profiles, which in the case of Th1 cells are IFNγ, TNFβ and IL-2 , while Th2 cells secrete IL-4, IL-5 and IL-10 (reviewed by Mosmann and Sad, 1996). Evidence suggests that the ability of UV exposed LC to induce a Th1 response is reduced (Simon *et al.*, 1990) and may preferentially induce anergy in these cells (Simon *et al.*, 1991). Conversely the ability to stimulate a Th2 response appears unaltered (Simon *et al.*, 1990). These changes may lead to alterations in the balance of Th1 and Th2 cytokines in DC:PBMC co-cultures. This study

therefore investigated the levels of IFNγ and IL-5, cytokines which are produced by Th1 and Th2 cells, respectively.

## 6.2 METHODS AND MATERIALS

### *6.2.1 Experimental Plan*

Sterile lymph collections were made as described previously (Section 2.3.2) and following centrifugation to collect the migrating cells, 10 ml of lymph supernatant was collected and stored at -80°C until required. The DC:PBMC co-cultures were set up in parallel to the functional assays discussed in chapter 5. Briefly, afferent lymph DC migrating from normal and UV exposed skin were collected and enriched by centrifugation over metrizamide. The DC were pulsed with antigen (OVA) and treated with mitomycin C to inhibit proliferation. Autologous PBMC was collected by venipuncture and enriched by centrifugation over Ficoll. After this $10^5$ DC were co-cultured with $10^6$ PBMC for 72 hours at 37°C/5% $CO_2$ (See Section 2.7). Following this, culture medium was collected from the cultures, placed in 1.5 ml tubes (Eppendorf) and microfuged at 12,000 rpm in to remove cells and debris, after which the supernatant was removed and placed in a second tube and stored at -80°C until analysed. All procedures for the isolation of lymph and cell culture supernatant were performed aseptic techniques.

### *6.2.2 Cytokine Assays*

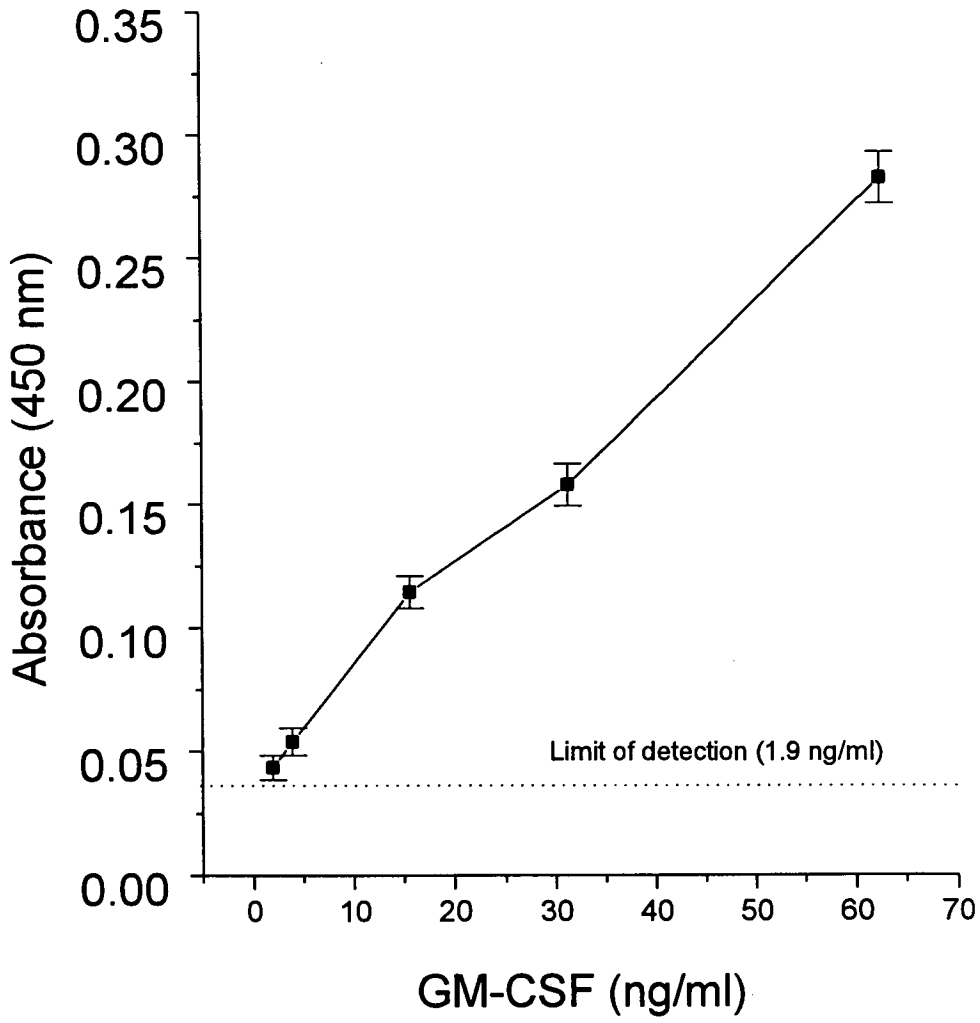
The assays and procedures for the detection and quantification of the cytokines GM-CSF, IL-1β, IL-5, IL-10, IFNγ and the soluble factor $PGE_2$ are presented in detail in Chapter 2, sections 2.9 - 2.11. It should be noted that the three experiments which are presented examining cytokine levels in DC:PBMC co-culture supernatants, all used the same three experimental subjects. The normal range of cytokine secretion was calculated using the previously described method (Section 2.8).

## 6.3 RESULTS

### *6.3.1 GM-CSF in lymph and Culture Supernatants*

The levels of GM-CSF in both lymph and cultures of afferent lymph DC were assayed by ELISA and quantified against the GM-CSF standard provided (Fig. 6.1). The assay proved not to be as sensitive as previously reported (Entrician *et al.*, 1996) and was only able to detect 1900 pg/ml of the

## Figure 6.1 GM-CSF Standard Curve



recombinant GM-CSF standard (Fig. 6.1). The ELISA was repeated and, despite increasing incubation times and the addition of further wash steps, no improvement in sensitivity was observed. No detectable levels of GM-CSF were found in any samples of either lymph or culture supernatants examined.

### *6.3.2 IL-1β Detection*

The levels of IL-1β in DC:PBMC co-cultures and supernatants from the macrophage inhibition assays were detected using a sandwich ELISA and compared to IL-1β standards (Fig. 6.2). The lower limit of IL-1β detection was 0.156 ng/ml (Fig. 6.2).

#### 6.3.2.1 IL-1β Detection in Lymph and DC-PBMC Co-Cultures

IL-1β was detected in all supernatants from afferent lymph DC and PBMC (Table 6.1). The results presented are the mean (and standard deviations) from three separate experiments. IL-1β concentrations for pre-treatment samples ranged between 0.3 ng/ml and 0.81 ng/ml, while the range of all post treatment samples was between 0.24 ng/ml and 0.99 ng/ml. There appeared to be little difference in the quantity of IL-1β present in all of the samples taken before and after exposure of ovine flank skin to 8.1 kJ/m$^2$ UVB (Table 6.1).

### *6.3.3 IL-10-like activity in Lymph after 2.7 kJ/m$^2$ UVB*

Lymph samples from two experimental subjects were assayed for IL-10-like activity before and after exposure of the flank skin to 2.7 kJ/m$^2$ UVB. Fig. 6.3 shows some variations in pre-treatment lymph levels of IL-10-like activity in samples from the two different experiments. 24 hours after UV exposure lymph sample from one sheep had decreased IL-10-like activity in comparison to pre-treatment levels (Fig. 6.3a). This experiment also showed sustained IL-10-like activity between 60 and 216 hours, peaking at 108 hours post-UV exposure, broken only by a transient return to pre-treatment levels at 132 hours. In the other experiment transient increases in IL-10-like activity were observed at 12, 48 and 192 hours post-UV exposure. Increased IL-10 like activity was observed at 48 hours and a further sustained increase between 120-144 hours post UV exposure (Fig. 6.3b). In both experiments IL-10-like activity was generally observed to increase after UV exposure of the skin to 2.7 kJ/m$^2$.

## Figure 6.2 IL-1β Standard Curve

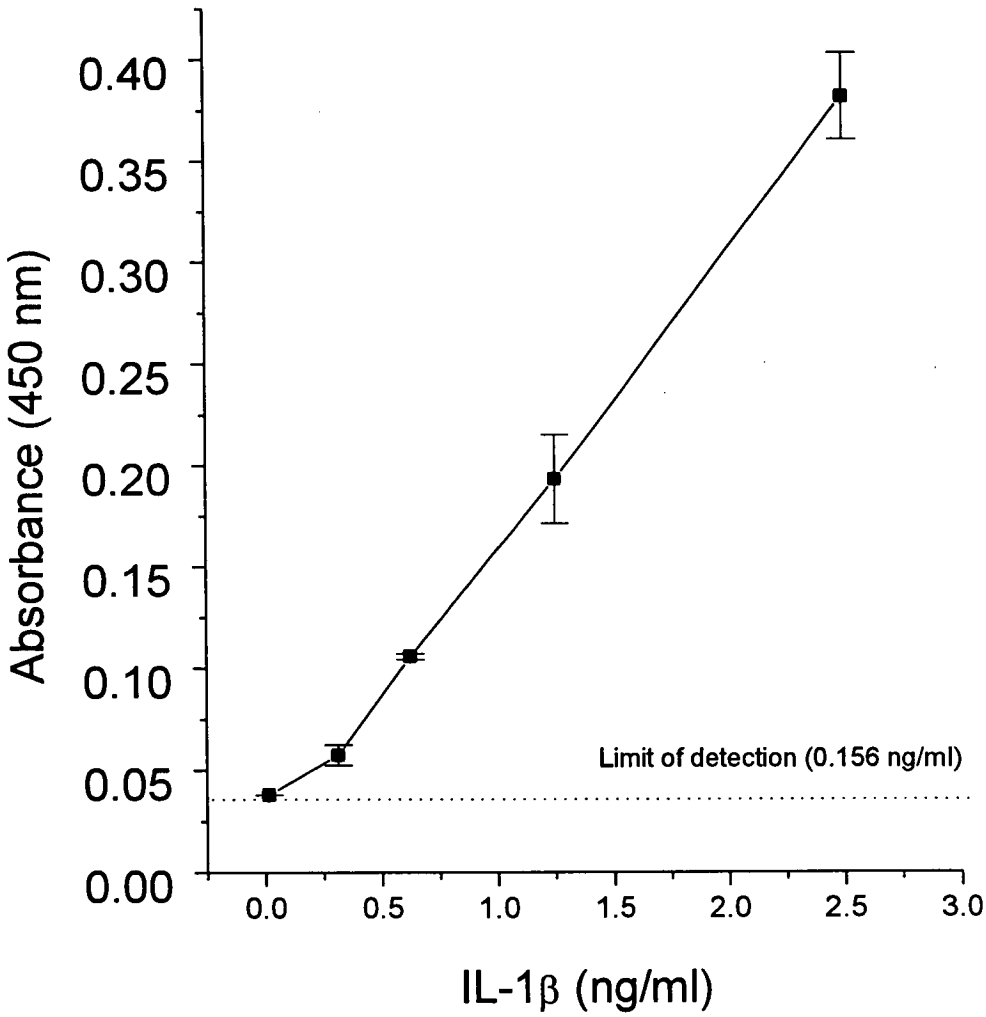
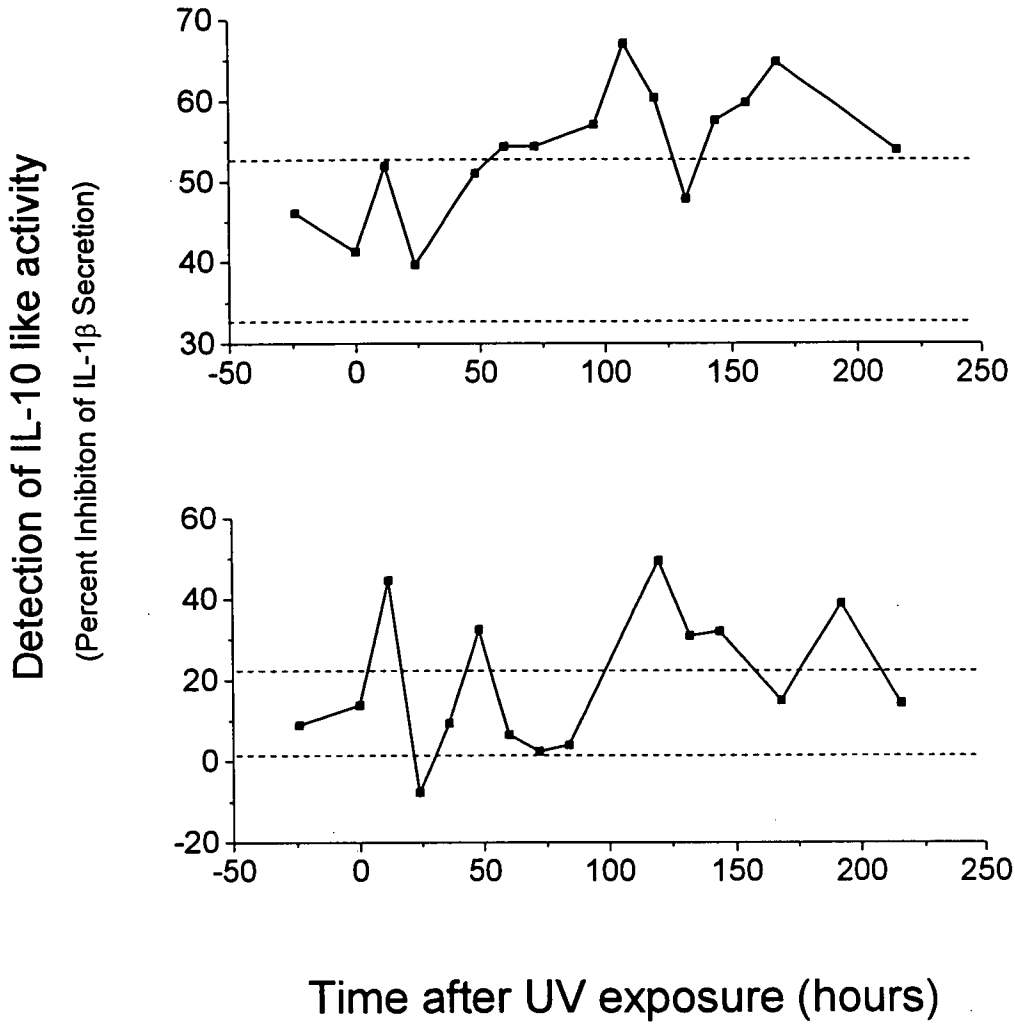


**Table 6.1 IL-1β in Supernatants from Afferent Lymph DC:PBMC Co-Cultures after Exposure to 8.1kJ/m$^2$ UVB**

| Time After UV Exposure (Hours) | IL-1β Levels (S.D) [ng/ml] |
|---|---|
| Pre-treatment | 0.52 (0.17) |
| 12 | 0.54 (0.13) |
| 24 | 0.42 (0.06) |
| 36 | 0.39 (0.08) |
| 48 | 0.45 (0.21) |
| 60 | 0.40 (0.11) |
| 72 | 0.37 (0.01) |
| 84 | 0.46 (0.02) |
| 96 | 0.48 (0.04) |
| 108 | 0.52 (0.09) |
| 120 | 0.63 (0.51) |
| 144 | 0.53 (0.23) |
| 168 | 0.34 (0.15) |
| 192 | 0.42 (0.14) |
| 216 | 0.52 (0.22) |
| 264 | 0.32 (0.10) |
| 288 | 0.58 (0.34) |
| 384 | 0.26* |
| 408 | 0.46* |

* Only 1 Experiment lasted to this timepoint

**Figure 6.3 Lymph IL-10 Assessed by Inhibition of Macrophage IL-1β Production from Two Individual Experiments after Exposure of Ovine Flank Skin to 2.7 kJ/m² UVB**




Dashed lines represent the normal range of IL-10 like activity from two pre-treatment samples.

### 6.3.4 *IL-10-like activity after 8.1 kJ/m² UVB*

#### 6.3.4.1 IL-10-like activity in Lymph

Lymph samples were assayed for IL-10 like activity after exposure of the flank skin to 8.1 kJ/m². Again some variation in the pre-treatment levels of IL-10-like activity were between experimental subjects (Fig. 6.4). Increased IL-10-like activity was detected in lymph collected after UVB exposure from two experimental subjects, occurring at 48-96, 192 (Fig. 6.4a) and 24 (Fig. 6.4b) hours respectively. While similar decreases were observed in the final experiment at 24 and 192 hours post UV exposure. While the final experiment was not observed to vary outside the normal range, possibly to greater level of variation in pre-treament samples. However, the general trend observed was that sustained increases in IL-10-like activity occurred in lymph after exposure of the skin to 8.1 kJ/m² UVB.

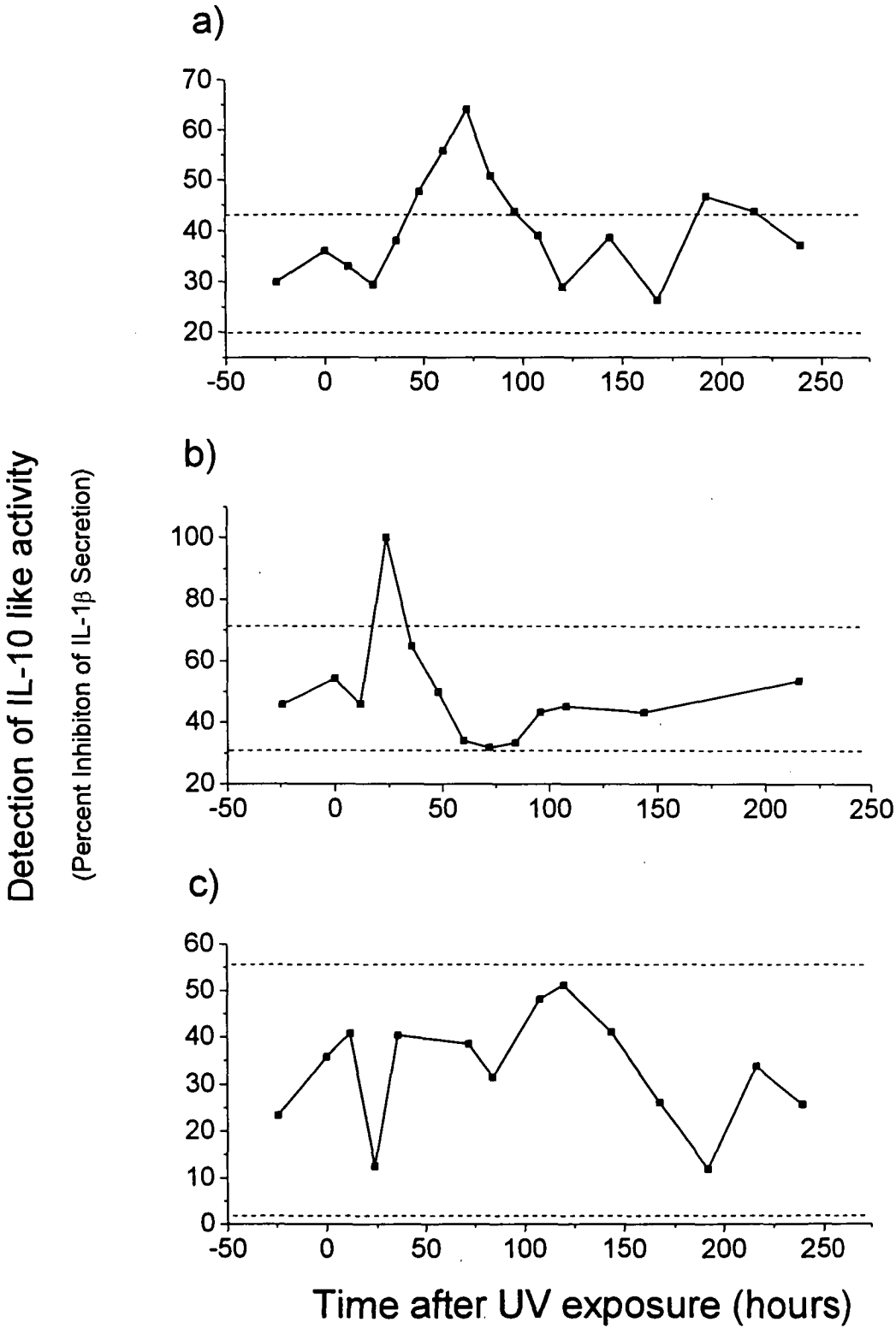
#### 6.3.4.2 IL-10-like activity in Culture Supernatants

Since functional studies were performed on DC migrating from UV exposed skin, IL-10-like activity in supernatants from DC:PBMC co-cultures was also assessed using the macrophage inhibition assay. Generally, supernatants from pre-treatment cultures displayed IL-10-like activity in comparison to control samples though the extent of this varied between individual experiments (Fig. 6.5). In one experiment (Fig. 6.5a) the IL-10-like activity was decreased below that of the controls containing only LPS at 12, 84 and 192 hours post treatment. While in one experiment the trend was a later decrease in the IL-10-like activity in culture supernatants (Fig. 6.5b). While from Fig. 6.5c it can be observed that from this experimental subject there was no variation outside the normally range. Reduced or unchanged IL-10-like activity was generally observed in DC:PBMC culture supernatants after UV exposure of flank skin to 8.1 kJ/m².

### 6.3.5 *IL-10-like activity in Lymph after 20.1 kJ/m² UVB*

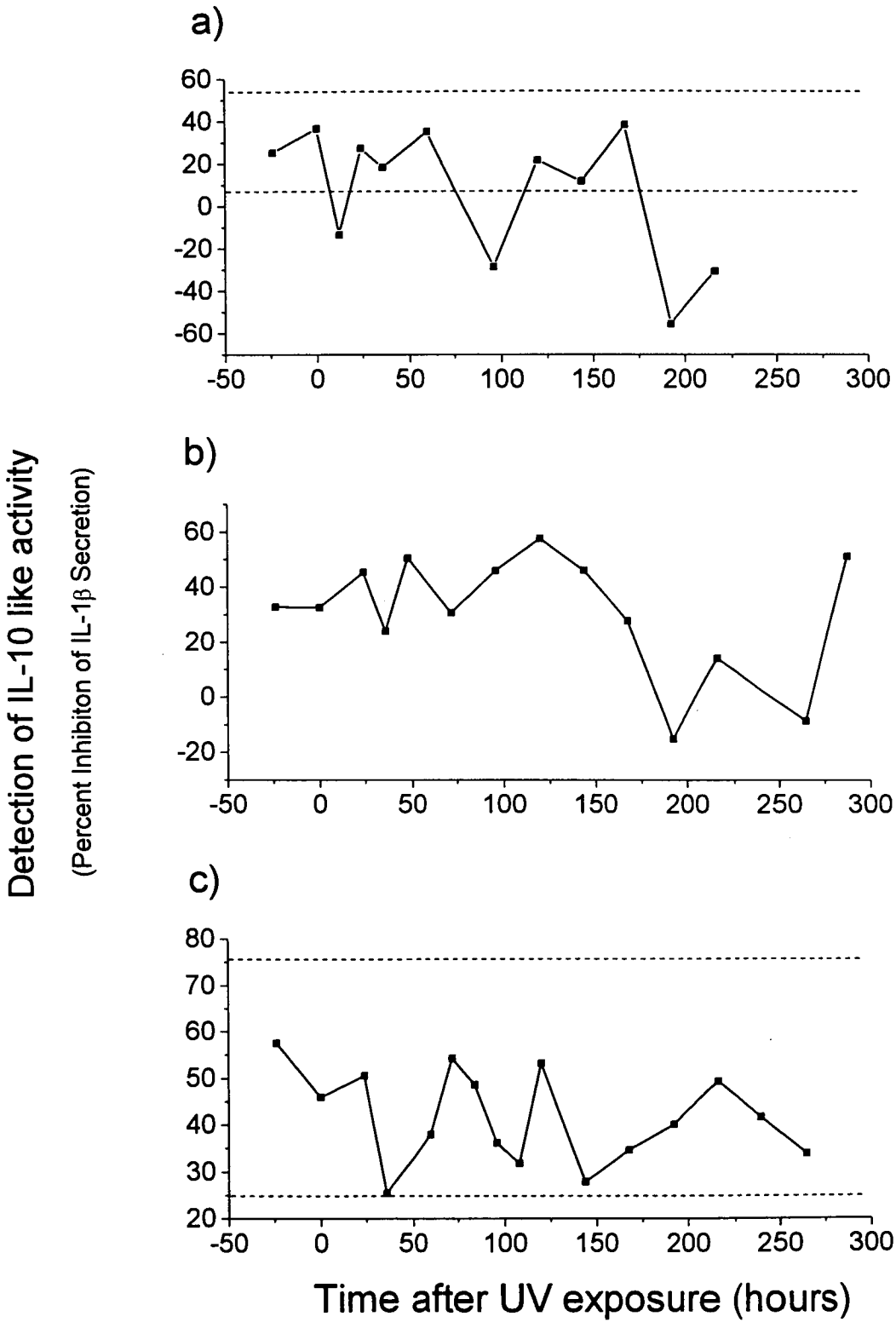
The IL-10 like activity contained in lymph from skin exposed to 20.1 kJ/m² was examined. Again, the levels of IL-10-like activity present in pre-treatment samples appeared to vary when individual experiments were compared. Unusually, one experimental was observed to have lymph that appeared to stimulate the secretion of IL-1β from macrophages. A definite trend of gradually increasing levels of IL-10-like activity was observed in two experiments (Fig. 6.6 a&b). Increased levels of IL-10-like activity were also

**Figure 6.4 Lymph IL-10 Assessed by Inhibition of Macrophage IL-1β Production from Three Individual Experiments after Exposure of Ovine Flank Skin to 8.1 kJ/m² UVB**



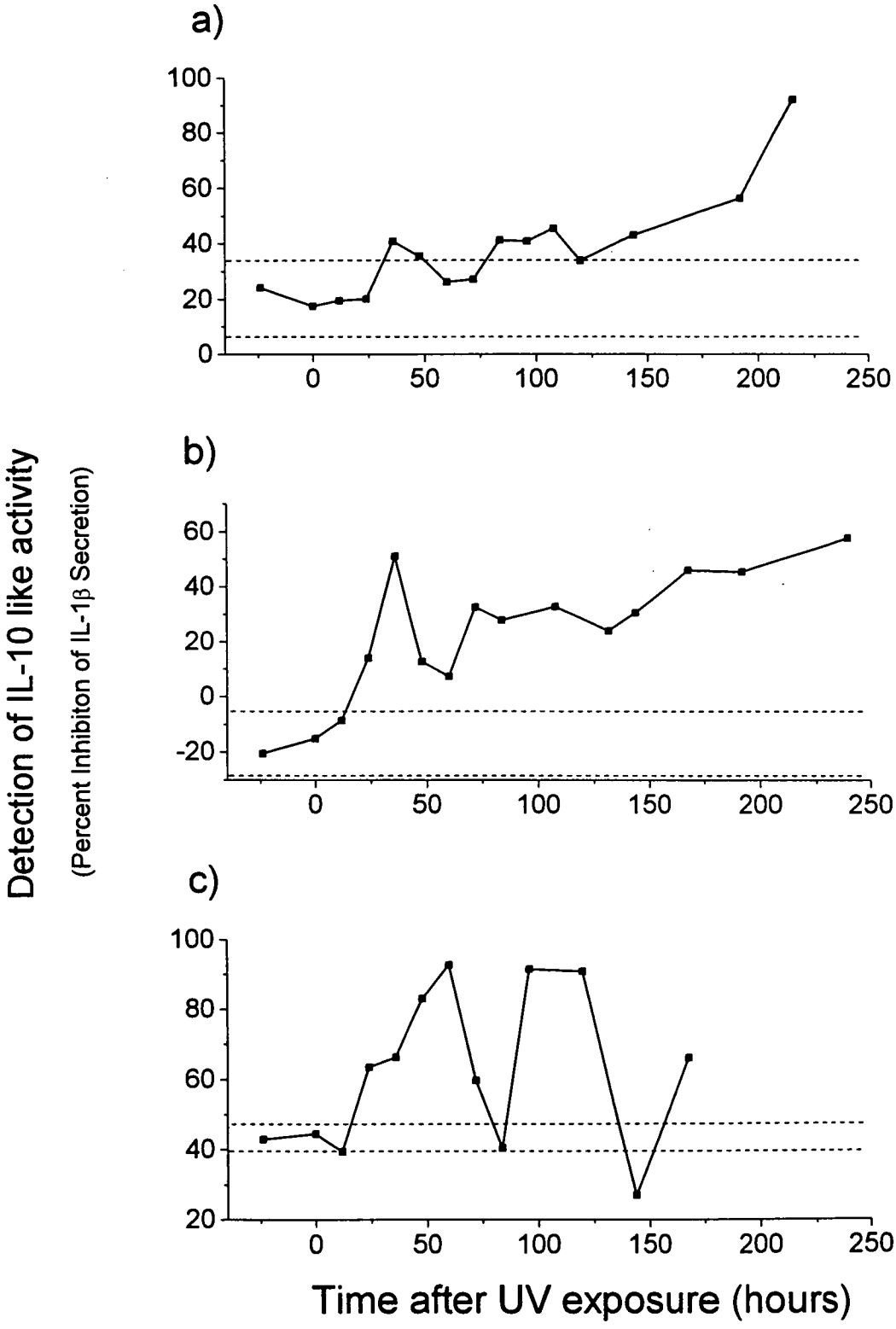

Dashed lines represent the normal range of IL-10 like activity from two pre-treatment samples.

**Figure 6.5 IL-10 Detected by Macrophage Inhibition of IL-1β synthesis by DC:PMBC Co-Cultures from Three Individual Experiments after Exposure of Ovine Flank Skin to 8.1 kJ/m² UVB**




Dashed lines represent the range of IL-10 like activity from two pre-treatment samples.

**Figure 6.6 Lymph IL-10 Assessed by Inhibition of Macrophage IL-1β Production from Three Individual Experiments after Exposure of Ovine Flank Skin to 20.1 kJ/m² UVB**




Dashed lines represent the normal range of IL-10 like activity from two pre-treatment samples.

observed in the other experiment for all time points 24 hours after UV exposure, apart from a return to the normal range at 84 hours post-UV exposure and one dip below pre-treatment levels, at 144 hours post treatment (Fig. 6.6c). This third experiment was of shorter duration thus trend towards the increased IL-10-like activity was observed towards the end of the time course in lymph from other experimental subjects could not be assessed for this experiment. However, it is apparent that lymph which drained skin exposed to 20.1 kJ/m² UVB contained a sustained level of IL-10-like activity.

### 6.3.6 *Detection of IL-10 Protein in Lymph after UV Exposure*

Western blotting was used to detect the presence of IL-10 protein in the lymph samples, which were shown to be positive for IL-10 like activity in the macrophage inhibition assay. Figs. 6.7, 6.8 and 6.9 show lanes from western blots performed on lymph samples from 2.7 kJ/m², 8.1 kJ/² and 20.1 kJ/m² respectively. IL-10 protein was detected using an anti-ovine-IL-10 polyclonal antibody and was found to have an apparent molecular weight of approximately 20 kD. While some of the bands on the gels appear to be of a slightly lower molecular weight, this was most likely an artefact of the western process, as the samples which were nearest the molecular weight markers were observed to be slightly distorted. Despite changing many variables it was not possible to overcome this problem without degradation of the molecular weight markers. While it can be seen that there were faint bands in normal (pre-exposure samples) from both the 8.1 kJ/m$^2$ (Fig. 6.8) and the 20.1 kJ/m$^2$ (Fig. 6.9), the normal sample from 2.7 kJ/m$^2$ (Fig. 6.7) had a quite intense band. Further, samples which were previously found to have inhibitory effects on IL-1β secretion by macrophages had intense bands. The detection of this protein was reduced 144 hours after UV exposure in the 2.7 kJ/m$^2$ and 20.1 kJ/m$^2$ doses (Figs. 6.7 and 6.9 respectively), however, it was still apparent in the lymph draining skin exposed to 8.1 kJ/m² (Fig. 6.8). It should be noted that no band was detected in the control samples which contained culture media only (RPMI+Supplements). Accordingly it is concluded that IL-10 is found in lymph draining from both normal skin and UV irradiated skin, although the quantity of IL-10 protein appears to be elevated after UV exposure.

### 6.3.7 *Prostaglandin $E_2$ in Lymph and Culture Supernatants*

The levels of secretion of $PGE_2$ in DC:PBMC co-cultures were assayed by a competitive immunoassay. The levels detected were compared to a range of

Figure 6.7 Detection of IL-10 Protein in Lymph after Cutaneous Exposure to 2.7 kJ/$m^2$ UVB

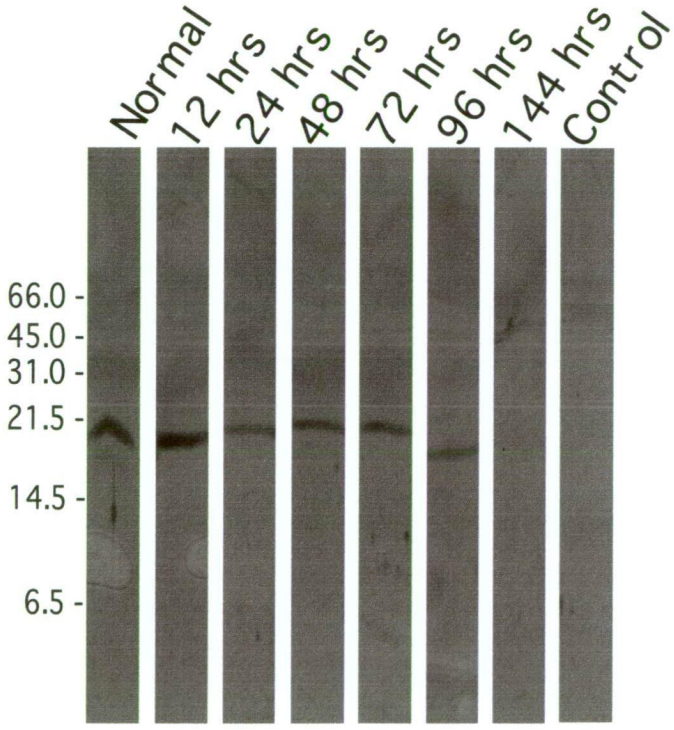


Figure 6.8 Detection of IL-10 Protein in Lymph after Cutaneous Exposure to 8.1 kJ/$m^2$ UVB

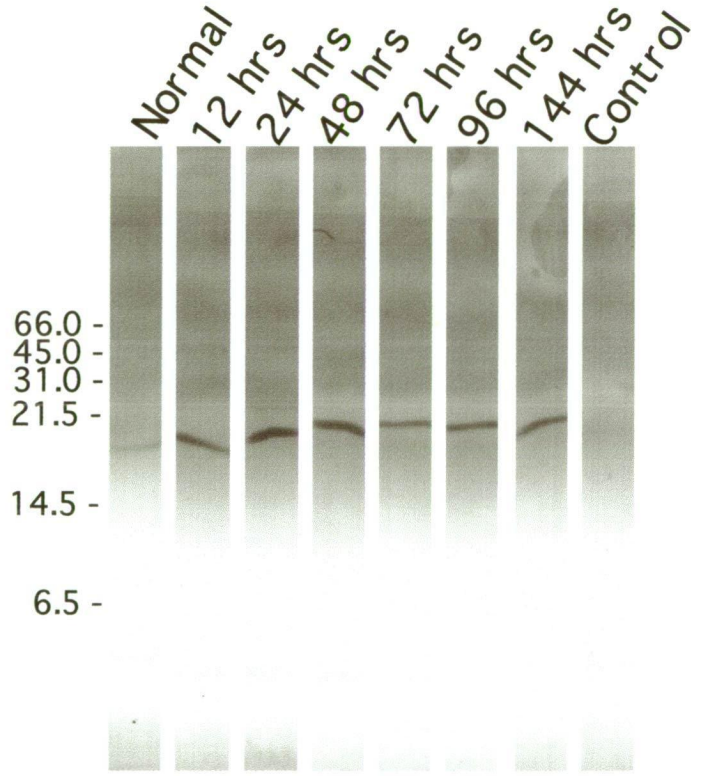
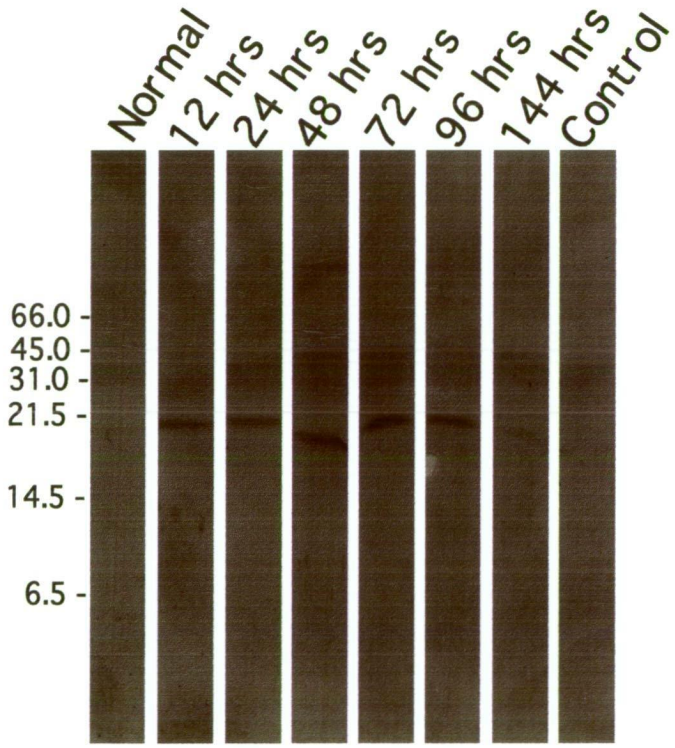


Figure 6.9 Detection of IL-10 Protein in Lymph after Cutaneous Exposure to 20.1 kJ/m$^2$ UVB



$PGE_2$ standards (Fig. 6.10). $PGE_2$ was detected in pre-treatment samples of three experimental subjects at levels ranging from 345 pg/ml to 889 pg/ml (Fig. 6.11). The levels of $PGE_2$ were dramatically increased to 3980 pg/ml and 7227 pg/ml respectively for two of the experiments 24 hours after exposure (Fig. 6.11a & b) while the remaining experiment (Fig. 6.11c) showed an increase to 1220 pg/ml at the same time point. These increases represent six, ten and two fold increases respectively above the mean background levels of $PGE_2$ for each of the three experiments. After 24 hours the levels of $PGE_2$ returned to approximately pre-treatment levels where they remained for the duration of two experiments (Fig. 6.11a&b). The remaining experiment (Fig. 6.11c) showed increased $PGE_2$ levels towards the end of the time course, peaking at 264 hours with $PGE_2$ concentration of 3220 pg/ml.

### 6.3.8 *IFNγ in Culture Supernatants*

IFNγ levels in DC:PBMC co-culture supernatants were measured in comparison to recombinant bovine IFNγ standards (Fig. 6.12). The range of IFNγ in the DC:PBMC co-cultures prior to UV exposure of the skin to 8.1 $kJ/m^2$ was between 0-21 pg/ml (Fig. 6.13a,b&c). However, in co-cultures set up using DC collected after UV exposure of the skin of the skin, substantial increases in IFNγ levels were detected in all experimental subjects. This increase in IFNγ secretion occurred within 48 hours of exposure were observed for two out of three sheep. Differences in the timing and magnitude of these increases were noted. In one experiment there was a transient peak at 24 hours of 391 pg/ml (Fig. 6.13b) while the other experiment showed a longer duration of effect and while IFNγ production was increased at 24 hours, it did not peak until 36-48 hours after exposure (Fig. 6.13c). Sustained detectable levels of IFNγ were present for these two sheep for an extended period after UV exposure (Fig. 6.13b&c). One experimental subject showed a more substantial peak in IFNγ levels to 226 pg/ml at the end of the time course. However, an increase in IFNγ concentration to 41 pg/ml was evident in lymph samples 24 hours after UV exposure (Fig. 6.13a). Smaller increases occurred at 96, 168 and 216 hours while the final collection point at 240 hours was found to contain 96 pg/ml of IFNγ. In general increased IFNγ production in DC:PBMC co-cultures occurs primarily within the first 24-48 hours after UV exposure. It should be noted that the experimental subject which did not show substantial increases initially in IFNγ concentration was not the same experimental subject in which a relatively smaller initial increase in $PGE_2$ levels was observed (Section 6.3.5).

## Figure 6.10 $PGE_2$ Standard Curve

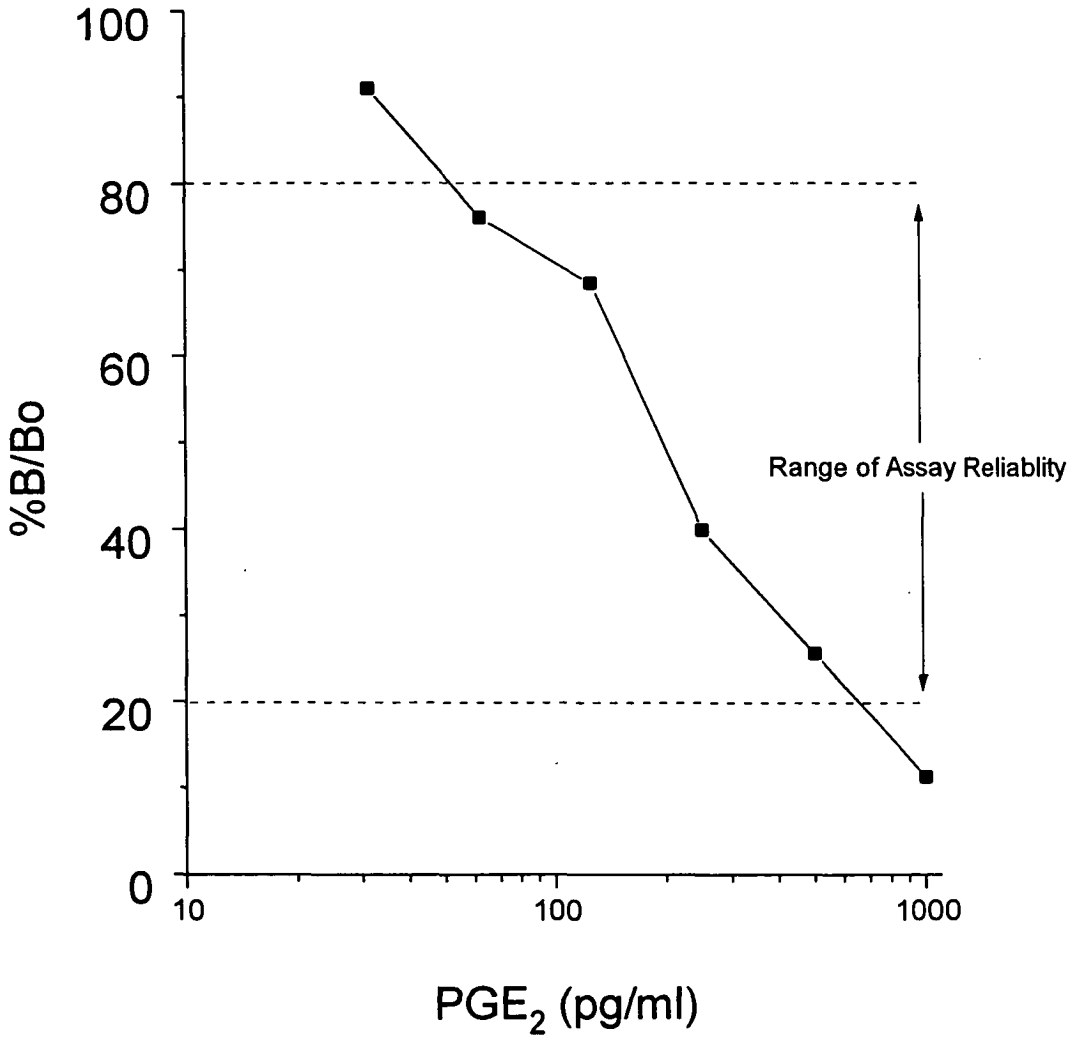
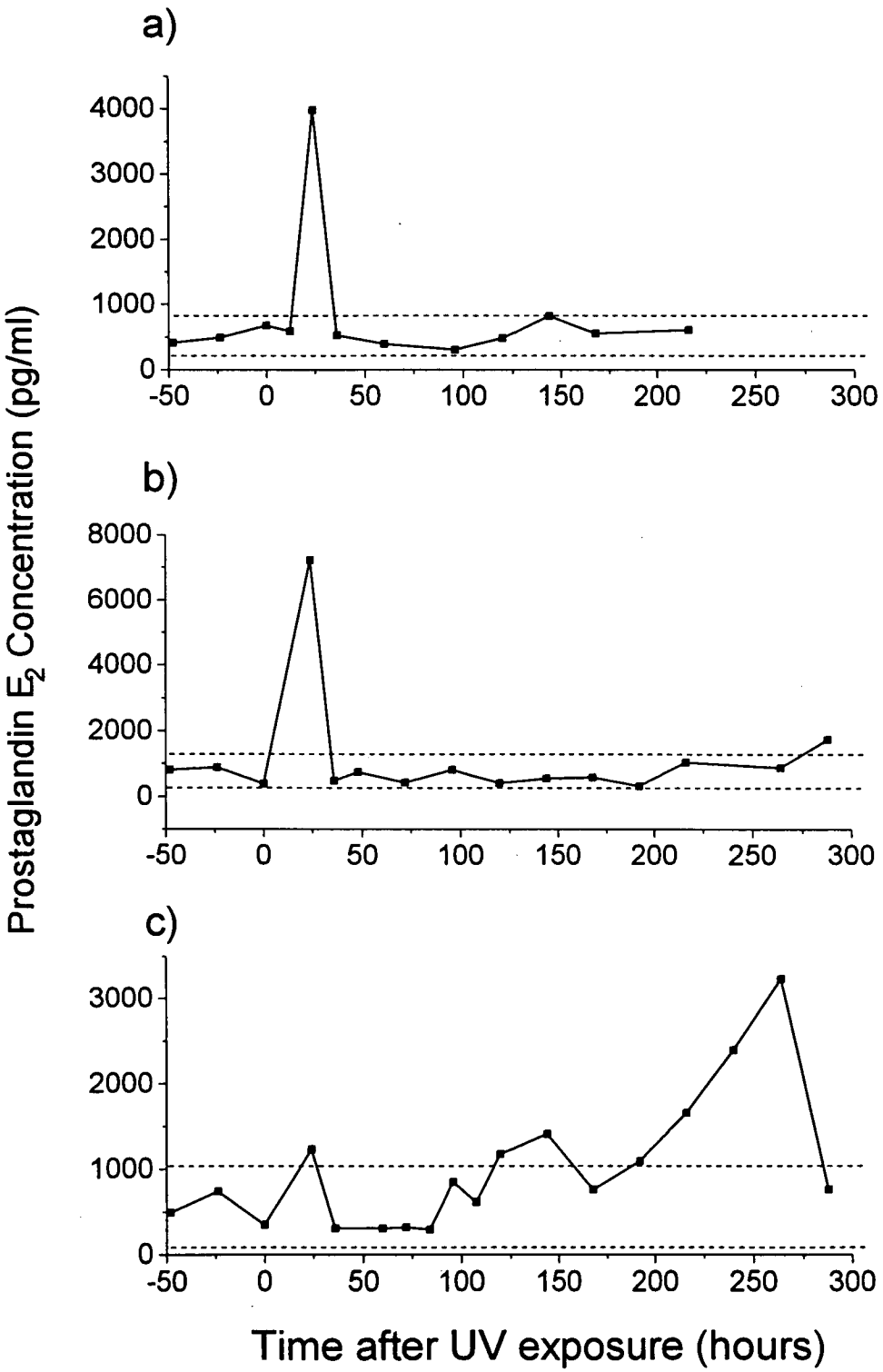


**Figure 6.11** **Prostaglandin $E_2$ in DC:PMBC co-cultures from Three Individual Experiments after Exposure of Ovine Flank Skin to 8.1 kJ/m² UVB**




Dashed lines represent the normal range for the experiments

## Figure 6.12 IFNγ Standard Curve

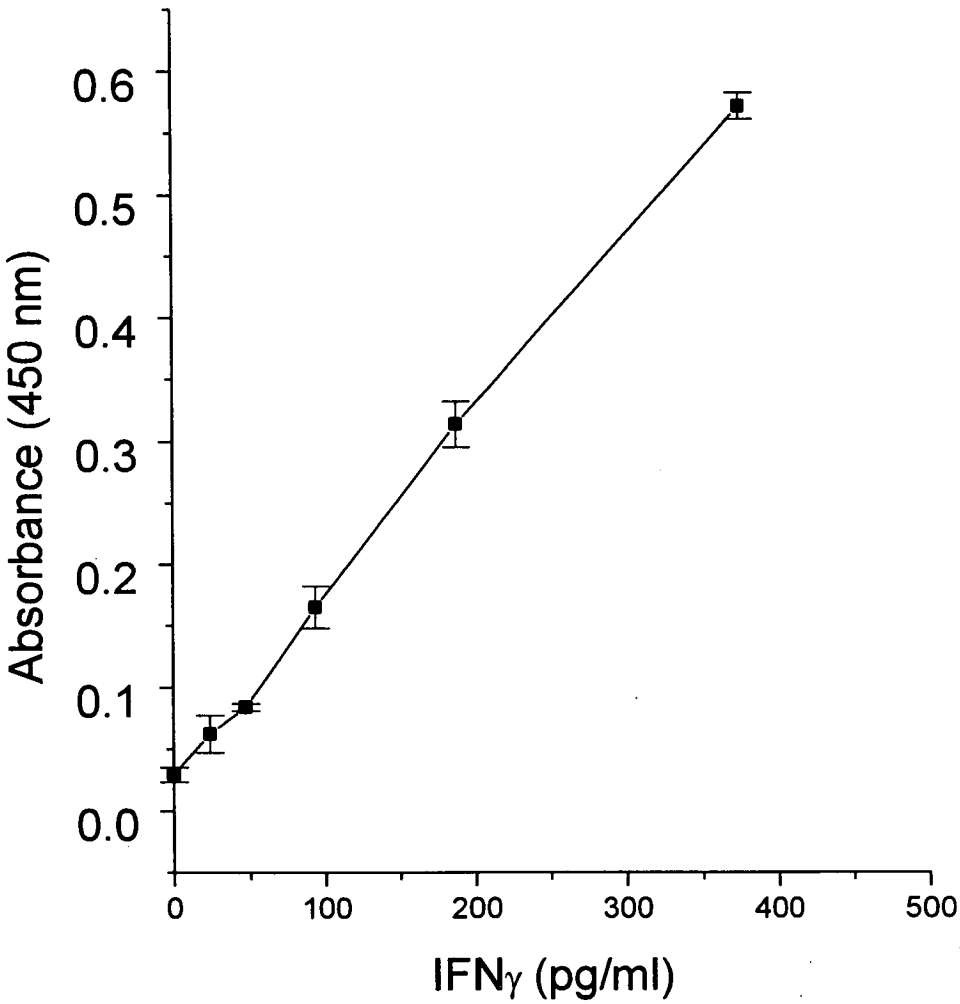
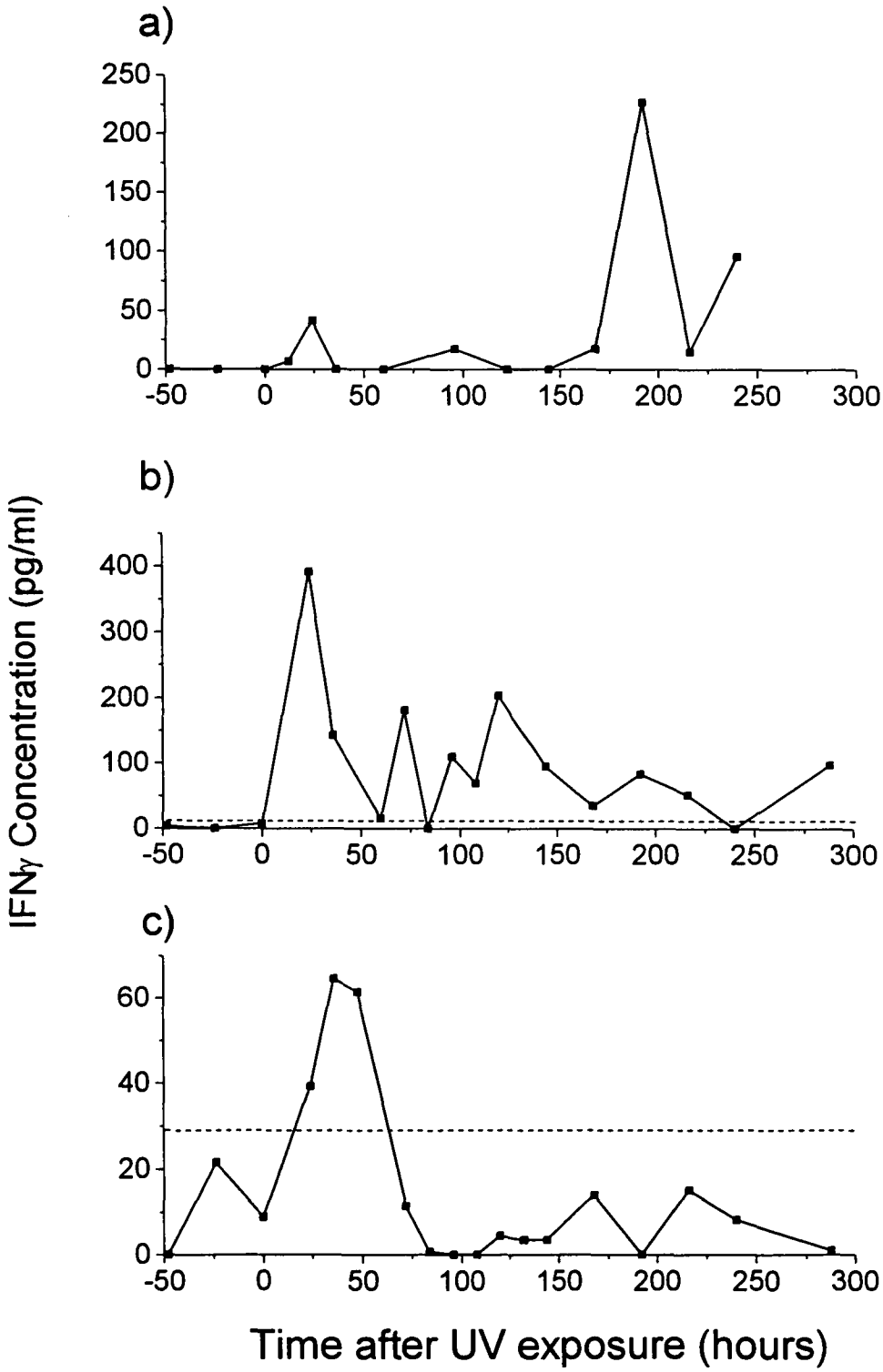


**Figure 6.13 IFNγ in DC : PMBC co-cultures from Three Individual Experiments after Exposure of Ovine Flank Skin to 8.1 kJ/m² UVB**




Dashed lines represent the normal range for the experiments.

### 6.3.9 *IL-5 in lymph and DC-PBMC Co-Cultures*

The levels of IL-5 in samples was assessed by a bioassay in which proliferation of the BAF cell line is IL-5 dependent. Supernatant from the X63 cell line was used a source of IL-5 for the initial growth of the BAF cell line and was also used as a positive control. Substantial proliferation of BAF cells was observed when incubated with 5% X63 supernatant as a positive control giving a mean [$^3$H]thymidine uptake of 28,500 CPM for the positive controls for one experiment. Little proliferation was observed in the negative control sample (355 CPM). In a separate experiment, incubation of the cell line with the mitogen PHA, produced a mean of 192 CPM. The majority of test samples, either lymph or culture supernatants caused similar rates of BAF proliferation to that of the negative controls. Some samples were observed to cause BAF cells to proliferate but this was inconsistent and bore no apparent relationship with UV exposure of the skin or other parameters controlled by these experiments

## 6.4 DISCUSSION

UV irradiation of skin and the subsequent production of cytokines and soluble factors by its constituent cells has been the subject of intense study (reviewed by Granstein,1996, Bergstresser and Takashima, 1996). The current investigation expands these studies by exploiting the properties of the ovine pseudoafferent system to examine the effects of UV exposure on skin cytokine production. Specifically, tests were conducted to identify the presence of cytokines after UV exposure of skin, in lymph fluid draining the skin and from cultures containing DC migrating from skin. The exposure of DC to cytokines, prior to their interaction with T cells can influence the nature of subsequent immune response generated (Cua *et al.*, 1996, Dai and Streilein, 1995, Steinbrink *et al.*, 1997). Thus DC migrating from UV exposed skin will be influenced by the cytokines present not only within the skin but also those in the lymph in which they migrate. In turn, the result of interaction between migrating DC and T cells in the DLN is influenced by a number of factors, including cytokines and soluble factors present in the local micro-environment. In the culture system used, it is likely that DC are a major source of cytokines which influence the development of T cell immune responses. The culture supernatants were harvested after three days, a time period previously shown to allow detection of immunoregulatory cytokines in DC:T cell co-cultures (Teunissen *et al.*, 1991, Schrieber *et al.*, 1992). The method used in the gathering and storage of supernatants was identical to that used by Ragg *et al.*, (1997) to assess IL-1β levels after carcinogen treatment of ovine skin.

### *6.4.1 GM-CSF*

While no GM-CSF was detected in either lymph or culture supernatants before or after UV exposure of skin, this result may be inconclusive. The ELISA used to detect the cytokine was much less sensitive than previously reported. Entrician *et al.*, (1996) indicated that the assay was sensitive to 100 pg/ml GM-CSF, however the present study found the assay only sensitive to 1900 pg/ml. The loss of sensitivity may have occurred in transit or during storage prior to use. Since GM-CSF has been shown to be present in lymph draining normal human skin, as have cells secreting the cytokine (Yawalker *et al.*, 1996b). The lack of detection may therefore be solely due to the insensitivity of the assay system used in this study, although species differences in the patterns of cytokine production between humans and sheep cannot be excluded.

### 6.4.2 *IL-1β Production*

The majority of IL-1β in the skin is produced by LC (Shevach *et al.*, 1992, Heufler *et al.*, 1992) and this cytokine plays a crucial role in the initiation of immune responses occurring within the skin (Enk *et al.*, 1993a). It has previously been shown in the sheep model that IL-1β is secreted by migrating DC cultured with PBMC (Ragg *et al.*, 1997). IL-1β is produced by activated DC as part of an antigen recognition process (Enk and Katz, 1992, Enk *et al.*, 1993). This activation process may be partially dependent on T cells, as XS52 a LC-like cell line requires interaction with T cells and, more specifically interaction through B7 or MHC II to produce IL-1β (Kitajima *et al.*, 1995). In the antigen presenting assay from which the supernatants were isolated, it appears that antigen is also crucial to this process as IL-1β production was not detected unless DC were pulsed with OVA prior to incubation (Ragg, unpublished observations). Macrophages are another possible source of IL-1β in the DC:PBMC cultures supernatants as ovine macrophages also produce IL-1β (Egan and Nash, 1996). From evidence already presented in this thesis, it appears that even after exposure to higher doses of UV, the proportion of migrating DC is greater than macrophages suggesting migrating DC are likely to be the main source of this cytokine, both before and after UV exposure of the skin.

The levels of IL-1β secreted by DC migrating from normal skin in this study were slightly lower in magnitude than the normal values reported previously by Ragg *et al.*, (1997). Ragg *et al.*, (1997) found that the loss of antigen presenting function observed in migrating ovine DC after cutaneous exposure to carcinogens (DMBA and BP) was closely associated with loss of IL-1β secretion. Initially, it was considered that the loss of antigen presenting activity of ovine DC migrating from UV exposed skin may have been similarly associated with a loss of IL-1β production. However, IL-1β was consistently present in supernatants from DC:PBMC after exposure to 8.1 kJ/m$^2$. Thus it is concluded that IL-1β secretion by migrating DC is unaffected by UV exposure. Secondly it provides evidence that migrating DC were viable after UV exposure, as they were still able to secrete the cytokine.

### 6.4.3 *IL-10 production*

This study attempted to detect IL-10 in lymph and in DC:PBMC co-cultures before and after cutaneous UV exposure. It is stressed that the macrophage inhibition assay could only detect IL-10-like activity as measured

by the inhibition of IL-1β synthesis by LPS exposed macrophages. This study also used an anti-ovine IL-10 polyclonal antibody to detect a protein having the expected molecular weight of ovine IL-10 (20 kD as found by Martin *et al.*, 1995). However the lack of a positive control (ovine IL-10) during the time of these experiments means that some small element of doubt may be associated with this data. While it is highly likely that the protein in lymph is IL-10, it remain possible that the activity and protein observed may be due to another unrelated cytokine or soluble factor having a similar molecular weight.

Generally lymph appears to suppress the ability of LPS stimulated macrophages to produce IL-1β. This may be due to inhibitory properties of cytokines which are normally present in ovine lymph, or may be due to the nutritive properties of lymph in comparison to culture media. In one case, lymph appeared to stimulate the production of inflammatory cytokines again illustrating the inherent variability of the outbred sheep model. The levels of IL-10-like activity increase in lymph samples after UV exposure to 2.7 kJ/m², 8.1 kJ/m² and 20.1 kJ/m². While the presence of an IL-10-like protein was detected in lymph samples prior to UV exposure of the skin, it would appear that cutaneous UV exposure was associated with increased levels of this protein.

Low levels of IL-10 have previously been demonstrated in normal lymph draining human skin and have been shown to be up-regulated in after elicitation of a primary allergic reaction such as the elicitation of allergic contact dermatitis.(Brand *et al.*, 1997). UV exposure of the skin has been shown to cause dramatic up-regulation in IL-10 levels in the skin and serum (Rivas and Ullrich, 1992), thus it is not unexpected that IL-10-like activity was detected in ovine lymph. The source of cutaneous and hence lymphatic IL-10 appears to be due to the secretion by macrophages (Kang *et al.*, 1994) and/or keratinocytes (Rivas and Ullrich, 1992, Enk *et al.*, 1995).

No increases in IL-10-like activity after UV exposure were observed in DC:PBMC co-cultures after exposure of the skin to 8.1kJ/m² UVB. In fact, it was more common for the loss of this activity to occur. This suggests that the cells within the DC or PBMC populations have not been induced to secrete increased levels IL-10 after UV exposure. This result ties in with the finding that IL-1β secretion in the DC:PBMC co-cultures was not inhibited after UV exposure of the skin. Thus it appears that DC migrating from UV exposed skin do not cause increased IL-10 production. It would therefore appear that the IL-10 detected in these experiments was produced in the skin or by non-dendritic cells in the lymph

### 6.4.4 *Prostaglandin $E_2$ Production*

This investigation found there were detectable levels of $PGE_2$ secreted in DC:PBMC co-cultures under normal conditions which ranged from 345 pg/ml to 889 pg/ml. $PGE_2$ has been shown to be secreted from organ cultured guinea pig skin at levels of approximately 4 ng/ml (Imokawa *et al.*, 1989). Therefore, the finding of $PGE_2$ in pre-treatment cultures was not surprising. This cytokine is likely to be derived from dendritic cells which have migrated from the skin as LC produce this soluble factor (Ruzicka and Aübock, 1987, Rosenbach *et al.*, 1990, Teunissen *et al.*, 1991). The finding that $PGE_2$ is present in normal DC:PBMC co-cultures provides a possible explanation for the lack of detection of IFNγ as discussed in the next section, as $PGE_2$ is known to inhibit IFNγ production by T cells (Hasler *et al.*, 1983). Further, exposure of T cell clones to $10^{-8}$ M $PGE_2$ reduces the production of IL-2 and IFNγ by Th1 and TH0 clones but not IL-4 and IL-5 production by Th2 and Th0 clones (Betz and Fox, 1991, Snijdewint *et al.*, 1993). While the molarity of $PGE_2$ found normally in the cultures was slightly lower at approximately $2x10^{-9}$ M, it might be possible that this amount may be sufficient to shift T cells towards the Th2 subtype and reduce IFNγ production to undetectable levels. This may reflect microenvironmental differences *in vitro* in comparison to *in vivo* conditions, where the presentation of antigen takes place in the lymph node.

24 hours after UV exposure of skin, the amount of $PGE_2$ present in DC:PBMC co-cultures increased by two, six and ten fold for the three experiments after exposure. As culture conditions were identical for the three experiments, the source of variation may be due to the outbred nature of the ovine model. Increased production of prostaglandins in the skin following UV exposure is a well established phenomena (Black *et al.*, 1978a, Black *et al.*, 1978b) and was found to reach its maximum at the same time point in the current study (24 hours). It should be noted that normally, the major arachidonic acid metabolite secreted by LC is $PGD_2$ (Ruzicka and Aübock, 1987, Rosenbach, 1990), however the ratio of $PGD_2$ to $PGE_2$ is reversed after UV irradiation of the skin (Ruzicka *et al.*, 1983). Thus suggesting that the cycloxygenase pathway after UV exposure appears to favour $PGE_2$ production. Extensive studies have shown that inhibitors of PG synthesis, such as indomethacin and aspirin reduce the erythemal response to UV light (Miller *et al.*, 1967, Greenberg *et al.*, 1975, Synder *et al.*, 1975, Black *et al.*, 1978b, Lim *et al.*, 1983). Thus it appears the nature and timing of the increase in $PGE_2$ observed in DC:PBMC co-cultures reflects acute inflammatory events. The

short duration of this increase probably rules out increased $PGE_2$ levels in DC:PBMC co-cultures playing a role in the reduction of DC or PBMC function. The increase in $PGE_2$ levels to $>10^{-8}$ M observed 24 hours after UV exposure of the skin observed for 2/3 experiments, would be sufficient to skew cytokine secretion by T cells towards that of the Th2 subtype (Betz and Fox, 1991, Snijdewint *et al.*, 1993).

### *6.4.5 IFNγ*

This study attempted to ascertain whether DC migrating from UV exposed skin caused alterations in the production IFNγ by responding T cells. Little or no IFNγ production was observed prior to UV exposure of the skin, suggesting that, under normal circumstances OVA pulsed ovine DC, do not appear to stimulate IFNγ production by responding T cells. However, increased production was observed following UV exposure. This is contrary to previous findings, which after cutaneous UV exposure followed by FITC sensitisation, showed that DLN cells produced less IFNγ (Sajio *et al.*, 1996). However, with Sajio's study the FITC sensitisation took place three days after irradiation and the DLN were not isolated until a further 18 hours. In the present study the increased IFNγ production occurred between 24-48 hours after UV exposure, this would have occurred prior to the sensitisation of the skin in Sajio's investigation and so would not have been detected. However, the lack of IFNγ production prior to UV exposure may represent differences between the ovine and murine models. In the ovine system the production of IFNγ by PBMC in response to OVA is crucially dependent on the adjuvant in which the protein is inoculated (Emery *et al.*, 1990). The use of alum as an adjuvant did not induce production of IFNγ by PBMC from immune sheep (Emery *et al.*, 1990), whether this is due to a Th2 immune response is unknown.

Variations in the time course of production IFNγ were noted between individual experimental subjects. This may be due to the outbred nature of the sheep used. Major increases in IFNγ production occurred within the short time period after treatment (24-48 hours), perhaps reflecting a response to the acute inflammatory effects of UV exposure. IFNγ has been shown to be primarily produced by activated Th1 cells, and is thought to skew T cells towards the production of Th1 cytokines. Further, it has been shown to promote macrophage production of cytocycidal soluble factors TNFα, Nitric oxide and reactive oxygen intermediates (reviewed by Farrar and Schrieber, 1993). This suggests a possible role for IFNγ in the elimination of damaged cells. IFNγ and LPS induction of NOS synthetase activity in murine DC was found to be closely

associated with DC apoptosis and sub-optimal T cell proliferation. (Lu *et al.*, 1996). Additional studies would be required to further elucidate if any the role of IFNγ in apoptotic cell death after UV exposure. However, the unchanged production of IL-1β would appear to reduce the possibility that DC are prematurely undergoing apoptosis.

### *6.4.6 IL-5*

This study utilised a recently developed bioassay to detect the secretion of IL-5 in DC:PBMC co-cultures before and after UV exposure of the skin. The BAF cell line has been manipulated so it requires IL-5 to proliferate (D Emery, personal communication). This study revealed that there appeared to be no IL-5 production in the co-cultures, suggesting under normal circumstances this cytokine is not secreted in amounts detectable using the above bioassay. Further, samples from DC:PBMC co-cultures after UV irradiation also did not contain detectable levels of the cytokine. While Simon *et al.*, (1990) found that UV irradiated LC preferentially activated Th2 cell clones in comparison to Th1 cell clones, he did not find increased stimulation of Th2 clones. In subsequent studies it was found that while restimulated hapten primed lymph node cells had a reduced Th1 response, no detectable Th2 response was generated (Simon *et al.*, 1994). This is supported by the present studies and so far in the ovine model IL-5 production has been only found in parasitised and DNA vaccinated sheep (D. Emery, personal communication). Sajio *et al.*, 1996, were able to show that loss of T cell proliferation after UV exposure, was due to a defect in antigen presentation and was associated *in vivo* with a down-regulation of both Th1 and Th2 cytokines, IL-4 and IL-10, respectively. IL-4 is perhaps the most well characterised Th2 cytokine but as yet no specific bioassay or ELISA has been developed for its detection in the ovine system. The development of such an assay would help to clarify the Th1/Th2 status of ovine immune responses. The results presented in this chapter suggest that DC migrating from UV exposed skin do not stimulate increased levels of Th2 cytokines by primed T cells (IL-5 and IL-10).

## 6.5 SUMMARY

This chapter describes investigations of alterations in the secretion of cytokines after UV exposure of ovine skin. Attempts to detect GM-CSF and IL-5 were unsuccessful using the assays available. IL-1β, an important T cell co-stimulatory factor produced by migrating ovine DC was found to be unaltered after cutaneous UV exposure. Normally IFNγ was undetectable or found in low

levels in cultures containing antigen pulsed DC and primed T cells. However, after UV exposure of the skin short term increases in the levels of IFNγ were noted in the culture supernatants. Similar short term increases in $PGE_2$ production were also found in culture supernatants after UV exposure of the skin. However, in contrast to IFNγ, $PGE_2$ was detected in supernatants from unexposed skin and the suppressive activity of this soluble factor may provide a reason for the lack of IFNγ production under normal circumstances. The detection of increased IL-10-like activity and an increase in IL-10 protein in lymph draining UV exposed skin, suggests that increased IL-10 production was occurring in the skin. No increased production of IL-10 was observed in culture supernatants, suggesting that preferential stimulation of Th2 response was not occurring *in vitro*. These changes may have important ramifications for the immunological reactions occurring after UV exposure of the skin which are discussed further in Chapter 7.

# CHAPTER 7: GENERAL DISCUSSION

Ultraviolet rays within the solar spectrum are arguably the most ubiquitous carcinogen to which humans regularly are exposed. Substantial evidence from animal models shows that UVB portion of sunlight fulfils the three steps of carcinogenesis, initiation, promotion and progression (reviewed by Kraus, 1996) The crucial role of the immune system in inhibiting tumour growth is highlighted by the findings that patients on immunosuppressive therapy have increased incidence of skin tumours (Marshall, 1974). Similarly loss of immunity has been shown after UV exposure, as UVB irradiated mice fail to reject highly immunogenic tumours, which were rejected by non-irradiated littermates (Fisher and Kripke, 1978, Kripke *et al.*, 1990). UVB exposure also down-regulates DTH responses in mice and humans (Toews *et al.*, 1980, Cooper *et al.*, 1992). Further the induction of long lasting antigen specific unresponsiveness occurs when antigen is applied through UV irradiated skin due to the formation of suppressor cells (Fisher and Kripke, 1978, Okamoto and Kripke, 1987)

One mechanism by which UV light alters immune function in the skin is via the modulation of LC, the local antigen presenting cells of the skin. LC are known to be depleted by exposure of the skin to UV irradiation, including LC in the ovine skin (Lyne and Chase, 1966). The experiments presented in this thesis have utilised an ovine model, where cannulated lymphatic vessels allowed the investigation of migration kinetics and function of *ex vivo* migrating DC, draining a defined area of UV exposed skin. The key findings of these studies were that UV exposure of the skin caused; increased migration of functionally aberrant LC, the appearance of macrophages in lymph and a reduction in the proliferative capacity of circulating cells. Investigations revealed that the loss of normal function of migrating DC was not related to B7 expression or the *in vitro* production of a number of cytokines. However, the increased *in vivo* production of the suppressive cytokine IL-10 was found.

The UV exposure of ovine flank skin triggered enhanced migration of LC ($CD1w1^+$ DC). This effect was noted for a range of doses between 2.7 and 80.4 $kJ/m^2$, in a dose dependent manner. The migrating LC were viable, thus eliminating the possibility that they were dead cells flushed from the skin in a non specific manner. These findings confirm previous observations in the murine system where increased numbers of DC were present in the lymph

nodes draining UV areas of skin (Moodycliffe *et al.*, 1992). The increase in the number of lymph node DC detected in those experiments where similar in magnitude to the increase in LC migration observed in these experiments. However, the increased migration of ovine LC contrasts with the findings of Richters *et al.*, (1996) and Kremer *et al.*, (1997) who used a human skin explant model. It can be argued that the migration of LC found in ovine pseudoafferent lymph more accurately reflects what occurs *in vivo* than an explant model wher a number of factors are abnormal such as the complete disruption of neural input. This may be particularly relevant as recent data has shown that the nervous system plays a role in UV induced LC depletion (Niizeki *et al.*, 1997).

While LC were readily stimulated to migrate by low doses of UV light, higher doses of UVB ($\geq$ 20.1 kJ/m$^2$) led to non-specific increases in the migration of other cell populations including T lymphocytes and CD14$^+$ cells. The increased migration of CD4, T19 and to a lesser extent CD8 T cell subsets may reflect the increased cellular traffic of these cells through UV inflamed skin. Similar effects have been observed in other ruminant models of cutaneous inflammation (Egan *et al.*, 1996, Mwangi *et al.*, 1990, Flynn *et al.*, 1994). This is most likely due to the up-regulation of adhesion molecules such as E-Selectin, on vascular endothelial cells which are directly affected by UV exposure (Strickland *et al.*, 1997). It has been shown in this thesis that migrating ovine DC do not express the CD14 surface molecule, which is expressed by macrophages migrating from UV exposed skin (Richters *et al.*, 1996). After exposure of ovine flank skin to UVB increased migration of CD14$^+$ cells occurred, supporting the findings that macrophages, like LC, migrate from UV irradiated skin (Muller *et al.*, 1994, Richters *et al.*, 1996). However, migration of macrophages extended out to 168-264 hours after UVB exposure, a much longer period than that previously reported by Richters *et* al., (1996) which may again indicate the limitations of the skin explant system. However, it should be noted that the CD14$^+$ cell population was minor in comparison to migrating DC, only comprising 13% of the DC fraction in the pseudoafferent lymph during its maximum migratory efflux. Hence, T cell interactions occurring within the lymph node are still likely to be dominated by migrating DC.

The subsequent reclassification of established monoclonal antibodies used to assess DC and macrophage migration has affected the interpretation of studies in this thesis (OM1, SBU-T6 & CC-G33). The recent reclassification of the SBU-T6 mAb which is a marker for LC in ovine skin (Mackay *et al.*, 1985 and thought to be equivalent to the human CD1c marker (Ragg *et al.*, 1994) is

now classified as CD1w1 (a workshop definition) and its reaction with a specific equivalent human CD1 subtype is now uncertain (Naessens *et al.*, 1997). However, as the SBU-T6 mAb undoubtedly reacts with ovine LC in the skin (Mackay *et al.*, 1985) and DC in the draining lymph, where CD1a and CD1c are both expressed (reviewed by Romani *et al.*, 1991, Hart *et al.*, 1997), it seems likely that SBU-T6 is reacting with an equivalent ovine surface molecule or molecules. During the course of these studies, the mAb (CC-G33) used to detect CD14 molecules on macrophages was accepted as a marker in the ovine/bovine system (Sopp *et al.*, 1996, Berthon and Hopkins, 1996, Naessens *et al.*, 1997). These ongoing changes in the development and classification of ovine reagents should make the future study ovine cell populations more easy to relate to the phenotype of cells in other species.

This study has shown enhanced migration of LC to the lymph node still occurs after cutaneous UV exposure and evidence exists that the functional ability of LC within the skin is reduced (Stingl *et al.*, 1981). It was therefore hypothesised that the functional ability of the migrating ovine DC would also be reduced. The capacity of migrating ovine DC to present antigen to primed T cells was evaluated after cutaneous exposure to 8.1 kJ/m$^2$ UVB. This dose was chosen in an attempt to minimise the gross inflammatory effects observed at higher doses, as it was the lowest dose to consistently cause an increased proportion of LC in the draining lymph. The functional capacity of the migrating DC was found to be reduced after UV exposure of the skin. The assay used in this study required active antigen uptake, processing and presentation by DC to the primed T cells. Not all these steps are required for allostimulation which appears unaffected by UV light exposure (Lappin *et al.*, 1996, Kremer *et al.*, 1997). Functionally, DC migrating from UV exposed skin are capable of inducing specific suppressor cells (Okamoto and Kripke, 1987). The DC studied in this thesis and by others have a reduced capacity to stimulate T cells (Dai and Streilein, 1995, Sajio *et al.*, 1996). How the suppressor cells are generated still remains a subject of debate. The cells are generally thought to be of the CD4 Th2 subtype (Yagi *et al.*, 1996) or CD8 equivalent (Tc2) (Schwarz *et al.*, 1998) and act via a suppression of Th1 responses. Evidence is not yet available which relates UV induced down-regulation of Th1 response to tumour formation. It is proposed that neoantigens arising in UV exposed skin when APC function is aberrant, may result in the activation of neoantigen specific suppressor T cells. Such events may reduce the immune response against the transformed cells and so would allow tumours to evade elimination by the immune system (reviewed by Nishigori *et al.*, 1996).

Experimental evidence from bilateral cannulations indicated that the loss of function was a systemic effect of UVB exposure. To investigate if this was due to alterations of DC or the responding primed T cells, mitogen stimulation of PBMC was used as an indicator of the proliferative capacity of the responder cells in the functional assay. Exposure of ovine skin to 8.1 kJ/m² reduced the proliferative ability of PBMC for 72 hours. While the loss of responder cell function illustrated the serious nature of the alterations in immune response observed after UV exposure, it also generated a degree of uncertainty about any perceived loss of DC function occurring during the first 72 hours post-exposure. However, since one experimental subject did not show a loss of the functional capacity of DC until 48 hours after UV exposure of the skin this suggests that the immediate loss of function may be due to the loss of the proliferative capacity of PBMC. Evidence from murine studies also demonstrates a delay of 3 days is required before painting UV exposed skin with hapten leading to the induction of tolerance and generation of suppressor cells (Howie *et al.*, 1986). The loss of DC function in the antigen presentation assay lasted between 24-192 hours after exposure, much longer than the reduced proliferative ability of PBMC which was detected for only 72 hours. The difference in time course of these two effects indicates that the aberrant DC function and temporary loss of mitogen induced proliferation are two separate effects occurring after UV exposure of the skin.

It was decided to investigate possible mediators that may play a role in the loss of antigen presenting cell function by migrating ovine DC. Possibilities include the loss of the expression of co-stimulatory molecules on the surface of migrating LC, the production of an inhibitory cytokine(s) or the lack of production of stimulatory cytokine(s). No loss of expression of B7 molecules occurred on migrating ovine DC after UV exposure of the skin, in fact it was common for increases in the expression of B7 molecules to occur. Recent evidence agrees with the findings of this study. Lappin *et al.*, (1996) has shown that the expression of B7-2 molecule is increased on migrating DC and B7 molecule expression also appeared to be up-regulated within the skin after UV exposure (Laihia and Jansen, 1997). However, the expression of B7 molecules by migrating CD1w1$^+$ DC was generally stable after exposure of the flank skin to 8.1 kJ/m² UVB, the same dose shown to cause inhibition of DC antigen presenting function. It therefore seems unlikely that loss of the APC function after UV irradiation of the skin is caused by alterations in B7 expression. This observation lead to changes in the production and secretion

of various cytokines and their role in aberrant DC becoming a more important focus of this thesis.

The production of the prototype costimulatory cytokine IL1β by LC is crucial for the initiation of immune responses in the skin (Enk *et* al., 1993). The production of this cytokine was investigated in DC:PBMC co-cultures, which were set up in parallel with cultures used for the APC assay. These experiments revealed that migrating DC were still capable of *in vitro* production of this cytokine after UV exposure. Thus, any loss of DC antigen presenting function after UV exposure of the skin, unlike chemical carcinogens (Ragg *et al.*, 1997), appears not to be associated with a loss of IL-1β production. Further soluble factors were investigated in DC:PBMC co-cultures after UV exposure with the only changes detected being a temporary up-regulation of IFNγ and $PGE_2$. $PGE_2$ is known to be produced by cells in the skin, including LC (Teunissen *et* al., 1991, Rosenbach *et al.*, 1990) and is up-regulated in the skin after UV exposure (Imokawa and Tejima, 1989) contributing to some immunosuppressive events (Jun *et al.*, 1988). The up-regulation observed in cultures containing DC and primed T cells may be due to the increased $PGE_2$ synthesis by migrating LC in response to the acute inflammatory effects of UV exposure. This in turn may be an attempt to reduce the production of inflammatory cytokines, such as TNFα and IL-6 by activated macrophages, which has been shown to be linked with IL-10 production (Strassman *et al.*, 1994). Both $PGE_2$ and IL-10 have been shown to influence the ability of immature DC to induce Th1 or Th2 responses (Kalinski *et al.*, 1997). It is apparent from these studies that the magnitude of temporary increases in production and/or secretion of $PGE_2$ may be sufficient for this to occur (Betz and Fox, 1991, Snijdewint *et al.*, 1993). This effect may also be mediated by the lack of IL-12 production as sufficient $PGE_2$ was found in pre-treatment samples ($10^{-9}$ M) to inhibit IL-12 production by DC *in vitro* (Kalinski *et al.*, 1997) and this is likely to be exacerbated with the increased $PGE_2$ levels after UV exposure of the skin. Further, Kalinski *et al.*, (1997) showed the loss of IL-12 production by DC was stable even after the withdrawal of $PGE_2$ from culture and this was associated with IL-10 secretion by DC. It may be hypothesised that $PGE_2$ production after UV exposure could affect the capacity of LC *in vivo* to produce IL-12 for a sustained period, thus leading to the observed loss of ovine APC function *in vitro*. However, in this study the *in vitro* expression of $PGE_2$ did not correlate with a loss of APC function. So $PGE_2$ could not be directly related to the functional changes observed.

A further effect of the exposure of immature DC to $PGE_2$ is a reduction in expression of the CD1 surface molecule while increasing CD14 expression (Kalinski *et al.*, 1997). This requires exposure to $PGE_2$ at levels greater than $10^{-9}$ M, which were observed to occur after UV exposure. Both markers were used in this study to investigate DC and macrophage migration respectively. It might be hypothesised that increased $PGE_2$ and IL-10 levels may alter DC maturation, leading to the development of a more macrophage-like cell. This theory may warrant future investigation, as it suggests that $PGE_2$ cultured DC may be useful for immunotherapy where the induction of a Th2 response is desirable.

A temporary increase in IFNγ was detected in DC:PBMC co-cultures set up with cells collected within 24 hours of UVB exposure of the skin. This finding was unexpected as it has not been demonstrated in responses studied in the DLN, where the loss of this cytokine is commonly associated with UV irradiation (Simon *et al.*, 1994, Sajio *et al.*, 1996). However, a novel form of T cell activation has been noted *in vitro* associated with macrophages in UV irradiated skin. This involved up-regulation of IFNγ mRNA and deficient IL-2rα expression by responding T cells (Stevens *et al.*, 1995). Circumstantial evidence exists in the ovine model which supports this observation as the initial increase in migration of macrophages from ovine skin occurs at a similar time point as the temporary production of IFNγ *in vitro*. IFNγ and $PGE_2$ would appear to have opposing effects on the immune response, with these soluble factors known to skew immunity towards a Th1 or Th2 response respectively. The production of both cytokines simultaneously *in vitro* illustrates the level of complexity associated with the generation of an immune response.

A bioassay was used to assess IL-10-like activity. This assay measured the inhibition of IL-1β by LPS stimulated macrophages after the addition of lymph or DC:PBMC co-culture supernatant. Lymph samples and co-culture supernatants taken before UV of the skin were compared with those derived at various time points after irradiation. This revealed that there was little or no increase in IL-10-like activity in supernatants from DC: T cell co-cultures after cutaneous exposure to 8.1 $kJ/m^2$ UVB. The lack of any increase in IL-10-like activity in the DC:PBMC co-cultures suggested that the DC migrating from UV exposed skin did not stimulate primed T cells to produce detectable levels of this cytokine. So the increased IL-10 secretion may be limited to *in vivo* production within the skin after UV exposure, where likely sources of IL-10 include macrophages (Kang *et al.*, 1994) and keratinocytes (Ullrich *et al.*, 1994,

Enk *et al.*, 1995, Grewe *et al.*, 1995). The apparent lack of increased IL-10-like activity in DC:PBMC co-cultures, despite the increased migration of $CD14^+$ macrophages from UV exposed sheep skin, suggests these cells were not producing this cytokine *in vitro*. This indicates that the activity may be restricted to macrophages and keratinocytes within the environment of the skin. Therefore this study has been unable to find a suppressive factor within DC:PBMC culture supernatants, which directly correlates to the loss of APC function observed.

Because of the lack of *in vitro* changes in IL-10 production corresponding to the functional changes, it was decided to investigate the *in vivo* production of IL-10 in ovine lymph draining the UV irradiated sheep skin. The detection of IL-10 protein by western blotting of lymph samples was undertaken using specific polyclonal anti-IL-10 antibodies. This technique detected the presence of protein with the approximate molecular weight of ovine IL-10 (20 kD). This was detected in lymph draining from both normal and skin exposed to 2.7, 8.1 and 20.1 $kJ/m^2$ UVB. While IL-10 protein was barely detectable in pre-exposure samples, increases in the intensity of the bands were observed after UV exposure of the skin. Hence the quantity of IL-10 protein in lymph draining UV exposed skin was increased. This was further supported by increased IL-10-like activity in lymph draining UV exposed skin. IL-10 has been shown to affect the APC function of LC which lose their ability to present antigen to Th1 cells but not Th2 cells (Enk *et al.*, 1993). Further, the systemic effects of UV immunosuppression on antigen presenting cell function are blocked by the administration of IL-10 neutralising mAb's (Ullrich *et al.*, 1994) and DC exposed to IL-10 *in vitro* induce tolerance rather than immunity by inducing antigen specific anergy (Steinbrink *et al.*, 1997). The induction of anergy in T cells may account for the sustained loss in ability of migrating ovine DC from UV exposed skin to stimulate primed T cells *in vitro*. The importance of IL-10 in UV induced immunosuppression is highlighted by studies which have shown that IL-10 knockout mice are completely resistant to the immunosuppressive effects of UV exposure (Beissert *et al.*, 1996). Further, IL-10 has been found to reduce the presentation of tumour associated antigens by LC (Beissert *et al.*, 1995), thus allowing transformed cells to evade a protective cellular immune response *in vivo* and develop into a tumour.

From the results presented in this thesis, the most likely explanation for the UV induced loss of APC function by migrating DC would appear to be the exposure to IL-10 and possibly $PGE_2$ while present in the skin and lymph. This

in turn may lead to a lack of IL-12 production by antigen presenting cells. IL-12 production by migrating ovine DC after UV exposure of the skin was not investigated due to a lack of ovine reagents specific for this cytokine. However, IL-12 is undoubtedly important in the induction of cellular immune (Th1) responses (Seder *et al.*, 1993) and the addition of IL-12 after UV irradiation may overcome the induction of UV induced suppression and the activity of UV induced suppressor cells (Schmitt *et al.*, 1995, Schwarz *et al.*, 1996). This suggests a pivotal role for the inhibition of IL-12 production in UV induced immunosuppression. This lack of IL-12 may occur for a sustained period, due to an increased production of IL-10 and PGE2 (Kalinski *et al.*, 1997).

These studies have shown the effects UV light has on the migration and functional capacity of ovine pseudoafferent lymph DC and provides some insights into mediators of these effects. However some questions remain unanswered. For instance the photoreceptor mediating these effects is still unknown. Loss of APC may be due to DNA damage, *cis*-UCA production or a combination of both. The availability of liposomes containing the excision repair enzyme T4 endonuclease V (T4N5) (Yarosh *et al.*, 1990) and antibody against *cis*-UCA (El-Ghorr and Norval, 1995) would readily allow these parameters to be assessed. Investigations of the stage at which the loss of APC function occurs, whether in antigen uptake, processing or presentation are currently being undertaken and should provide valuable insights into the stage(s) at which UV irradiation effects APC function. The increasing availability of ovine reagents will allow detailed investigation into the role of IL-10 and IL-12 in the loss of APC function demonstrated here. While this thesis and many previous investigations have concentrated on the cellular mechanism of UV immunosuppression, little is known about the molecular mechanisms associated with these changes. This therefore remain a challenging area for future study.

## SUMMARY

This study has shown that cutaneous UVB exposure results in a dose dependant increase in LC migration from exposed skin for sustained periods after UV exposure. Increasing the level of UV exposure also increased the numbers of migrating T cell subsets and cells expressing the $CD14^+$ surface molecule characteristic of macrophages. Like LC, the enhanced migration of these cells lasted for a sustained period of time but these cells were only a relatively minor population when compared to migrating DC. The functional capability of migrating DC was investigated and found to be aberrant, as they failed to present exogenous antigen and induce the proliferation of autologous primed T cells. This loss of functional activity lasted for 6-8 days after UV exposure. Further, direct effects on circulating cells were found, with the mitogen induced proliferation of PBMC reduced for 72 hours after exposure. The cause of APC functional loss was investigated but did not correspond to any reduction in B7 molecule expression on migrating DC. While no sustained production of inhibitory cytokines was detected in the culture supernatants, which corresponded to the loss of APC function, immediate transient increases in the levels IFNγ and $PGE_2$ were observed. The detection of increased IL-10-like activity and IL-10 protein was found in lymph draining UV exposed skin. *In vivo* production of this cytokine in the skin and lymph may be responsible for the failure of the migrating DC to present antigen through possible down-regulation of IL-12 production. These alterations of LC from UV exposed skin have serious consequences for cutaneous immunity, as LC play a crucial role in immunosurveillance and their subsequent loss of antigen presenting activity upon migration may lead to reduced cellular immunity, resulting in the increased potential for viral and microbial infections. Further, LC migrating from UV exposed skin may be incapable of presenting tumour antigens which arise in UV exposed skin. This, in turn, may lead to tumours evading immune responses and progressing to malignancy. These findings add to and expand our knowledge showing the detrimental effects of UV light on the immune system.

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