UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Immunosuppressive effects of skin-related factors.

by

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ABSTRACT

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IMMUNOSUPPRESSIVE EFFECTS OF SKIN-RELATED FACTORS

By Rajinder Kaur Sihota

There are potential therapeutic benefits of being able to suppress immune responses, for example immunosuppressive agents can be useful in the treatment of many inflammatory / immune-mediated diseases in skin and in other organs. Alpha-melanocyte stimulating hormone (alpha-MSH) is a 13 amino acid peptide, which has been demonstrated to have immunosuppressive activity and some studies have suggested that related melanocortin peptides may similarly be able to suppress immune reactions. Therefore, NDP-alpha-MSH (a super potent analogue of alpha-MSH), MTII and SHU 9119 (cyclic analogues of alpha-MSH) and KPV (the c-terminal tripeptide of alpha-MSH) were investigated to determine whether these are more potent than alpha-MSH alone in suppressing human immune responses *in vitro*. Each of the compounds significantly suppressed mitogen- and/or antigen-induced lymphocyte proliferation at concentrations ranging from 10^{-13}M to 10^{-7}M. There was an amount of variability between individuals in the degree of suppression by the different compounds, with each of the compounds exhibiting up to greater than 50% suppression in some subjects. However, the mean suppression ranged from 19% to 26% at 10^{-13}M and 16% to 26% at 10^{-11}M for all five compounds. Thus, although each of the compounds have immunosuppressive activity, none of these analogues of alpha-MSH seem more potent than alpha-MSH alone at suppressing antigen presenting cell (APC)-mediated lymphocyte proliferation.

Ultraviolet radiation (UVR) can also cause immunosuppression and the benefits of knowing which cells and molecules are responsible for this could lead to the development of new immunosuppressive therapeutic approaches/agents as well as helping to explain how UVR works during treatment of inflammatory skin disease. Several mechanisms for UVR-induced immunosuppression have been postulated from mouse studies, but less is known about this topic in humans and, in particular, whether effects on APCs account for suppression of immune responses following UVR exposure. Therefore, an *in vitro* model, which employed human monocytes and T-cells purified from peripheral blood mononuclear cells, was developed to examine for effects of UVR on human APCs. This demonstrated that UVR can act via APCs to suppress mitogen-induced autologous T cell proliferation. Further studies indicated that the suppression was not due to UV-induced cell death nor as a result of sub-optimal antigen presenting capacity, but instead occurred secondary to the release of a soluble factor by the monocytes which significantly suppressed anti-CD3/anti-CD28- and PHA-induced T lymphocyte proliferation. Although the soluble factor present in the supernatant was not identified, the data indicates that this was unlikely to be IL-10 or PGE_2. However, the data suggest that UVR may have some of its immunosuppressive effects in humans via its actions on APCs and could provide a focus for future investigations aiming to discover biological agent(s) which may have the potential to be used as immunosuppressive therapies in man.

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Abbreviations.

Alpha-MSH	Alpha-melanocyte-stimulating hormone
Beta-MSH	Beta-melanocyte-stimulating hormone
cAMP	Cyclic adenosine monophosphate
CHS	Contact hypersensitivity
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CPD	Cyclobutane pyrimidine dimers
CPM	Counts per minute
DMSO	Dimethyl sulfoxide
FACS	Fluorescence activated cell-sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GPCRs	Guanine nucleotide-binding protein coupled seven transmembrane receptors
HDMECs	Human dermal micro-vascular endothelial cells
ICAM-1	Intercellular adhesion molecule
IFN-γ	Interferon Gamma
IL-1β	Interleukin 1 beta
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-8	Interleukin 8

IL-10	Interleukin 10
iNOS	Nitric oxide synthase
L-NAME	Nitro-L- arginine methyl ester
L-NMMA	N-monomethyl-L-arginine acetate
LPS	Lipopolysaccharide
LTT	Lymphocyte transformation test
MACS	Magnetic activated cell sorting
MC1R	Melanocortin 1 receptor
MC2R	Melanocortin 2 receptor
MC3R	Melanocortin 3 receptor
MC4R	Melanocortin 4 receptor
MC5R	Melanocortin 5 receptor
MIP-1	Macrophage Inflammatory Proteins
MRAP	MC2R accessory protein
mRNA	Messenger Ribonucleic acid
[Nle4-dPhe7]-MSH	NDP-alpha MSH
NF-κB	Nuclear transcription factor-κB
NKT cells	Natural Killer Cells
NO	Nitric Oxide
PAF	Platelet activating factor
PBMCs	Peripheral Blood Mononuclear Cell
PC1	Prohormone convertase 1
PC2	Prohormone convertase 2

PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PHA-P	Phytohaemagglutanin
PKA	Protein kinase A
POMC	Proopiomelanocortin
RANTES	Regulated on Activation, Normal T Expressed and Secreted
ROS	Reactive oxygen species
SEM	Standard error of the mean
SI	Stimulation index
SK/SD	Streptokinase-streptodornase
TNCB	Trinitrochlorobenzene
TNF-alpha	Tumour Necrosis Factor-alpha
UCA	Urocanic acid
UV	Ultraviolet radiation
VCAM	Vascualar cell adhesion molecule

Chapter 1

1.1 Structure and function of the skin.

The skin is the largest organ in the human body and acts as a barrier to the external environment. It ranges in thickness from 0.5mm to 4.0mm depending on its location (Haake *et al* 2000). The skin is composed of two layers the epidermis and the dermis; figure 1-1.

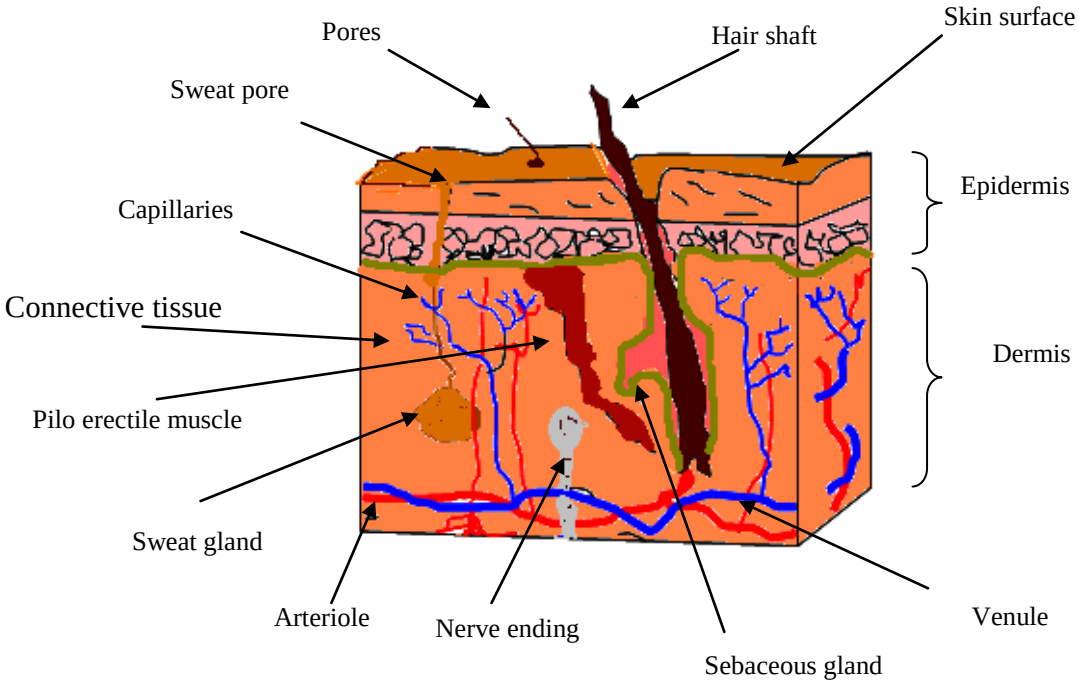


Figure 1.1: Cross section through human skin.

The epidermis consists of keratinocytes, melanocytes, Langerhans cells (the antigen presenting cells of the skin) and Merkel cells (have sensory and neuroendocrine functions) (Halata *et al*, 1993; Moll *et al* 1989). The layers of the epidermis are (starting with the innermost layer) the stratum basale, stratum spinosum, stratum granulosum and stratum corneum; on the soles of the feet and the palms of the hand the stratum lucidum is situated between the stratum granulosum and the stratum corneum (Haake *et al* 2000). The keratinocytes in the stratum basale layer divide and then move up through the epidermis changing from columnar keratinocytes to a

polygonal shape, and synthesise keratins which aggregate higher up in the epidermis. At the stratum granulosum the nuclei have degraded and lipids are released by membrane-coated granules attaching to the cell membrane. The stratum corneum makes up the outermost layer of the epidermis and serves as a barrier against UV, bacterial infection and chemicals and also keeps the skin hydrated (reviewed by McGrath *et al* 2004). The thickness of this layer varies according to location of the body, for example this layer is thicker on the palms of the hands and on the soles of the feet as these need more protection. Melanocytes are present in the basal layer and synthesise melanin (which determines skin colour). Merkel cells, which are involved in sensation are also found in this layer (Haake *et al* 2000).

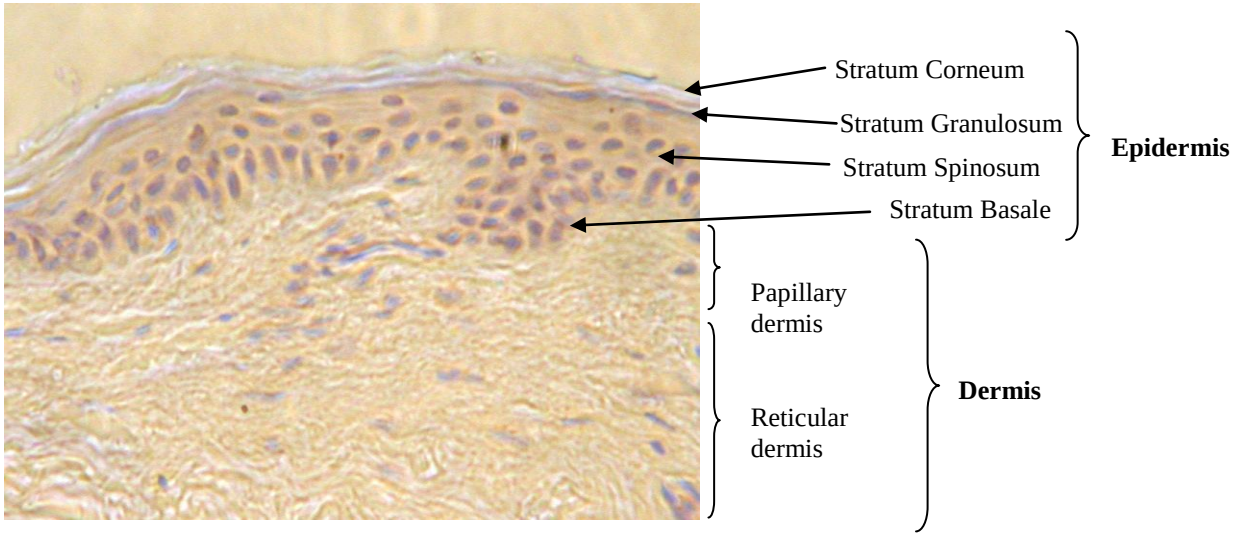


Figure 1.2: Cross section of healthy human skin from the buttock, showing the layers of the epidermis and the dermis.

The dermis is composed of connective tissue containing collagen and elastin fibres. It is connected to the epidermis by a basement membrane. The dermis varies in thickness from 3mm on the back to 0.6mm on the eyelids. The cells in the dermis include fibroblasts, macrophages, adipocytes (around sweat glands, sebaceous

glands), mast cells, lymphocytes and dendritic cells. Blood vessels, nerves, sweat glands and hair follicles are embedded in the dermis. The dermis is divided into two areas, the papillary region (adjacent to the epidermis) and the reticular region. The papillary region is composed of loosely interwoven collagen. Its surface area is increased by small finger like projections called dermal papillae, these indent the epidermis and many of these contain loops of capillaries. The reticular region is composed of dense irregular connective tissue containing coarser bundles of collagen fibres (Bolognia er al 2007). Spaces between the collagen fibres are occupied by, nerves, sweat glands, ducts and hair follicles (Haake *et al* 2000; McGrath *et al* 2004). The sub-cutaneous layer lies below the dermis and attaches the skin to the underlying fascia overlying bone and muscle. This layer supplies the dermis with blood vessels and nerves. In addition the tissue of this layer insulates the body (Haake *et al* 2000).

The functions of the skin include protection (barrier to physical agents, protects against mechanical injury, prevents the loss of body fluids, reduces penetration of UV radiation, and immunity), regulation of body temperature (thermoregulation via vasodilatation / constriction and sweating), excretion (via the sweat glands), acting as a sensory organ, and vitamin D production (Archer *et al* 1998).

1.1.1 Skin immunity.

The skin plays an important role in immunity through protecting the body against infections by acting as a barrier and by initiating immune responses when noxious foreign agents penetrate into the skin. The immune cells in the skin are the

Langerhans cells (epidermal dendritic cells) (Stingl *et al* 1977), T lymphocytes (Andrew *et al* 1949), dermal dendritic cells (Kripke *et al* 1990), macrophages (Toews *et al* 1980), B lymphocytes (David *et al* 1980), NKT cells (dermis) (Gober *et al* 2007; Askenase 2001), mast cells (papillary dermis) (Sondergaard *et al* 1971) and keratinocytes (Blauvelt *et al* 1996), (epidermis) (McGrath *et al* 2004). The Langerhans cells reside in the epidermis and are the main antigen presenting cells in the skin (Cumberbatch *et al* 1999). Immature Langerhans cells that are present in the epidermis ingest antigens and this activates and matures the Langerhans cells (Toews *et al* 1980). Langerhans cells are required to migrate to draining lymph nodes in order to present antigens they have encountered (Griffiths *et al* 2005). The cytokines TNF-alpha and IL-1β have been demonstrated to be important for Langerhans cell migration. After exposure to a contact allergen Langerhans cells release IL-1β, which acts in an autocrine fashion via the IL-1 receptor 1 (IL-1RI) expressed by Langerhans cells (Cumberbatch *et al* 1988). Furthermore IL-1β acts in a paracrine fashion on adjacent keratinocytes to stimulate the production of TNF-alpha, which in turns delivers a signal for Langerhans cell mobilisation via TNF-R2 (Cumberbatch *et al* 1999), figure 1.3.

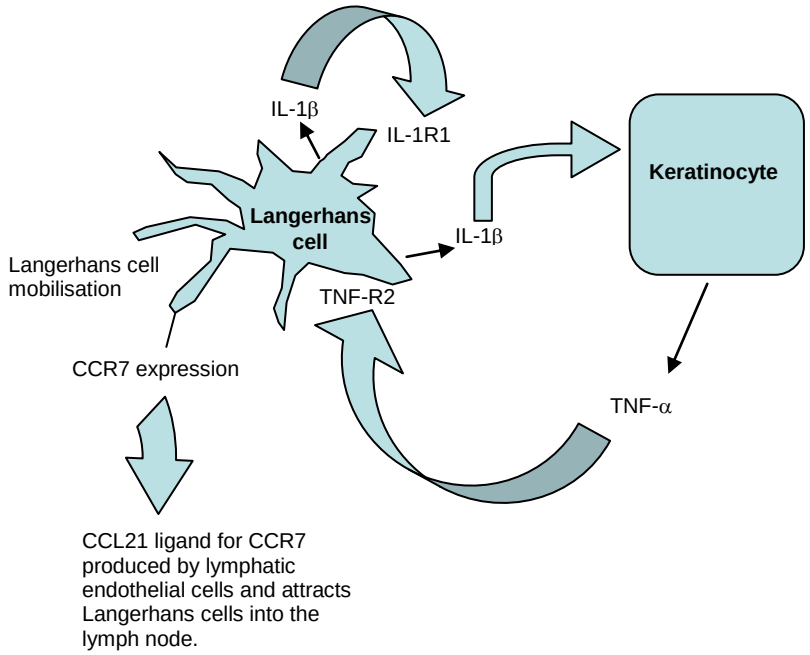


Figure 1.3: The role of Interleukin 1 (IL-1) and TNF-alpha (TNF-alpha) in Langerhans cell migration. IL-1 and TNF-alpha act in a paracrine and autocrine fashion to stimulate Langerhans cell migration.

Intrademal injection of TNF-alpha or IL-1β into murine skin results in Langerhans cell migration from the epidermis to the draining lymph node (Cumberbatch *et al* 1997; Griffiths *et al* 2005). In humans intradermal injection of TNF-alpha results in a reduction of the number of Langerhans cells in the epidermal sheets two hours later (Cumberbatch *et al* 1999). Furthermore, in mice, neutralizing antibodies against TNF-alpha or IL-1β results in an inhibition of Langerhans cell migration after applying topical antigen (Cumberbatch M *et al* 1997).

At the lymph node, the Langerhans cells present the antigen to T cells (Steinman *et al* 1995). Langerhans cells are responsible for initiating immune responses for example in allergic contact dermatitis and skin graft rejection (allogenic) (Toews *et al* 1980). Keratinocytes express MHC class II antigens (Lampert *et al* 1981) and

intercellular adhesion molecule (ICAM-1) on their surface and can therefore present antigens to infiltrating T lymphocytes (Dustin *et al* 1988), in addition keratinocytes secrete a wide variety of immunomodulatory cytokines (for example IL-1 and IL-10) that can regulate many immune responses (Enk *et al* 1993; Banerjee *et al* 2004; Parish, 1998).

Mast cells are present in most tissues, which have contact with the outside world, for example the skin, lungs, eye and digestive tract (Kitamura 1989). Mast cells contain granules rich in histamine, heparin and other mediators of inflammation, when activated they de-granulate releasing these mediators (Kitamura 1989). The release of inflammatory mediators is induced by various factors including; physical damage of the epidermis (for example due to mechanical trauma, high temperature, ionising radiation), endogenous mediators (tissue proteases derived from eosinophils and neutrophils), chemical substances (toxins-produced from bacteria or other micro-organism, proteases and venom) and finally de-granulation can be trigged by immune mechanisms (Theoharides TC & Kalogeromitros D, 2006). In allergy the IgE antibody for the antigen binds to receptors on the mast cell and cross-linking of these receptors causes de-granulation of the mast cell. This results in the wheal and flare reaction in skin, due to the release of histamine which dilates the blood vessels, activates the endothelium and increases blood vessel permeability, which causes local oedema (swelling), redness, warmth and attracts other inflammatory cells to the skin at that site (Church & Clough, 1999). In addition, de-granulation also causes itching or pain due to the stimulation of nerve endings (Eady *et al* 1998).

1.1.2 Contact allergic dermatitis.

Contact allergic dermatitis occurs when the skin is exposed to a hapten (a small molecule that is able to produce an immune response only after attaching to an endogenous protein); examples of haptens include hair dyes, fragrances and metallic compounds (for example nickel) (Anderson *et al* 1995). There are two stages in the development of contact allergic dermatitis. The first step is the sensitisation phase, the surface of the skin is exposed to the hapten, and for the hapten to be recognised by T lymphocytes it needs to be protein bound (Anderson *et al* 1995). The hapten is then ingested by the immature Langerhans cells present in the epidermis, IL-1β and TNF-alpha are released and CCR7 is up regulated on the Langerhans cells promoting the Langerhans cells to migrate towards the dermis (Yanagihara *et al* 1998; Saeki *et al* 1999). Expression of CCR7 is important for migration, as in mice lacking CCR7 there is no migration of activated Langerhans cells and subsequently no contact hypersensitivity response (Forster *et al* 1999). As the Langerhans cells migrate to the dermis and the lymph nodes they mature. CCL21, the ligand for CCR7, is produced by lymphatic endothelial cells and attracts the Langerhans cells into the T lymphocyte area of the draining lymph node (Saeki *et al* 1999). Furthermore lymph node cells produce CCL21 and CCL19 (both ligands for CCR7) which directs the Langerhans cells to the T cell area. Langerhans cells present the antigen to the T cells, in the naïve T cell area, and secrete IL-12 and IL-18 to drive TH1 polarization (Müller *et al* 1995), the specific T cells precursors expand clonally in the draining lymph node and diffuse to the blood stream, here they become memory/effector T cells. The effector T cells express CCR10 (Morales *et al* 1999), its ligand CCL27 is

produced by keratinocytes and other skin cells (Homey *et al* 2000), therefore the effector T cells are attracted to the skin and therefore migrate through the endothelial cells and into the skin. This sensitisation phase lasts up to 15 days in humans. The next phase is the elicitation phase. When the skin is exposed to the same hapten (after the sensitisation phase), the hapten is ingested by Langerhans cells and keratinocytes and these present the hapten to the effector T cells (Dustin *et al* 1988). The effector T cells are then activated in the dermis and initiate an inflammatory response by releasing type 1 cytokines (IFN-γ and IL-2) which activate resident skin cells resulting in the release of mediators, type 1 cytokines and chemokines including CCL2 and CXCL10, resulting in lymphocyte infiltration and cutaneous lesions (Parish, 1998; Wilkinson *et al* 1998; Wang *et al* 2000).

1.2 Melanocortin peptides.

Melanocortin peptides are pituitary hormones that include adrenocorticotropin (ACTH) and alpha, beta and gamma melanocyte stimulating hormones (MSH). The melanocortin peptides are involved in a range of physiological functions including pigmentation (Lerner & McGuire, 1961; Abdel-Malek *et al* 1995), modulation of inflammation (Robertson *et al* 1986; Vulliemoz *et al* 2006; Grabbe *et al* 1996), energy homeostasis, food intake (Huszar *et al* 1997; Chen *et al* 2000), temperature control (Murphy *et al* 1983; Huang *et al* 1997), exocrine gland secretion, analgesia, steroidogenesis (Mountjoy *et al* 1992; Penhoat *et al* 1989) and cardiovascular regulation (Bazzani *et al* 2001; Minako Yamaoka-Tojo *et al* 2006).

1.2.1 Proopiomelanocortin (POMC).

Melanocortin peptides are products of the cleavage of proopiomelanocortin (POMC). Although alpha-MSH and related POMC peptides had been identified previously, POMC expression was first observed in AtT-20 pituitary tumour cells in mice in 1977 (Mains *et al* 1977; Roberts & Herbert, 1977). Later in 1978 POMC was found in a human non-pituitary tumour, a cell line obtained from the pleural fluid of a patient with small cell anaplastic carcinoma of the lung (DMS-79 cell line), which secreted immunoreactive ACTH and beta-lipotrophin (Bertagna *et al* 1978). Since then it has been realised that one of the main sites of expression of the POMC gene under normal circumstances is the pituitary gland (Civelli *et al* 1982b). POMC gene expression has also been reported in other tissues, including in melanocytes and keratinocytes in the skin (Thody *et al* 1983; Slominski *et al* 1995; Chakraborty *et al* 1996), brain (Autelitano *et al* 1989; Civelli *et al* 1982a), testes, gastrointestinal tract, lung (Mechanick *et al* 1992b) and the spleen (Lolait *et al* 1986). Furthermore the POMC gene is expressed in various immune cells including lymphocytes and monocytes (Mechanick *et al* 1992a; Oates *et al* 1988; Buzzetti R *et al* 1989; Star *et al* 1995). Oates *et al* (1988) found low transcript levels of POMC in normal PBMCs and tonsillar lymphocytes, but detected the highest levels of POMC in B cells infected with human Epstein-Barr virus (EBV). An increased local production of POMC peptides has also been detected in arthritis, in viral and parasitic infections and in inflammatory skin diseases such as atopic eczema in humans (Blalock, 1989; Catania & Lipton, 1993; Smith *et al* 1992). Mutations in the *POMC* gene are rare

but are associated with obesity, alterations in pigmentation and adrenal insufficiency (Krude *et al* 1998).

Alpha-melanocyte-stimulating hormone (alpha-MSH) is a 13 amino acid peptide (Acetyl-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) which is formed by cleavage of POMC by prohormone convertases 1 and 2 (PC1, PC2) see figure 1.4. PC1 generates ACTH and PC2 cleaves the first 13 amino acids off from ACTH, yielding alpha-MSH (Benjannet *et al* 1991; Lipton & Catania, 1997). Mutations in PC1 are rare and are associated with obesity (Jackson *et al* 1997). Two forms of alpha-MSH exist in keratinocytes and in the epidermis of skin, acetyl-alpha-MSH and desacetyl-alpha-MSH. Acetyl-alpha-MSH is more potent than desacetyl-alpha-MSH in increasing adenylate cyclase in HEK 293 cells transfected with the melanocortin 1 receptor (MC1R) which is the receptor for alpha-MSH (Wakamatsu *et al* 1997). The functions of alpha-MSH are discussed in detail below.

Beta-melanocyte-stimulating hormone (beta-MSH) was discovered by Harris *et al* in 1959 (Harris, 1959). However, Scott and Lowry later demonstrated that beta-MSH is not present in the human pituitary and that the peptide isolated by Harris *et al* was in fact due to enzymic degradation of beta-liptrophin during extraction (Scott & Lowry, 1974). Bertagna *et al* later discovered that beta-MSH was present in human non- pituitary tissues including the hypothalamus (Bertagna *et al* 1986). In mice beta-MSH suppresses LPS-induced brain inflammation, by blocking NFκB translocation to the nucleus, inhibiting inducible nitric oxide synthase expression and decreasing nitric oxide production; this action of beta-MSH is thought to act via the

melanocortin receptor 4 (Muceniece *et al* 2005). Beta-MSH can also inhibit neutrophil migration in a mouse model of experimental gout (Getting *et al* 1999). Gamma-melanocyte-stimulating hormone (gamma-MSH) is expressed in the pituitary (Zhou *et al* 1993). Mice null for the *PC2* gene have low levels of circulating gamma-MSH and salt-sensitive hypertension, which suggests an important role of gamma-MSH in sodium metabolism and blood pressure control (Ni *et al* 2003). Gamma-MSH has also been demonstrated to have anti-inflammatory activity, for example gamma-MSH inhibits the increase in circulating IL-6 levels induced by endotoxin in monkeys (Vulliemoz *et al* 2006).

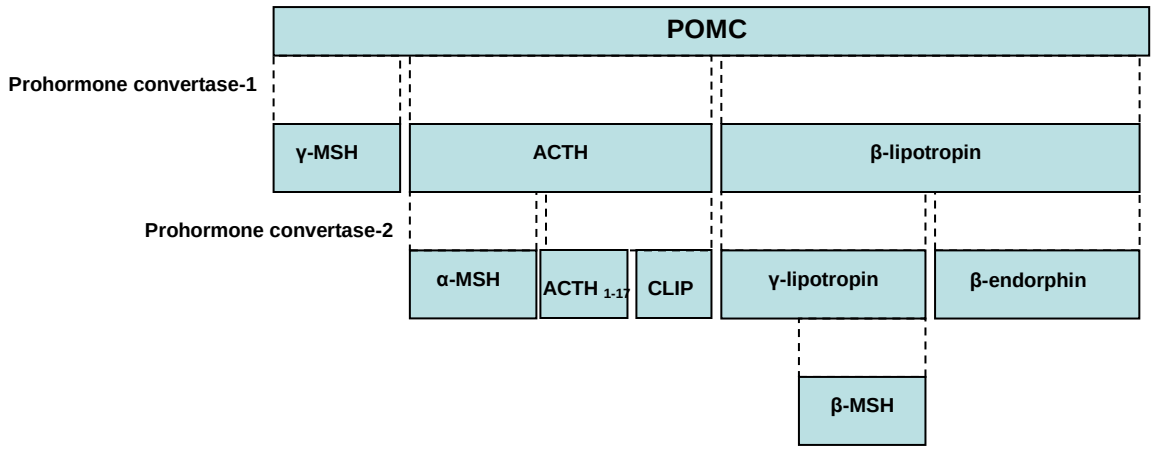


Figure 1.4: POMC is cleaved by the prohormone convertases 1 and 2 to generate the various melanocortins as depicted.

1.3 Melanocortin receptors

There are 5 melanocortin receptors, designated MC1R, MC2R, MC3R, MC4R and MC5R. These receptors belong to the class A of guanine nucleotide-binding protein (G-protein)-coupled seven transmembrane receptors (GPCRs). They are all linked to cAMP generation via the stimulatory Gαs protein and activation of adenylate

cyclase. The melanocortin receptors are expressed in a wide range of tissues (see table 1).

MCR subtype	Ligand affinity	Expression	Functions
MC1R	**Alpha-MSH> ACTH >> γ-MSH**	**melanocytes, monocytes, macrophages, neutrophils, endothelial cells, fibroblasts, mast cells and B lymphocytes**	**pigmentation antipyretic anti-inflammatory**
MC2R	**ACTH**	**adrenal cortex at sites of mineralcorticoid and glucocorticoid production**	**steriodogenesis**
MC3R	**γ-MSH = ACTH = alpha-MSH**	**Brain, placenta, heart, gut, human monocytes and mouse peritoneal macrophages**	**modulation of autonomic functions, feeding and inflammation**
MC4R	**Alpha-MSH = ACTH >> γ-MSH**	**all brain regions of mammals**	**regulation of food intake and energy expenditure**
MC5R	**Alpha-MSH > ACTH > γ-MSH**	**adrenal glands, fat cells, kidney, liver, lung, lymph nodes, bone marrow thymus, mammary glands, testis, ovary, uterus, oesophagus, stomach, duodenum, skin, lung skeletal muscle and exocrine glands. Also on B and T lymphocytes**	**regulation of exocrine secretions**

Table 1: Table of the 5 melanocortins receptors showing which cells/tissues they are expressed in and their functions, for references see text.

All of the melanocortin receptors recognise the core 4 amino acid sequence (HFRW) of the melanocortins, as shown in figure 1.5.

ACTH NH_2-SYSME**HFRW**GKPVGKKRRPVKVYPNGAEDESAEAFPLEF-OH

α-MSH Ac-SYSME**HFRW**GKPV- NH_2

β-MSH NH_2-AEKKDEGPYRME**HFRW**GSPPKD-OH

γ-MSH NH_2-YVMG**HFRW**DRF-OH

Figure 1.5: The amino acid structure of each of the melanocortins, with the common HFRW sequence highlighted.

1.3.1 Melanocortin 1 receptor (MC1R).

The human *MC1R* gene was first cloned in 1992 by Chhajlani & Wikberg and Mountjoy *et al* and encodes 317 amino acids (Chhajlani & Wikberg, 1992; Mountjoy *et al* 1992) and is located on chromosome 16q24.3 (Schioth *et al* 2003). The affinity of MC1R to its ligands is alpha-MSH ≥ ACTH > β-MSH >> γ-MSH (Chhajlani & Wikberg JE, 1992). Activation of MC1R by alpha-MSH in melanocytes stimulates adenylyl cyclase, increases intracellular cAMP and activates tyrosinase which is the enzyme important in eumelanin formation (Gantz *et al* 1993a). In addition MC1R gene expression is regulated by microphthalmia-associated transcription factor which also regulates the expression of the genes for the enzymes involved in melanin syntheses, tyrosinase and TRP-1 (Adachi *et al* 2000; Aoki & Moro, 2002). The MC1R receptor is expressed in keratinocytes (Chakraborty & Pawelek, 1993; Chakraborty *et al* 1999; Moustafa *et al* 2002; Bohm *et al* 1999a), fibroblasts, human dermal micro-vascular endothelial cells (HDMECs) (Bohm *et al* 1999b), melanocytes (Siegrist *et al* 1989; De Luca *et al* 1993), monocytes, macrophages (Bhardwaj *et al* 1997), neutrophils (Catania *et al* 1996), endothelial cells (Hartmeyer *et al* 1997), mast cells (Adachi *et al* 1999) and B-

lymphocytes and a subpopulation of the $CD8^+$ population (cytotoxic T-lymphocytes), (Neumann Andersen *et al* 2001), however, Cooper *et al* did not detect MC1R expression in T-lymphocytes cultured with streptokinase/streptodornase (Cooper *et al* 2005). Activation of MC1R by alpha-MSH on melanocytes leads to pigmentation and on immune cells activation of MC1R has an anti-inflammatory effect (discussed later).

The MC1R gene is highly polymorphic; over 100 variants of the MC1R gene have been identified, giving rise to a fair skin, red hair phenotype (discussed in detail later in this chapter).

1.3.2 Melanocortin 2 receptor (MC2R).

The *MC2R* gene encodes a 297 amino acid GPCR and is located on chromosome 18p11.2 (Mountjoy *et al* 1992). The MC2R (also known as the ACTH receptor) is activated by ACTH. Binding of ACTH to MC2R stimulates adenyl cyclase and induces increases in cellular cAMP which leads to the activation of protein kinase A (PKA) (Penhoat *et al* 1989). MC2R is expressed in the adrenal cortex at the sites of mineralocorticoid and glucocorticoid production (Mountjoy *et al* 1992; Noon *et al* 2002). Activation of MC2R stimulates steroidogenesis (Mountjoy *et al* 1992). Mc2r is also expressed in murine adipocytes (Boston & Cone, 1996) however MC2R is not expressed in human adipocytes (Boston, 1999). In addition, Chinese hamster ovary cells transfected with MC2R fail to export MC2R from the endoplasmic reticulum whereas cell surface MC2R was seen after transfection of a mouse adrenocortical cell line, Y6, with GFP-tagged MC2R, suggesting that there is a specific adrenal

accessory factor required for its surface expression (Noon *et al* 2002). Metherell *et al* 2005 later demonstrated that a MC2R accessory protein termed MRAP is required for correct expression of MC2R. Furthermore, in the presence of MRAP, non-adrenal cells lines such as fibroblasts are able to express MC2R in its active form properly inserted into the plasma membrane at levels similar to those found in mice adrenal cells (Forti *et al* 2006). Subjects with a mutation in MRAP have a deficient response to ACTH resulting in a rare autosomal recessive syndrome called familial glucocorticoid deficiency (FGD) type 1, which causes hypoglycaemia and hyperpigmentation (Sherpard *et al* 1959; Metherell *et al* 2005).

1.3.3 Melanocortin 3 receptor (MC3R).

The *MC3R* gene was first cloned by Gantz *et al* and was found to encode 361 amino acids and is located on chromosome 20q13.2-q13.3 (Gantz *et al* 1993a). This receptor is expressed in brain (hypothalamus and the limbic system) (Roselli-Rehfuss *et al* 1993a) placenta, heart and gut (Gantz *et al* 1993a). In addition, MC3R is expressed by monocytes (Taherzadeh *et al* 1999) and by mouse peritoneal macrophages (Getting *et al* 1999). Gantz *et al* found a similar dose dependant increase of cAMP production following stimulation of MC1R and MC3R with alpha-MSH, beta-MSH, NDP-alpha-MSH and ACTH but that MC3R was, unlike MC1R, also activated by gamma-MSH (Gantz *et al* 1993a). In fact, the MC3R receptor is the only melanocortin receptor that is activated by γ-MSH; the potency of its agonists are γ-MSH = ACTH ≥ α-MSH (Roselli-Rehfuss *et al* 1993a). Activation of MC3R causes an increase in intracellular calcium as a result of signalling via

inositol triphosphate, as well as an increase in cellular content of cyclic AMP (Konda *et al* 1994). MC3R participates in modulation of inflammation (anti-inflammatory) and feeding (decreases weight); mice knock-outs of Mc3r have increased fat mass, reduced lean body mass, hyperleptinaemia and hyperinsulinaemia (Chen *et al* 2000; Getting, 2002).

1.3.4 Melanocortin 4 receptor (MC4R).

MC4R was cloned in 1993 and was found to be localised on chromosome 18q22 by Gantz *et al* (Gantz *et al* 1993b) and encodes 32 amino acids (Gantz *et al* 1993b). The potency of activation of MC4R by the melanocortins is alpha-MSH = ACTH > β-MSH >> γ-MSH. Activation of MC4R results in an increase in intracellular cAMP production (Gantz *et al* 1993b). MC4R is expressed in the brain including the cortex, thalamus, hypothalamus, brainstem and spinal cord (Wikberg, 1999). MC4R inhibits food intake, regulates energy expenditure (Nargund *et al* 2006; Huszar *et al* 1997; Kask *et al* 1998) and modulates erectile function (Van der Ploeg *et al* 2002). Mc4r null mice are obese (and mice lacking both Mc3r and Mc4r are significantly heavier than Mcr4-/- mice) (Huszar *et al* 1997; Chen *et al* 2000). In humans, obesity is associated with polymorphisms in the coding region of *MC4R* (Vaisse *et al* 1998; Yeo *et al* 1998).

1.3.5 Melanocortin 5 receptor (MC5R).

MC5R was cloned by Gantz *et al* and found to encode 325 amino acids (Gantz *et al* 1994). *MC5R* is positioned on chromosome 18p11.2 (Gantz *et al* 1994). The

potency of activation of this receptor is alpha-MSH ≥ ACTH >> γ-MSH. Activation of MC5R results in cAMP signalling and calcium production; the calcium signalling through MC5R is thought to be modulated via ryanodine receptor interaction (Hoogduijn *et al* 2002). MC5R is expressed in the adrenal glands, fat cells, kidney, liver, lung, lymph nodes, bone marrow, thymus, mammary glands, testis, ovary, uterus, oesophagus, stomach, duodenum, skin, skeletal muscle and exocrine glands (Catania *et al* 2004). MC5R has also been shown to be expressed on the surface of mouse pro-B-lymphocyte cells (Ba/F3) (Buggy J J, 1998). In addition, Taylor & Namba have demonstrated that alpha-MSH induces $CD25^+$ $CD4^+$ regulatory T cells through MC5R expressed on primed T cells (Taylor & Namba, 2001). Mc5r knockout mice have a widespread dysfunction of the exocrine glands and a marked reduction in sebum (Chen *et al* 1997). Furthermore MC5R regulates the release of pheromone in urine which suppresses aggression in male mice as demonstrated by the fact that MC5R knockout mice are more aggressive when placed in urine from a MC5R knockout scented cage than MC5R knockout mice placed in a urine scented cage from wild type MC5R mice (Caldwell & Lepri, 2002).

1.4 The anti-inflammatory actions of alpha-MSH.

Alpha-MSH has been reported to exhibit potent anti-inflammatory activity in several tissues including skin. In the late 1980s Rheins *et al* reported that, when topically applied to mice, alpha-MSH inhibits contact hypersensitivity (CHS) responses to the contact sensitiser dinitrofluorobenzene (Rheins *et al* 1989). Grabbe *et al* (1996) demonstrated that systemic alpha-MSH induces hapten-specific tolerance in mice, with a reduction in the initial CHS response to trinitrochlorobenzene (TNCB) and

subsequent failure to develop a CHS response to TNCB. This tolerance is prevented by anti-IL-10 administration, indicating that the immunosuppressive effects of alpha-MSH could be mediated via the anti-inflammatory cytokine IL-10 (Grabbe *et al* 1996).

Alpha-MSH has been demonstrated to have anti-inflammatory activity in a range of inflammatory disorders. Alpha-MSH has been detected at high levels in the synovial fluid of patients with rheumatoid arthritis with an inverse correlation between the severity of inflammation and the amount of alpha-MSH detected (Catania *et al* 2004; Catania *et al* 1994; Ceriani *et al* 1994),). In addition, rats with adjuvant induced arthritis (which is a preclinical model of rheumatoid arthritis) have been demonstrated to have a significant reduction in joint pathology after the administration of alpha-MSH (Catania *et al* 2004). Similarly, Getting *et al* (2002) reported that local (but not systemic) ACTH suppressed inflammation in a rat model of gouty arthritis; in this model the MC3R/MC4R antagonist SHU 9119 blocked anti-inflammatory actions of ACTH but not the anti-inflammatory action of the selective MC3R agonist γ-MSH. Raap *et al* (2003) demonstrated in a mouse model of OVA-induced airway inflammation that alpha-MSH decreased levels of allergen specific IgE, IgG1 and IgG2a, decreased eosinophil infiltration and lowered IL-4 and IL-13. In addition, alpha-MSH did not reduce OVA-induced airway inflammation in IL-10 null mice, suggesting that its effects were mediated via IL-10 (Raap *et al* 2003). In a mouse model of inflammatory bowel disease (dextran sulphate induced colitis), alpha-MSH reduced the appearance of faecal blood, inhibited weight loss and decreased production of TNF-alpha after stimulation of the lower colon with the

mitogen concanavalin A (Rajora *et al* 1997b). Furthermore, alpha-MSH reduced trinitrobenzene sulfonic acid induced-colitis in rats; this effect was reversed when the rats were pre-treated with indomethacin (cyclooxygenase-1 selective antagonist) or sodium nitroprusside (a nitric oxide donor) suggesting that this anti-inflammatory activity of alpha-MSH involved nitric oxide and prostaglandins (Oktar *et al* 2000).

Stimulation of cultured human neutrophils with interferon or lipopolysaccharide (LPS) results in the accumulation of MC1R mRNA (Catania *et al* 1996). Furthermore, alpha-MSH is able to inhibit the chemotactic peptide FMLP (f Met-Leu-Phe) and IL-8 gradient induced neutrophil migration (Catania *et al* 1996). Similarly, alpha-MSH was able to prevent the IL-1-α and TNF-alpha-mediated accumulation of neutrophils in sponges implanted subcutaneously in mice (Mason & Van Epps, 1989). Related to this, Chiao *et al* demonstrated in a rat model of renal ischemia that alpha-MSH reduced kidney tubular necrosis, neutrophil plugging and capillary congestion, principally by reducing the neutrophil chemokine KC/IL-8 and the adhesion molecule ICAM-1 at the transcriptional levels and the induction of NO synthase in the outer medulla (Chiao *et al* 1997).

1.4.1 Mechanism of action

The mechanisms through which alpha-MSH exerts its anti-inflammatory effects seem to be through a combination of inhibition of nuclear transcription factor-κB (NF-κB) signalling, inflammatory mediator production and co-stimulatory molecule expression.

1.4.2 Inhibition of intracellular NF-κB signalling by alpha-MSH.

NF-κB is retained in an inactive form bound to IκBα in the cytoplasm, but phosphorylation of IκBα (as a result of stimulation by drugs, cytokines, bacterial products and viruses) leads to the degradation of IκBα and translocation of NF-κB to the nucleus where it binds to NF-κB-responsive genes and induces production of cytokines (TNF-alpha, IL-1, IL-2, IL-3), chemokines (IL-8, MIP-1, RANTES) and adhesion molecules (ICAM-1, VCAM, E-selectin) (Ghosh & Karin, 2002). Manna and Aggarwal (1998) demonstrated that alpha-MSH inhibits NF-κB activation by inflammatory agents (LPS, okadaic acid and ceramide) in a human U937 monocytic cell line. Similarly, Kalden *et al* (1999) showed that alpha-MSH inhibits LPS-induced NF-κB activation in human microvascular endothelial cells, resulting in down regulation of adhesion molecule expression, including vascular cell adhesion molecule 1 (VCAM-1) and E-selectin. Inhibition of NF-κB by alpha-MSH has also been documented in normal human keratinocytes and in human dermal fibroblasts (Moustafa *et al* 2002; Hill *et al* 2006). However, although the addition of alpha-MSH to human dermal fibroblasts inhibited NF-κB activation, it had no effect on IκB-α localisation as determined by immuofluorescent microscopy (Hill *et al* 2006).

1.4.3 Inhibition of cytokines and adhesion molecules by alpha-MSH.

Alpha-MSH has been shown to inhibit the release of IL-1β and TNF-alpha by PBMCs after stimulation with bacterial LPS and the HIV envelop glycoprotein 120 (Catania *et al* 1998). Furthermore, central or systemic injection of alpha-MSH suppresses the increase in TNF-alpha levels in the brains of mice following

intracerebroventricular injection of LPS (Rajora *et al* 1997a). Alpha-MSH has also been shown to significantly inhibit TNF-alpha stimulated up regulation of ICAM-1 in both melanocytes and melanoma cells (Hill *et al* 2006); indeed IBMX (an inhibitor of phosphodiesterase, which causes an increase in cAMP) and forskolin (which activates adenyl cyclase, raising levels of cAMP) similarly cause ICAM-1 down-regulation, suggesting that alpha-MSH may have its inhibitory effects via cAMP signalling (Hedley *et al* 1998). Alpha-MSH inhibits IL-1 induced murine thymocyte stimulation and inhibits IL-1 induced production of prostaglandin E in human fibroblasts (Cannon *et al* 1986). In addition i.v infusion of NDP-alpha-MSH (a potent analogue of alpha-MSH) into rhesus monkeys decreases endotoxin induced IL-1, IL-6 and TNF-alpha plasma levels (Vulliemoz *et al* 2006).

Alpha-MSH can down regulate CD86 (a T cell co stimulatory molecule) expression on monocytes (Bhardwaj *et al* 1997) and also induce the release of IL-10 (Bhardwaj *et al* 1996). However, Cooper *et al* (2005) demonstrated that anti-IL-10 blocking antibodies failed to prevent the suppression of antigen-induced lymphocyte proliferation by alpha-MSH, suggesting the suppression in that system was not due to monocytes releasing IL-10. Alpha-MSH is able to reduce NO production in RAW 264.7 cultured murine macrophages stimulated with LPS and IFN-γ by inhibiting the production of NO synthase (Star *et al* 1995). Microglia cells are specialised macrophages in the CNS and alpha-MSH was found to dose-dependently decrease the production of NO, TNF-alpha and IL-6 by N9 cells (a murine microglial cell line) stimulated with LPS (Delgado *et al* 1998). Conversely, Tsatmali *et al* (2000) noted an enhanced increase in NO production by LPS stimulated B16 mouse and

FM55 human melanoma cells when preincubated with alpha-MSH, but that higher doses of alpha-MSH resulted in a decrease in NO levels. In the same investigations, when the melanoma cells were preincubated with LPS the addition of alpha-MSH resulted in less NO production. These effects were mirrored by alterations in the levels of inducible nitric oxide synthase (iNOS); in the presence of alpha-MSH the expression of iNOS mRNA was increased, but in the presence of LPS and alpha-MSH the melanoma cells had lower levels of iNOS mRNA expression compared with LPS alone.

1.5 Analogues of alpha-MSH.

There are a wide range of synthethic analogues of alpha-MSH. NDP-alpha-MSH, SHU 9119, MTII and KPV are some of the analogues of alpha-MSH that have been demomstrated in many studies to have similar or more potent effects than alpha-MSH.

1.5.1 NDP-alpha-MSH.

[Nle4-dPhe7]-MSH (NDP-alpha MSH) is a linear synthetic analogue of alpha-MSH where the methionine at position 4 of alpha-MSH is replaced with norleucine and the phenylalanine racemized to the $_{D}$-isomer at position 7 (Sawyer *et al* 1980). NDP-alpha-MSH is a potent agonist at all the melanocortin receptors except MC2R (Schiöth HB *et al* 1995) and 26 times more potent than alpha-MSH in terms of stimulating pigmentation (Sawyer *et al* 1980). There are two types of melanin in skin and hair, phaeomelanin (red-yellow) and eumelanin (brown-black). In human melanocytes NDP-alpha MSH increases the eumelanin content in melanocytes (Hunt *et al* 1995). Injection of NDP-alpha MSH into healthy human volunteers causes

darkening of the skin (Levine *et al* 1991). In subjects with *MC1R* variant alleles NDP-alpha MSH increases the melanin content to a greater degree than that in patients with a wild type *MC1R* genotype, thus it has been suggested that NDP-alpha-MSH could be useful in providing photoprotection to individuals with fair skin as there is a relatively greater increase in melanin content with NDP-alpha-MSH (FitzGerald *et al* 2006). NDP-alpha MSH also has effects on other organ systems, for example administration of NDP-alpha MSH reduces the size of the ischemic area induced by permanent coronary occlusion in a rat model (Bazzani *et al* 2001). In addition NDP-alpha MSH has been shown to decrease leukocyte infiltration into, and inflammatory mediators in, the allograft following heart transplantation in rats, thus reducing the chance of rejection (Gatti *et al* 2002; Colombo *et al* 2005). Furthermore, injection of NDP-alpha-MSH reduces LPS-induced proinflammatory cytokine production (TNF-alpha, IL-1 and IL-6) in primates (Vulliemoz *et al* 2006). Similarly, NDP-alpha MSH decreases TNF-alpha production by LPS stimulated human PBMCs (Gatti *et al* 2006).

1.5.2 Cyclic analogues; MTII and SHI9119.

MTII (Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]α-MSH (4-10)-$NH_{2)}$ and SHU9119 (Ac-Nle^4-c[Asp^5,D-Nal(2)^7,Lys^{10}]α-MSH (4-10)-$NH_{2)}$ are both cyclic analogues of alpha-MSH (Al-Obeidi F, 1989). Transfection studies of COS-1 cells transfected with either MC1R, MC3R, MC4R or MC5R show that both MTII and SHU9119 have high binding affinity for all these receptors. MTII is an agonist at MC1R, MC3R, MC4R and MC5R (Schioth *et al* 1997; Haskell-Luevano *et al* 2000). The administration of MTII causes melanogenesis via MC1R (Dorr *et al* 1996; Hruby VJ

et al 1995), inhibition of food intake via MC4R (Fan *et al* 1997), induction of anorexia via MC4R (Clegg *et al* 2003) and grooming in rats via MC5R (Adan *et al* 1999). MTII has also been shown to display anti-inflammatory effects, for example MTII suppresses monosodium urate induced neutrophil influx by suppressing the chemokine KC (a neutrophil attractant chemokine) in a murine model of gout (Getting *et al* 1999). Furthermore MTII, acting via MC3R, inhibits monosodium urate induced IL-1β, KC and neutrophil accumulation in the peritoneal cavity of mice (Getting *et al* 2001). Conversely, MTII causes penile erections in humans via MC4R (Dorr *et al* 1996).

SHU9119 is a potent agonist at MC1R and MC5R (Haskell-Luevano *et al* 2000) and an antagonist at MC3R and MC4R (Hruby VJ *et al* 1995; Schioth *et al* 1997). SHU9119 blocks MTII induced inhibition of food intake via MC3R (Fan *et al* 1997), prevents the antipyretic affects of alpha-MSH via MC3R and MC4R (Huang *et al* 1997) and prevents alpha-MSH induced protection of myocardial ischemia via MC3R (Salvatore Guarini *et al* 2002).

SHU9119 blocks MTII induced inhibition of feeding in mice, hence preventing anorexia (Clegg *et al* 2003). In the monosodium urate murine model of gout SHU9119 inhibits the anti-inflammatory action of alpha-MSH (Getting *et al* 1999). In addition SHU9119 prevents MTII inhibition of monosodium urate induced IL-1β, KC and neutrophil accumulation in the peritoneal cavity of mice (Getting *et al* 2001).

Vulliemoz *et al* demonstrated that SHU 9119 has no effect on pro-inflammatory cytokine responses in primates. These results are very interesting as they are one of

the first authors to give evidence that alpha-MSH and its analogues can modulate pro and anti-inflammatory cytokine responses in primates *in vivo*. (Vulliemoz *et al* 2006).

1.5.3 KPV

KPV is the carboxyl terminal (11-13) end of alpha-MSH and has anti-inflammatory actions but no pigmentary actions (Elliott *et al* 2004). Cultured human dermal fibroblast cells pre-treated with alpha-MSH or KPV respond with an inhibition of TNF-alpha stimulated NF-κB, (Hill *et al* 2006; Getting *et al* 2003). In addition KPV has been shown to antagonize the binding of IL-1β to its receptor, suggesting that alpha-MSH may also function as an IL-1 receptor antagonist (Getting *et al* 2003). KPV also inhibits the production of NO and the pro-inflammatory cytokines TNF-alpha and IL-6 in a microglial cell line after stimulation with LPS (Delgado *et al* 1998). Conversely, KPV improves corneal epithelial wound healing in rabbits through the production of NO, as pre-treatment with the NO inhibitor nitro-L-arginine methyl ester (L-NAME) inhibits the repairing effect of KPV (Bonfiglio *et al* 2006).

1.6 *MC1R* variants.

Alpha-Melanocyte stimulating hormone activates MC1R on melanocytes to stimulate the production of melanin. Valverde *et al* demonstrated that 80% of individuals with a red hair and/or fair skin phenotype have at least one variant in the *MC1R* gene whereas less than 20% with a black/brown hair phenotype have a *MC1R* variant (Valverde *et al* 1995). Box *et al* further investigated *MC1R* gene variants,

by studying *MC1R* gene variants in twins and found the three variants Arg151Cys, Arg160Trp and Asp294His to be most frequent and to be associated with red hair (Box *et al* 1997). Smith *et al* found 75% of Irish subjects contained a variant *MC1R* allele and 30% of these had two variant alleles, with all of the individuals with red hair having at least one of the three Arg151Cys, Arg160Trp and Asp294His variant (Smith *et al* 1998). Subsequently, Healy *et al* reported that individuals who had a wild type *MC1R* gene were able to tan more than people with one or two variant alleles (Healy *et al* 2000). Transfection and transgenic studies have identified the mechanisms responsible for these associations. For example, Schioth *et al* carried out transfection studies in COS-1 cells with the Val60Leu, Arg142His, Arg151Cys, Arg160Trp and Asp294His variants and found that these receptors were unable to stimulate cAMP production as much as the wild type receptor in response to alpha-MSH, despite alpha-MSH being able to bind to these variant receptors (Schioth *et al* 1999). However the transfection in Schioth *et al* 1999 experiments were transient, therefore the number of receptors varied per cell. Robinson and Healy confirmed Schioth *et al* 1999 experiments in stable cell transfections (Robinson *et al* 2000). Healy *et al* further confirmed these variant receptors in Mc1r deficient transgenic mice (Healy *et al* 2001).

There are 3 major types of skin cancer, melanoma, squamous cell carcinoma and basal cell carcinoma. Darker more pigmented skin is a protective factor in UV induced skin cancer. Eumelanin (brown/black) protects skin from UV induced skin damage by absorbing UV and neutralising free radicals, whereas UV induces free radical formation by the yellow/red pheomelanin (Kricker *et al* 1991). Furthermore

subjects carrying MC1R gene variants have been shown to have an increased risk of melanoma (Valverde *et al* 1996; Healy *et al* 1999). In addition Palmer *et al* found that 72% of patients with melanoma had a MC1R variant (Palmer *et al* 2000). This suggests that MC1R variants have an increased risk of skin cancer (Rees *et al* 1997). In addition Smith *et al* found that the Asp294His variant is associated with non-melanoma skin cancer (Smith *et al* 1998).

MC1R variants also have been shown to have more freckling sites (face, chest, legs, arms, abdomen, back and shoulders) than non MC1R variant individuals (Flanagan *et al* 2000). Box *et al* also found that more heavily freckled individuals had variants of MC1R (Box *et al* 2001). Furthermore Bastiaens *et al* demonstrated that the number of MC1R gene variants determined the degree of freckling (Bastiaens *et al* 2001).

Mogil *et al* demonstrated that MC1R variants have greater pain relief (analgesia) from the x-opiod, pentazocine, than non variants (Mogil *et al* 2003; Mogil *et al* 2005).

Recent work by Cooper *et al* has shown that one mechanism by which alpha-MSH may suppress immune responses to exogenous antigens might be via MC1R on monocytes/macrophages but that *MC1R* variants do not prevent this action (Cooper *et al* 2005).

1.7 Ultraviolet radiation-induced immunosuppression

Ultraviolet radiation (UV) from the sun comprises UVA, UVB and UVC, however only UVA and UVB are able to pass through the ozone layer (McKenzie et al 2003).

Exposure to UV causes a variety of effects in human skin, including sunburn, tanning, photoaging, vitamin D synthesis, immunosuppression and skin cancer (Autier et al 1999; Kligman et al 1985; Malvy et al 2000; Grant & Gruijl, 2003; Sayre et al 2007; Whiteman et al 2001). One of the earlier studies to show that UV causes immunosuppression and permits cancer growth was in 1977, where UV-treated mice rejected H-2 -incompatible tumor allografts and H-2-compatable skin allografts (Kripke et al 1977). Furthermore in the 1980s Romerdahl et al demonstrated that murine skin tumours transplanted onto naïve unirradiated mice are rejected, but not if these mice are exposed to UV prior to receiving the transplant (Romerdahl et al 1989).

There is evidence that exposure to UVB results in immunosuppression, and that UVA may also cause immunosuppression, although some controversy surrounds the effects of UVA on the immune response. Dittmar *et al* (1999) found that Langerhans cells isolated from UVB irradiated skin failed to activate naïve T cells but that Langerhans from UVA irradiated skin stimulated T cell activation. In addition, Dittmar *et al* (1999) demonstrated that exposure of mice to UVB alone results in an increase in IL-10, whereas exposure to UVA results in an increase in IFN-γ (one day after UVA) and IL-12 (three days after UVA) and that exposure to UVA and UVB together causes less IL-10 production than UVB alone. However, UVA has been reported to reduce contact hypersensitivity to nickel in human skin (Damian *et al* 1999; Nghiem *et al* 2001) which is similar to the reduced contact hypersensitivity observed following exposure to UVB (Toews *et al* 1980; Friedmann 1981; McLoone *et al* 2005; Macve *et al* 2004; Palmer *et al* 2004).

1.7.1 Mechanisms of UV-induced immunosuppression

There seem to be a number of mechanisms involved in UV-induced immunosuppression, which can be listed according to the cell involved (T regulatory cells, keratinocytes, Langerhans cells) or according to the relevant molecules (urocanic acid, cyclobutane pyrimidine dimers, ICAM-1, reactive oxygen species, nitric oxide, platelet activating factor, prostaglandins, CD80 and CD86).

1.7.2 Urocanic acid.

Urocanic acid (UCA), a metabolic product of histidine, is abundant in the stratum corneum. UCA cannot be broken down as there are no enzymes present for the catabolisation of UCA in the stratum corneum (Scott, 1981). After exposure to UVB trans-UCA is isomerised to cis-UCA and tape-stripping the epidermis to remove the stratum corneum (and so removing UCA) prevents UVB induced immunosuppression (De Fabo & Noonan, 1983). In addition Kondo *et al* demonstrated that injection of cis-UCA into the dermis of mice results in a decrease of CHS, this mimics the immunosuppressive affect of UVB (Kondo *et al* 1995).

Furthermore Moodycliffe *et al* have demonstrated that administration of neutralising antibodies to TNF-alpha before UVB results in a decrease in the number of dendritic cells in the draining lymph node as well as preventing UVB induced suppression of CHS to oxazolone (Moodycliffe *et al* 1994), demonstrating the important role of TNF-alpha in migrating Langerhans cells. In addition, injection of neutralising anti-TNF-alpha antibodies intraperitoneally and injection of cis-UCA acid intradermally

results in a CHS response to DNFB, therefore this implies that UVB irradiation converts trans-urocanic acid to cis-urocanic acid which stimulates the local release of TNF-alpha which impairs the induction of CHS (Kurimoto & Streilein, 1992).

1.7.3 Cyclobutane pyrimidine dimers.

The cytokine interleukin-12 (IL-12) is able to prevent UVB-induced suppression of the CHS response in mice (Schwarz *et al* 2005). In contrast, IL-12 is not able to overcome the UVB induced reduction in the CHS response in xpa deficient mice which are unable to repair DNA photoproducts after UV exposure (Schwarz *et al* 2005). UVB causes DNA damage by generating cyclobutane pyrimidine dimers (CPD) in DNA (Stege *et al* 2000; Kulms *et al* 1999) and it has been shown that IL-12 reduces the number of CPDs in UVB irradiated skin, which taken in combination with the lack of effect in xpa deficient mice suggests that IL-12 may have its actions by promoting DNA repair. In addition, the application of liposomes containing the dimer-specific DNA repair enzyme T4 endonuclease V to UV irradiated mice decreased the number of CPD in the epidermis and prevented the suppression of CHS (Kripke *et al* 1992). Taken together, these studies suggest that UV causes immunosuppression via the generation of CPDs. In a related study, Vink *et al* used fluorescein isothiocyanate (FITC) as a contact sensitiser to follow the migration of antigen presenting cells in the skin to the draining lymph node in mice and found that the draining lymph node dendritic cells contained CPD following UV exposure (Vink *et al* 1996). This raises the question as to whether UVB induced DNA

damage in Langerhans cells is the main mechanism underlying UV-induced immunosuppression or whether the DNA damage of the epidermal keratinocytes plays a greater role in this process. Related to this, a decrease in ICAM-1 expression in antigen presenting cells following UVB has been reported (Fujihara *et al* 1996; Tang & Udey, 1991). This decrease in ICAM-1 expression was greater when more CPD were present and the addition of liposomes containing photolyase, an enzyme that removes CPD, after UVB lead to ICAM-1 expression being restored to control levels (Grewe *et al* 2000).

1.7.4 Reactive oxygen species.

UV irradiation of skin results in the generation of reactive oxygen species (ROS). ROS are very unstable, they react aggressively with other molecules resulting in cross linking between proteins and DNA, single-strand breaks in DNA, peroxidation of lipids and loss of sulfhydryl groups in protein, resulting in inactivation of enzymes and increased proteolysis (Halliwell, 1999). UVB radiation results in increased ROS production in skin cells (Masaki *et al* 1995) but can also cause reductions in some enzymes (catalase and superoxide dismutase) which deal with ROS (Masaki *et al* 1995). It has been reported that free radical scavengers prevent UV-induced Langerhans cell depletion in the epidermis, suggesting that ROS are important in mediating UV-induced immunosuppression (Yuen & Halliday, 1997).

1.7.5 Nitric Oxide.

Nitric oxide is generated in increased amounts after UV exposure (Yuen *et al* 2002). Yuen *et al* noted that topical application of the nitric oxide inhibitor, N-monomethyl-L-arginine acetate (L-NMMA), to mice prior to UV irradiation resulted in an inhibition of UV-induced suppression of CHS (Yuen *et al* 2002). Furthermore Halliday *et al* demonstrated in humans that topical application of L-NMMA before UV irradiation also resulted in less suppression of the CHS to nickel (Halliday *et al* 2004). Therefore, UV induced nitric oxide production appears to be an important factor in UV-induced immunosuppression.

1.7.6 Platelet activating factor.

Lymph nodes cells transferred from UVB irradiated FITC-sensitised mice to naive mice show a transfer of immune tolerance (Matsumura *et al* 2006; Gorman *et al* 2006). UVB stimulates keratinocytes to release platelet activating factor (PAF) and injection of a PAF antagonist prior to transfer of lymph node cells from UV irradiated mice prevents this transfer of tolerance (Matsumura *et al* 2006). In addition, as stated above, UV radiation results in generation of ROS which leads to lipid peroxidation and Walterscheid *et al (2002)* have reported that UV irradiation of keratinocytes results in oxidation of phosphatidylcholine, a membrane lipid, leading to the generation of PAF-like lipids, which bind to PAF receptors and activate cytokine synthesis. Furthermore the same group showed that injection of PAF-like lipids into mice resulted in an upregulation of IL-10 and COX-2 gene transcription to a similar extent to that following UV irradiation. Furthermore, the addition of a PAF

antagonist prior to UV irradiation prevented the upregulation of IL-10 and COX-2 suggesting that PAF may play a role in UV-induced immunosuppression (Walterscheid *et al* 2002).

1.7.7 CD80 and CD86

CD80 and CD86 are important co-stimulatory molecules expressed on monocytes; after activation they cause an upregulation of the IL-2 receptor promoting T lymphocytes to proliferate. Previous experiments have shown that UVB irradiated monocytes have a decreased expression of CD80 and CD86 (Fujihara *et al* 1996).

1.7.8 Prostaglandins.

Prostaglandins have many effects, including pro-inflammatory and anti-inflammatory activities. After UVB irradiation there is an increase in prostaglandin E_2 (PGE_2) in human skin and this is one of the major mediators of UVB-induced immunosuppression (Black AK *et al* 1978; Rhodes *et al* 1995). PGE_2 is also released by monocytes after UVB radiation *in vitro* but causes a decrease in pro-inflammatory cytokines including IL-1 and TNF-alpha (de Waal Malefyt *et al* 1991; Hart *et al* 1993). Furthermore, the addition of PGE_2 to non-UVB irradiated monocytes causes a similar inhibition of ICAM-1 expression to that seen after UVB exposure (Grewe *et al* 2000). Therefore prostaglandins may play a part in UV-induced immunosuppression.

1.7.9 Effects on T cells.

T cells isolated from UV irradiated-antigen-immunised mice release IL-4 and lower levels of IFN-γ and IL-2 when stimulated with antigen *in vitro* indicating UV radiation results in a shift toward a Th2 type immune response (Araneo *et al* 1989). Furthermore, UV irradiated Langerhans cells were not able to present antigens to Th1 clones (Simon *et al* 1990), suggesting that UV alters the normal Th1 and Th2 balance. IL-10, which has been considered a Th2-derived cytokine, has been reported to be elevated in the serum of mice after UV exposure (Rivas & Ullrich, 1994) with UV-induced immunosuppression absent in mice lacking IL-10 (Beissert *et al* 1996). In addition, the injection of anti-IL-4 or administration of anti-IL-10 antibodies abrogated UV induced immunosuppression of CHS suggesting that Th2 cells are important for the reduced immunity following UV (Rivas & Ullrich, 1994; Shreedhar *et al* 1998a). However, UV irradiated keratinocytes produce significant amounts of IL-10, but do not produce IL-4 (Rivas *et al* 1994). Through a combination of experiments, Shreedhar *et al*. (1998a) went on to show that UV causes the formation of PGE_2 which induces the production of IL-4 which in turn induces IL-10 production resulting in immunosuppression.

1.7.10 T regulatory/suppressor cells.

Another source of IL-10 are T regulatory cells, which are a subset of T cells (FOXP3 CD25hi) that suppress the immune response. There is an increase in T regulatory cell numbers after UV irradiation (and there is some evidence that they can

immunosuppress via release of IL-10 (Schwarz *et al* 2005). Earlier, Elmets *et al* demonstrated that UVB irradiated mice develop hapten-specific tolerance and that these mice cannot be resensitised against the same hapten several weeks later (even if the hapten is applied on a non-UVB irradiated skin site); no CHS response to the hapten was seen after the adoptive transfer of spleen cells from UVB-irradiated hapten-exposed mice to naive mice suggesting the development of suppressor cells (now known as regulatory T cell) following UV (Elmets *et al* 1983). Furthermore Shreedhar *et al* (1998b) UV irradiated mice and exposed them to FITC and then transferred T cell clones from these mice to naïve recipient mice, which resulted in suppression of the induction of CHS against FITC, consistent with these clones being regulatory T cells. Injection of UV-induced T regulatory cells into the ears of mice sensitised to another antigen did not cause a suppression of CHS demonstrating that the T regulatory cells are hapten specific, however, when the ears of oxazolone sensitised mice were injected with DNFB specific T regulatory cells and painted with DNFB prior to challenge with oxazolone, the CHS was suppressed, suggesting that once the T regulatory cells are activated antigen-specifically they can suppress in an antigen independent manner (Shreedhar *et al* 1998b). Schwarz *et al* also demonstrated that UV irradiation of mice lacking expression of FAS or FAS-L results in no UV induced immunosuppression. Furthermore they showed that after UV irradiation of normal mice, culture of their T lymphocytes with hapten pulsed bone marrow derived dendritic cells results in cell death of the dendritic cells, but that if the dendritic cells are isolated from mice lacking FAS or FAS-L there is no induction of cell death of the dendritic cells by the T cells (Schwarz *et al* 1998).

This suggests that UV induced regulatory T cells may exert their suppressive function by inducing cell death of antigen presenting cells via the Fas/ Fas-L Fas pathway.

1.7.11 Keratinocytes

Schwarz *et al* (1986) demonstrated that injection of cell supernatants from UV irradiated keratinocytes, but not from fibroblasts or macrophages, into mice results in a suppression of CHS and that addition of indomethacin prior to UV exposure does not abolish the suppression suggesting that prostaglandins are unlikely to be the relevant immunosuppressive factor (Schwarz *et al* 1986). In the 1990s several groups demonstrated that UVB promotes the release of IL-10 from keratinocytes *in vitro* and *in vivo* (Kang *et al* 1998; Beissert *et al* 1995). Furthermore IL-10 mRNA is increased in keratinocytes that have been UVB irradiated, and supernatants from UVB cultured keratinocytes are able to suppress IFN-γ production by antigen activated TH1 cell clones (Rivas & Ullrich, 1992). In addition Rivas *et al* reported that injection of supernatants from UVB irradiated keratinocytes into mice inhibits CHS, but that injection of anti-IL-10 antibodies along with UVB irradiated keratinocyte supernatant, resulted in no immunosuppression (Rivas & Ullrich, 1992). Enk *et al* further demonstrated that UVB irradiated mouse skin produces high levels of IL-10 which is further enhanced with the application of a hapten to the skin (Enk & Katz, 1992). However, Kang *et al* demonstrated that the most potent secretion and production of IL-10 in the epidermis was from UVB irradiated macrophages

(Kang *et al* 1994). Krutmann *et al* also reported that human keratinocytes have reduced expression of ICAM-1 after UV irradiation (Krutmann *et al* 1992). In addition UV irradiation of keratinocytes induces IL-1 (Kupper *et al* 1987), which has been shown to enhance PGE_2 synthesis (Pentland & Mahoney, 1990) which can suppress CHS.

1.7.12 Langerhans cells.

Langerhans cells are present in the epidermis, and are the primary antigen presenting cells in the skin. Langerhans cells present foreign molecules to T cells by migrating through the dermis into the draining lymph nodes (Yanagihara *et al* 1998). UV irradiation of Langerhans cells *in vivo* results in DNA damage in these cells and DNA damaged Langerhans cells are present in the skin draining lymph nodes after UV irradiation (Toews *et al* 1980). It is well known that UVB triggers a decrease in Langerhans cell numbers in skin of mice and humans; (Toews *et al* 1980; Freidmann 1981; McLoone *et al* 2005; Macve *et al* 2004; Cumberbatch M *et al* 1997; Moodycliffe *et al* 1994). There is some evidence that these UV-irradiated Langerhans cells are functionally altered in that Dittmar *et al* (1999) found that Langerhans cells isolated from UVB irradiated skin failed to activate naïve T cells.

1.8 Aims.

Alpha-MSH is known to have anti-inflammatory activity (Catania & Lipton, 1993; Catania *et al* 2004; Getting, 2006). Cooper *et al* demonstrated that alpha-MSH was able to suppress antigen induced lymphocyte proliferation in human individuals

however the potency of alpha-MSH varied between individuals from 0% to 80% suppression (Cooper *et al* 2005). In the same study, some preliminary evidence suggested that the SHU9119 and MTII analogues of alpha-MSH might be more potent immunosuppressives than alpha-MSH alone. However, NDP-alpha-MSH is a potent analogue of alpha-MSH and previous work has demonstrated that the tri-terminal peptide sequence of alpha-MSH, KPV, has anti-inflammatory activity Therefore, the aim of the first part of this thesis was to determine whether any or all of these analogues of alpha-MSH had greater immunosuppressive effects than alpha-MSH alone.

In the second part of the thesis, the aim was to examine UV-induced immunosuppression in more detail. It is appreciated from animal models that UV causes cutaneous immunosuppression through a variety of mechanisms, but there is limited information on what happens in humans and in particular whether the effects of UV are via its actions on antigen presenting cells or via another mechanism. Thus the specific question here was to identify whether UV directly suppressed the function of human antigen presenting cells. It was acknowledged that in order to address this, an *in vitro* UV / cell culture system was required, therefore an additional aim was to develop a culture system which would allow the direct and indirect effects of UV on antigen presenting cells to be investigated.

Chapter 2

2.1 Chemicals and solutions.

Glassware was sterilised by heating to 160^{o}C for one hour. Sterile plastics including universal and Falcon centrifuge tubes were purchased from Fisher Scientific. Disposable 1.5ml and 0.5ml polypropylene eppendorfs and pipette tips were autoclaved at 18psi for 30 minutes.

All chemicals were stored and handled as recommended by the manufacturer.

Human AB serum (Sigma-Aldrich, Dorset) was heat inactivated by placing in a 56^{o}C water bath for 20 minutes.

2.2 Cell culture.

S91 melanoma cells were maintained in DMEM (Invitrogen, Paisley, UK) supplemented with 10% heat inactivated foetal bovine serum (FBS Invitrogen, Paisley, UK), 2mM L-Glutamine (Invitrogen, Paisley, UK) and 100 U/ml penicillin and 100μg/ml streptomycin (Invitrogen, Paisley, UK) at 37^{o}C in 5% CO_2 (DMEM supplemented with the above solutions will be referred to as supplemented DMEM), appendix a.1.1.2. Cells were cultured in T75 flaks. Cells were frozen by adding DMEM containing 10% Dimethyl sulfoxide (DMSO) 20% FBS into a cyron tube and stored at -80^{o}C and finally in liquid nitrogen in storage media (appendix a.1.1.3). Cells were revived from storage by defrosting the frozen cells in a 37^{o}C water bath for 1 minute and adding the cells to 12mls of DMEM (supplemented with 10% heat inactivated FBS) and placing in a T75 flask and incubating at 37^{o}C in 5% CO_2.

Peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640 medium enriched with L-glutamine (Invitrogen Paisley, UK) supplemented with 5% heat inactivated human AB serum (Sigma-Aldrich, Dorset), 100U/ml penicillin, 100µg/ml streptomycin and 1mM sodium pyruvate (Invitrogen Paisley, UK) at 37^{o}C in 5% CO_2 (RPMI supplemented with the above solutions will be referred to as total RPMI medium), see appendix a.1.1.1.

The S91 melanoma cells were passaged at 70% confluence. The cells were passaged by firstly removing the medium from the flask and then washing the flask with room temperature, sterile PBS to remove any residual media. S91 cells were detached from the base of the flask by adding 3ml/flask of dissociation solution (non-enzymatic) (appendix a.1.1.2) and incubating at 37^{o}C for 5 minutes. Twelve ml of supplemented DMEM was added to the T75 flask and then transferred to a Falcon tube and centrifuged at 500g for 5 minutes at 4^{o}C. The supernatant was discarded and the cell pellet re-suspended in 1ml of supplemented DMEM prior to counting on a haemocytometer. 10µl of the 1ml cell suspension was placed in a haemocytometer and the cells were counted on a 4 x 4 square grid. The cell volume was adjusted to an appropriate number for subsequent culture, for example 2 x 10^5 cells in 12ml of DMEM per T75 flask.

2.3 Isolation of PBMCs.

Blood from healthy volunteers was collected in vacutainer tubes containing tripotassium EDTA (an anticoagulant, BD Biosciences, Oxford). The PBMCs were

separated from the blood by gradient centrifugation, using Lymphoprep (Axis-Shield, Norway). Ten mls of blood was layered over 10mls of lymphoprep in a 30ml universal tube and then centrifuged at 1300g for 20 minutes at room temperature. After centrifugation the PBMCs form a distinct band at the medium interface. The PBMCs were then removed from this interface using a Pasteur pipette and placed in a 50ml Falcon tube (Nunc, Germany). The PBMCs were then washed in PBS by centrifuging at 500g for 7 minutes at 4^{o}C, the supernatant was removed and the PBMCs were washed again by re-suspending the cell pellet in fresh ice cold PBS and centrifuging at 500g for 7 minutes at 4^{o}C. The supernatant was then discarded and the cell pellet was re-suspended in total medium (RPMI, supplemented with 100U/ml penicillin and 100µg/ml streptomycin and 1mM sodium pyruvate and 5% human serum, as described in 2.2). The cell viability was determined by the exclusion of 0.04% trypan blue (Sigma-Aldrich, Dorset); dead cells appear blue under the microscope. The cell number was adjusted to 1 x 10^{6} cells/ml by counting 10µl of the cell suspension on a haemocytometer, where at least 100 cells were counted to ensure accuracy.

2.4 Lymphocyte proliferation assay.

4.5 x 10^{5} PBMCs were seeded in 450µl per well in a 48 well plate (Nunc, Germany) and incubated with or without alpha-MSH, NDP-MSH, SHU 9119, MTII or KPV (all from Bachem, Germany) at the following concentrations; 10^{-13}M, 10^{-11}M, 10^{-9}M and 10^{-7}M. All cultures were performed either in duplicate or triplicate. At the start of the experiment, the PBMCs were stimulated with either 0.5/0.125 U/ml of the

antigenic mixture of streptokinase-streptodornase (SK/SD; Varidase™ Wyeth, Berkshire) or 2μg/ml of the mitogen PHA-P (Sigma-Aldrich, Dorset). Cultures were incubated at 37^{o}C in 5% CO_2 for 48 hours if stimulated with PHA, or for 6 days if stimulated with SK/SD. Six hours before the end of the culture period, to assess the cell proliferation [^{3}H] thymidine was incorporated to the cell cultures at 1μCi per ml and incubated for the reminder of the culture time at 37^{o}C in 5% CO_2. The cells in each well were mixed by pipetting up and down. 450μl from each well was divided into 3 volumes of 150μl each of which were placed in separate wells of a 96 well plate (Nunc, Germany), therefore 3 wells from the 96 well plate correspond to 1 well of the 48 well plate. The cells were then transferred onto a filter plate using a harvester (PerkinElmer's FilterMate^{TM}Universal Harvester) and the filters allowed to dry at room temperature Forty μl of scintillation fluid was added to each well and the plate sealed using an adhesive film (PerkinElmer). Counts per minute were (cpm) recorded on a Top Count NXT Microplate Scintillation Counter.

Lymphocyte proliferation was expressed as a stimulation index (SI), which was calculated as follows:

$$\frac{\text{Cpm in presence of stimulus – cpm in absence of stimulant}}{\text{cpm in absence of stimulant}}$$

the stimulus being either SK/SD or PHA.

2.5 Melanin pigmentation assay.

The melanin pigmentation assay was performed to assess the potency and viability of the alpha-MSH. Melanin is a brown pigment therefore the amount of pigmentation can be analysed using a spectrometer. S91 melanoma cells were used in this study as they are easy to grow and they pigment when stimulated through the MC1R. S91 cells were detached from the flask by discarding the medium in the flask and adding 3ml of dissociation solution for 5 minutes. 7ml of supplemented DMEM (Invitrogen, Paisley, UK) was then added to the flask and the cells were transferred to a Falcon tube and counted using a haemocytometer. The cell concentration was adjusted to 4 x 10^5 cells/ml. 1800µl of the cells (at 4x10^5 cells/ml) and 200µl of the compound (either alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 or KPV) at concentrations of 10^{-6}M and 10^{-8}M (all compounds were diluted to the correct concentration in DMEM) were added in triplicate wells (per concentration per compound) to each well of a 6 well plate (Nunc, Germany). In the control plate 200µl of DMEM media was added in replace of the compound. The cultures were then incubated at 37^{o}C in 5% CO_2.

After 5 days of culture the medium was removed and the cells were detached from the base of the flask using 500µl of cell dissociation solution. The cells were then transferred to their respective labelled eppendorfs. Taking 10µl of the solution from each eppendorf and placing this volume onto a haemocytometer determined the cell number, such that at least 100 cells were counted per eppendorf. The cells were then centrifuged at 1300g at 4^{o}C for 5 minutes. The supernatant was removed and 200µl of 1M NaOH was added to each pellet (to disrupt the cell membrane so the melanin

content could be analysed). The cells were then vortexed to ensure each pellet was completely dissolved by the NaOH.

A series of incremental concentrations (1mg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 10µg/ml, 5µg/ml and 1µg/ml) of synthetic melanin was used to correlate the spectrophotometry reading with melanin concentration.

The standard melanin concentrations and the samples were placed in separate wells of a 96 well flat-bottomed plate (Nunc Germany) and placed in a spectrometer.

2.6 MC1R sequencing.

2.6.01 Isolation of genomic DNA from blood samples.

A Qiagen DNA extraction kit was used to isolate genomic DNA from blood samples (Qiagen, W. Sussex, UK). The blood samples were left at room temperature for 10 minutes and then 200µl of blood was added to a lysis tube containing 20µl of Qiagen protease before 200µl of AL lysis buffer was added, and the solution mixed by pulse-vortexing for 15 seconds. The lysates were incubated at 56^{o}C for 10 minutes, and centrifuged for 5 seconds at 500g in a micro-centrifuge to remove droplets from the inside of the lid. Two hundred µl of absolute ethanol was then added and mixed thoroughly by pulse-vortexing for 15 seconds. Following centrifugation in a micro-centrifuge at full speed for 5 seconds, the lysate was transferred to a QIAamp mini spin column containing a membrane to which DNA adheres, thus allowing DNA to be separated from the rest of the mixture. The column was centrifuged at 600g for 1 minute, the filtrate discarded and the QIAamp mini spin column placed in a clean wash tube before 500µl of AW1 wash buffer was added and the column centrifuged

at 600g for 1 minute. The filtrate was then discarded and the mini spin column placed in a second clean wash tube, whereupon 500µl of AW2 wash buffer was added and the tube centrifuged at 1500rpm for 1 minute. The filtrate was again discarded and the QIAamp mini spin column placed in a third clean wash tube and centrifuged at full speed for 3 minutes to dry the membrane. The QIAamp mini spin column was then air-dried for 5 minutes at room temperature and placed in a clean elution tube before 50µl of elution buffer was added to the centre of the membrane. The QIAamp mini spin column was then incubated at room temperature for 1 minute and centrifuged at 600g for 1 minute to elute the DNA. A sample of the eluted DNA was electrophoresed on a 4% agarose gel to determine the integrity of the sample.

2.6.02 Gel electrophoresis.

40mls of 1x TAE (from 50x stock solution) and 1% of agarose (Bio-Rad, Hermal Hempstead, UK) was heated in a microwave oven at full power (700w) for 5 minutes. Once dissolved, the agarose was left to cool to approximately 50^{o}C before the addition of 2µl of ethidium bromide. The mixture was then poured into a Bio-Rad Perspex tray containing a comb and on polymerisation, the gel was placed in the electrophoresis tank containing 1 X TAE. Eight µl of sample DNA with 2µl of Orange G (see appendix a.1.2.2) and 10µl of a 1KB DNA ladder (Gibco-Invitrogen, Paisley) was loaded into the wells. The samples were electrophoresed for 20 minutes at 70 V and the resolved DNA was visualised under UV illumination at 305nm using a UV dual intensity transilluminator (UVP, Cambridge, UK).

2.6.04 Polymerase chain reaction (PCR)

PCR was used to amplify the genomic DNA *MC1R* sequence using specific primers for *MC1R*; *MC1R-165* forward (fw) 5'-AGAGGGTHTHAGGGCAGATCTG-3') and MC1R + 33 reverse (rev) (5-'CACACTTAAAGCGCGTGC-3'). Amplification was performed using 1x reaction buffer (Biplane, London, UK), 2mM $MgCl_2$ (Biolone, London, UK), 200mM dNTPs (Bioline, London, UK), Bio*Taq* polymerase (Bioline, London, UK), nuclease free water and 2µl template of DNA and 50ng/µl MC1R+33 primer and 50ng/µl) MC1R-165. Once this was mixed the DNA was amplified in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Warrington, UK), by denaturing for 5 minutes at 94^oC, followed by 35 cycles of 94^oC for 1 minute, 62^oC for 1 minute (annealing of primers to DNA template), 72^oC for 2 minutes, (elongation of DNA primers) followed by a 1 cycle at 72^0C for 7 minutes. To ensure the PCR had yielded the correct product (*MC1R*) DNA, a sample of the DNA was then run in an agarose gel (2.62). The predicted molecular weight of the *MC1R* amplicon was approximately 954bp.

2.6.05 Purification of DNA.

A QIAquick purification kit was used to purify the PCR products (Qiagen, W. Sussex, UK). An eppendorf centrifuge 5417R was used for each centrifugation step. Five volumes of Buffer PB were mixed with one volume of the PCR sample. This mixture was then transferred to a QIAquick spin column, which was placed in a collection tube and centrifuged for 1 minute at 500g, to bind the DNA to the membrane. The flow through was discarded and 750µl buffer PE was added to the

QIAquick column and centrifuged for 1minute at full speed to wash the column. The flow through was discarded and centrifuged for 1 minute at full speed to remove any residual ethanol from buffer PE. The QIAquick column was then placed in a 1.5ml micro centrifuge tube and air dried for 5 minutes. To elute the DNA, 30µl of Buffer EB was added to the QIAquick column and was left to stand for 1 minute and then was centrifuged, thus allowing the elution of the purified DNA into the collection tube. A sample of the DNA was resolved on an agarose gel (see above) to ensure the *MC1R* encoding DNA was still present.

2.6.06 Sequencing of PCR products

The *MC1R* gene sequence was determined with primers MC1R322fw (5'-GCGGTGCTGCAGCAGCTGG-3'), MC1R344rev (5'-TGCTGCAGCACCGCAGCC-3'), MC1R581rev (5'-ACCACGAGGCACAGCAGG-3') and MC1R715FW (5'-GGCGCTGTCACCCTCACC-3'). Sequencing was performed using a dye terminator cycle sequencing kit (Amersham Pharmacia Biotech) and a model ABI 377 automated DNA sequencer (Applied Biosystems).

2.7 Detection of cell markers using FACS.

The following antibodies were used to stain the PBMCs. Monocytes or T cels; FITC anti-Human FOXP3 (eBioscience), PE anti-CD25 (Caltag-Medsystems), APC CD4 (Caltag-Medsystems), FITC CD14 (BD Biosciences) and CD3-PE (BD Biosciences). PBMCs were treated as 2.3. Following treatment, approximately 5x 10^5 cells were added to 1ml of FACS buffer (appendix a.1.4) in FACS tubes and pelleted at 500g

for 7 minutes at 4^0C. The supernatant was removed by suction, the pellet resuspended in 50µl of block buffer (1% human serum in FACS buffer) and the tubes placed on ice for 30 minutes to block any non-specific binding. Three µl of the antibody was then added to the tubes and the cells were incubated on ice for 2 hours. The cells were then washed in 1ml FACS buffer, pelleted at 300g for 5 minutes at 4^oC and the supernatant removed. The cell pellet was re-suspended in 500µl FACS buffer and analysed on a Becton Dickson FACScan flow cytometer.

For intracellular staining of cells, 1ml of fixation/permeabilization buffer (eBioscience) was added to the cells and the cells were then left on ice for 20 minutes, this was to permeabilize the cell so the antibody could enter. The cells were washed with 2ml cold FACS buffer and centrifuged at 300g for 5 minutes at 4^oC, the supernatant was removed. The cells were washed again with cold 1x permeabilization buffer and centrifuged at 300g for 5 minutes at 4^oC. The cells were then resuspended in 80µl of cold 1x permeabilization buffer. 20µl of block buffer was then added. The cells were then mixed and incubated for 5 minutes in the refrigerator (4^oC). 10µl of the antibody to stain the intracellular marker was added to the tubes (anti-FOXP3 antibody). The cells were mixed and incubated for 30 minutes in the refrigerator. The cells were washed with 1ml of permeabilization buffer and centrifuged at 300g for 5 minutes at 4^oC. Finally the cell pellet was re-suspended in 500µl FACS buffer and analysed on a Becton Dickson FACScan flow cytometer. The florescence of 10,000 events (cells) was recorded for each sample

and the data was analysed using Win MDI5.8 computer software (Verity House Software, inc).

2.8 Isolation of monocytes from PBMCs

2.8.01 Method 1

PBMCs were isolated from human blood as described above and adjusted to $1x10^6$ cells/ml in RPMI (supplemented with 100U/ml penicillin and 100μg/ml streptomycin and 1mM sodium pyruvate with no serum). The PBMCs were then incubated in 35mm Petri dishes (NUNC) for 1 hour at 37°C in 5% CO_2. The non-adherent cells were then gently removed and pooled into a 50ml falcon tube (NUNC) centrifuged for 6 minutes at 500g 4°C and re-suspended in RPMI (supplemented with 100U/ml penicillin and 100μg/ml streptomycin, 1mM sodium pyruvate and 5% serum) to a concentration of 1million cells/ml. The adherent cells were washed twice in PBS warmed to room temperature (21°C).

2.8.02 Method 2

PBMCs were isolated as described above. Monocytes were isolated from the PBMCs using CD14 microbeads (Miltenyi Biotec) by positive selection using the autoMACS separator (see below). T lymphocytes were isolated from the non-CD14 cell population by negative selection using a T cell separation kit (Miltenyi Biotec) via the autoMACS separator (see below).

2.8.03 Positive selection of $CD14^+$ PBMCs using the autoMACS.

PBMCs were isolated from healthy humans as described above. The PBMCs were then centrifuged at 300g for 10 minutes. After removing the supernatant, the cell pellet was resuspended in 80µl of buffer (see appendix a.1.5.1) per 10^7 total cells. 20µl of CD14 Microbeads (Milteni Biotec) we added per 10^7 total cells, and the cells were mixed well and incubated for 15 minutes in the refrigerator (4^oC). The cells were then washed by adding 2ml of buffer (see appendix a.1.5.1) per 10^7 of cells and then centrifuged at 300g for 10 minutes. The supernatant was then removed and the cells were resuspended in 500µl of buffer (see appendix a.1.5.1). The cells were magnetically separated with the autoMACS Separator. This was done by firstly preparing and priming the instrument for separation (see appendix a.1.5.3). The tube containing the sample was placed under the uptake port and the 2 collection tubes were placed under the collection ports at port neg1 and port pos1. For positive selection the program 'Possel' was used on the autoMACS. The positive collection was collected from outlet port pos1.

2.8.04 Negative selection of T lymphocytes from PBMCs using the autoMACS.

The two main components of the T cell separation kit from Milteni Biotec is firstly a Biotin-Antibody Cocktail containing a cocktail of biotin-conjugated monoclonal antibodies against CD14, CD16, CD19, CD36, CD56, CD123 and Glycophorin A. Secondly anti-Biotin microbeads containing microbeads conjugated to a monoclonal anti-biotin antibody (isotope mouse IgG1). Therefore all cells except T cells will be labelled in the PBMC population.

To separate the T cells firstly**,** the number of PBMCs was determined. The PBMCs were then centrifuged at 300g for 10 minutes and the supernatant pipetted off. The cell pellet was resuspended in 40µl of buffer (see appendix a.1.5.1) per 10^7 total cells. 10µl of Biotin-Antibody Cocktail (Milteni Biotec) was added per 10^7 total cells. The cells were mixed and incubated for 10 minutes in the refrigerator (4^oC). 30µl of buffer (see appendix a.1.5.1) and 20µl of Anti-Biotin Microbeads (Milteni Biotec) per 10^7 total cells were added. The cells were then incubated for 15 minutes at 4^oC and then washed with buffer (see a.appendix a.1.5.1) by adding 10x the labelling volume and centrifuging at 300g for 10 minutes. The supernatant was then removed by pipetteing and the cells resuspended up to 10^8 cells in 500µl of buffer (see appendix a.1.5.1). The cells were then separated with the autoMACS Separator. The autoMACS was firstly prepared and primed (see appendix a.1.5.3). The tube containing the magnetically labelled cells was placed under the uptake port of the autoMACS Separator. The program used was 'Deplete'. The negative fraction (outlet port 'neg1') contained the enriched T cell population and the outlet port 'pos1' contained the magnetically labelled non-T cell population.

It was difficult to determine the cell number of the monocytes using method one and the non-CD14 population had other antigen presenting cells (for example B lymphocytes) therefore the effect of UV on APC would not be fully known therefore method 2 was used to isolate the monocytes and T cells.

2.9 UV irradiation

Human monocytes were employed as a model antigen-presenting cell to investigate the direct effects of UVR on antigen presentation. Monocytes and T lymphocytes were isolated from human PBMCs using the autoMACS system (described above). Monocytes were UV irradiated using a Philips T12 lamp at 0, 5, 10, 20, 40, 80 160 or 320mJ/cm^2. UV irradiated monocytes were cultured (i) with PBMCs in the presence of PHA, and (ii) with T cells using PHA and separately anti-CD3/anti-CD28 as stimuli (R & D systems). In addition, supernatants from UV irradiated monocytes (at 24 hours) were added to PHA/PBMC and to anti-CD3/anti-CD28 T lymphocyte cultures. Lymphocyte proliferation was assessed after 48 hours culture by [^{3}H] thymidine incorporation (described above). Trypan blue exclusion was used to determine the viability of the monocytes 24 hours and 48 hours after UVR.

2.10 Statistical analysis.

Analysis of the data was preformed using SPSS for Windows and conducted using two-way ANOVA, means, the medium, ranges, and standard errors were calculated using Statsdirect, as recommended by a statistician. P values less than p= 0.05 were regarded as significant.

Chapter 3

3.1 Introduction

There have been many reports on the anti-inflammatory/immunomodulatory activities of alpha-MSH *in vitro* and *in vivo*. For example, in the late 1980s Daynes and co-workers demonstrated that alpha-MSH was able to inhibit a range of inflammatory and immunomodulatory activities induced by IL-1 and TNF-alpha, including reducing body temperature, reducing blood levels of circulating neutrophils and suppressing contact hypersensitivity responses (Daynes et al., 1987; Robertson et al., 1988). Subsequent studies in mice confirmed that alpha-MSH could decrease contact hypersensitivity responses (Grabbe et al., 1996; Rheins et al., 1989). In addition, Sarker et al demonstrated that alpha-MSH inhibits LPS induced NFκB induction in monocytes, a T-cell line and in neutrophils isolated from human blood, as well as being able to inhibit LPS induced nitric oxide production, proteolytic enzyme release, reactive oxygen intermediate generation and expression of adhesion molecules and CD14 on monocytes (Sarkar et al., 2003). Gatti et al also demonstrated that alpha-MSH suppresses the inflammatory effects of endotoxin by inhibiting the production of TNF-alpha in LPS stimulated PBMCs (Gatti et al., 2006). Similarly, rats injected with LPS and alpha-MSH have decreased TNF-alpha plasma levels compared to those administered LPS alone (Grieco et al., 2005). More recently in monkeys alpha-MSH has been demonstrated to decrease LPS induced increases in plasma TNF-alpha, IL-1 and IL-6 cytokine levels (Vulliemoz et al., 2006). In humans, Cooper et al (2005) investigated the anti-inflammatory/immunomodulatory effects of alpha-MSH on human PBMCs and demonstrated that alpha-MSH suppresses antigen induced lymphocyte proliferation,

but found no difference in suppression between subjects with wild type, heterozygous variant, or homozygous/compound heterozygous *MC1R* alleles (Cooper et al., 2005).

Many structural analogues of alpha-MSH show activity at melanocortin receptors. NDP-alpha-MSH, an agonist at several melanocortin receptors, has been demonstrated to be more potent than alpha-MSH in terms of pigmentation (Sawyer et al., 1980). Two cyclic analogues of alpha-MSH are MTII and SHU9119, of which MTII is an agonist at MC1R, MC3R, MC4R and MC5R whereas SHU9119 is an agonist at MC1R, MC5R and an antagonist at MC3R and MC4R. KPV is the tri-terminal peptide of alpha-MSH and has been shown in many studies to be a potent suppresser of the inflammatory response, but this may not act via the melanocortin receptor system because KPV does not have the amino-acid sequence HFRW of alpha-MSH that is required for melanocortin receptor binding/activation (Hruby et al., 1987). Although it is recognised that alpha-MSH has anti-inflammatory effects in humans, it is not clear whether these analogues have greater anti-inflammatory/immunomodulatory effects than alpha-MSH alone. Furthermore, in the Cooper et al (2005) paper, preliminary evidence suggested that MTII and SHU9119 might be more potent than alpha-MSH in terms of anti-inflammatory activity, but this was based on a limited number of experiments. Hence in this current project it was decided to investigate and compare the immunomodulatory activities of alpha-MSH NDP-alpha-MSH, MTII, SHU 9119 and KPV with a view to determining whether one of these analogues might offer better potential for future development of a “melanocortin-immunosuppressive” agent for use in humans.

Binding of alpha-MSH to MC1R. MC3R, MC4R or MC5R results in an increase in intracellular cAMP (Gantz et al., 1993). Forskolin also increases intracellular cAMP levels via adenylate cyclase (i.e. independently of melanocortin receptor signalling) and has many physiological effects including suppression of mitogen induced T-lymphocyte proliferation (Kaever & Resch, 1985; Bryce et al., 1999). Therefore, the effects of forskolin were also investigated to see if this was more potent than alpha-MSH in suppressing immune responses. In addition, leptin is a peptide hormone that is produced in adipocyte cells and whose expression has been shown to be regulated/reduced by alpha-MSH (Soukas et al., 2000; Hoggard et al., 2004). Furthermore, leptin has a helical cytokine structure similar to IL-2, the leptin receptor shows sequence homology to members of the cytokine receptor super family and leptin has been reported to have an ability to stimulate immune responses (Tartaglia et al., 1995; (Santos-Alvarez et al., 1999; Martin-Romero et al., 2000). Therefore, the effects of leptin on human immune responses *in vitro* were also examined in this study.

3.2 Methods

3.2.1 Melanin pigmentation assay.

The melanin pigmentation assay was used to ensure that alpha-MSH and related compounds (NDP-alpha-MSH, MTII, SHU 9119) could stimulate pigmentation of melanoma cells, and thus to demonstrate that any lack of potency in subsequent immunosuppression assays was not due to a poorly manufactured batch of

melanocortin receptor compound; the pigmentation assay methodology is described in chapter 2, section 2.5.

3.2.2 Lymphocyte transformation assay / effects of alpha-MSH and analogues.

As described in chapter 2, section 2.3, PBMCs were isolated from the blood of healthy volunteers and stimulated with either PHA or SK/SD (streptokinase/streptodornase) in the presence or absence of alpha-MSH, NDP-alpha-MSH, MTII, SHU9119 or KPV. The concentrations of alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 and KPV used to examine for effects on suppression of lymphocyte proliferation ranged from 10^{-15}M to 10^{-7}M for PHA and (based on the Cooper et al, 2005, publication) from 10^{-13}M to 10^{-7}M for SK/SD. The effect of repeated doses of each peptide was also investigated on PHA induced lymphocyte proliferation by re-addition of the peptides to the corresponding culture wells at 2, 18, 20, 22, 24, 26, 42, 44 and 46 hours after the initial dose of the peptide; in these experiments 50µl of the medium was removed from each well and replaced with 50µl of the medium containing peptide at each time point, whereas in control wells 50µl of medium was replaced. During all LTT assays, the outer wells of each culture plate contained PBS in order to minimize edge effects and to avoid evaporation of medium in the outer test wells giving inaccurate results.

3.2.3 Leptin and forskolin experiments.

For the leptin and forskolin investigations, the experiment was performed as above except that forskolin (Sigma-Aldrich) or leptin (R & D systems) were added in

replace of the alpha-MSH. The forskolin concentrations used were 10^{-13}M, 10^{-11}M, 10^{-9}M and 10^{-7}M. Leptin was used at concentrations of 0.01ng/ml, 0.1ng/ml, 1ng/ml, 10ng/ml and 100ng/ml. In the forskolin experiments PHA or SK/SD used as a stimulant and PHA was used as a stimulant in the leptin experiments.

3.2.4 Stimuli.

PBMCs from the blood of healthy volunteers were stimulated with either the antigen SK/SD (streptokinase/streptodornase, Phoenix Pharmaceuticals), which activates $CD4^+$ T-cells or the mitogen PHA which activates $CD4^+$ and $CD8^+$ T cells. The concentrations of SK/SD and PHA were calculated by doing a titration experiment for every new batch; the concentration that was found to be non-toxic, yet stimulated lymphocyte proliferation was 1:500 and/or 1:1000 of SK/SD and 2μg/ml of PHA, therefore these concentrations were used.

3.2.5 Flow cytometry / FOXP3 experiments.

To determine whether alpha-MSH suppressed SK/SD or PHA-induced lymphocyte proliferation by increasing T regulatory cell production, FOXP3 (the T-regulatory cell transcription factor) expression was investigated in lymphocytes, stimulated with SK/SD or PHA. This was examined in the presence and absence of alpha-MSH, by intracellular staining for FOXP3 and documented using fluorescence activated cell-sorting (FACS) analysis.

3.2.6 Statistical analysis.

Statistical analysis was performed using a two-way analysis of variance, ANOVA; p-values below 0.05 were considered significant.

3.3 Results

3.3.1 Alpha-MSH and analogues containing His-Phe-Arg-Trp stimulate pigmentation of S91 melanoma cells.

S91 melanoma cells were cultured separately in the presence of alpha-MSH, NDP-alpha-MSH, MTII, SHU or KPV. As expected all the analogues containing His-Phe-Arg-Trp (alpha-MSH, NDP-alpha-MSH, MTII and SHU 9119) stimulated melanin synthesis but KPV (which only contains the 3 C-terminal amino acids of alpha-MSH) did not stimulate pigmentation (figures 3.01 and 3.02; n=3 separate experiments per compound). This is consistent with the results of previous papers that have shown that the His-Phe-Arg-Trp sequence is required to activate MC1R and to stimulate pigmentation in melanocytes/melanoma cells peptides (Hruby VJ *et al* ., 1987; Ying J *et al* ., 2003). The melanin pigmentation assay confirmed that alpha-MSH, NDP-alpha-MSH, MTII and SHU 9119 are potent inducers of pigmentation, but indicated that each of these four compounds were similar in their ability to stimulate pigmentation in S91 cells despite the fact that NDP-alpha-MSH has been reported as being a super-potent analogue of alpha-MSH (Sawyer et al., 1980). Furthermore, the pigmentation results suggested that there were no problems with the batches of the alpha-MSH, NDP-alpha-MSH, MTII and SHU 9119 compounds and that these batches were suitable to use in subsequent experiments on immunosuppression.

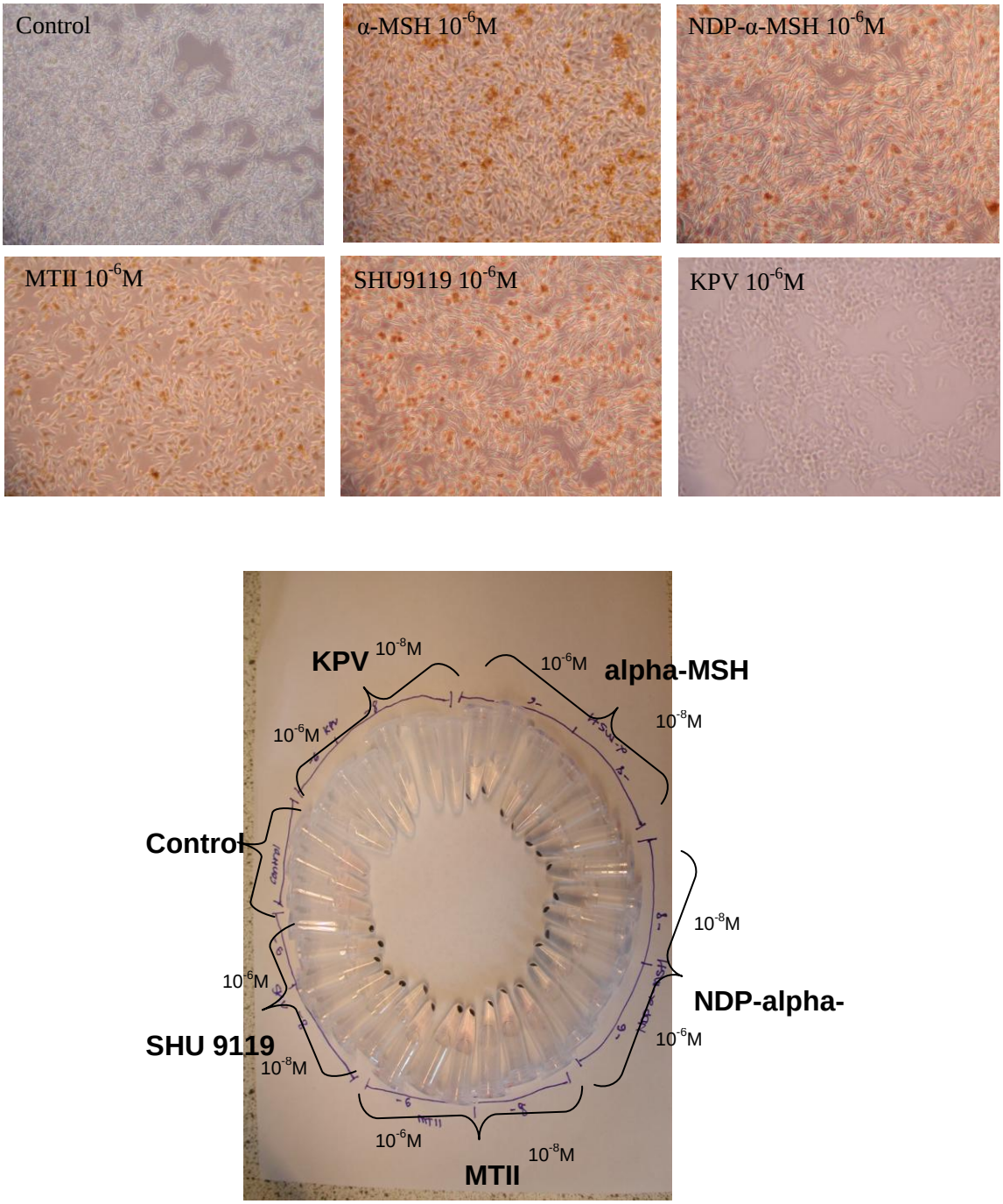


Figure 3.01: Representative photographs of S91 cells and cell pellets cultured in the presence of alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119, KPV or media alone (control); alpha-MSH, NDP-alpha-MSH, MTII and SHU 9119 stimulated melanin synthesis in these cells.

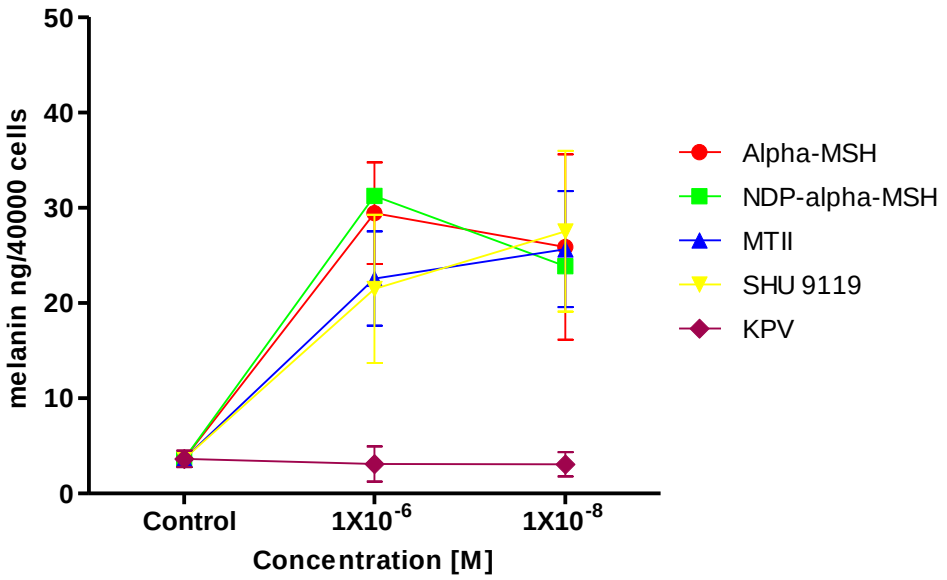


Figure 3.02: Melanin production in S91 melanoma cells. S91 cells were cultured in the presence of medium alone (control) or with alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 or KPV at 10^{-8}M and 10^{-6}M for 5 days. Quantification of the amount of pigmentation (using a spectrometer) after culture with the compounds shows that alpha-MSH, NDP-alpha-MSH, MTII and SHU 9119 stimulated melanin synthesis; n=3 experiments with triplicate wells per compound per experiment, values represent mean with SEM.

3.3.2 Alpha-MSH and its analogues inhibit SK/SD and PHA induced lymphocyte proliferation.

PBMCs were isolated from healthy human volunteers and cultured in the presence or absence of alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 and KPV at 10^{-15}M, 10^{-13}M 10^{-11}M 10^{-9}M and 10^{-7}M concentrations. The PBMCs were stimulated with either the antigen SK/SD for 6 days or the mitogen PHA for 48 hours; both of these agents require antigen presentation and cause T cell proliferation, however, PHA is a more non-specific stimulus than SK/SD which is thought to model antigen presentation *in vivo* better but which may be affected by recent clinical / sub-clinical infections with SK/SD. It was possible to detect some alterations in the cultures by

microscopy following stimulation with these agents, for example the cells tended to form clumps (thought to be proliferation of lymphocytes around monocytes/macrophages which act as antigen presenting cells, figure 3.03). Uptake of tritiated thymidine (as per chapter 2, section 2.4) was used in the cultures in order to quantify the degree of proliferation because this is incorporated into newly synthesised DNA during the S-phase of the cell cycle as the cells replicate their DNA.

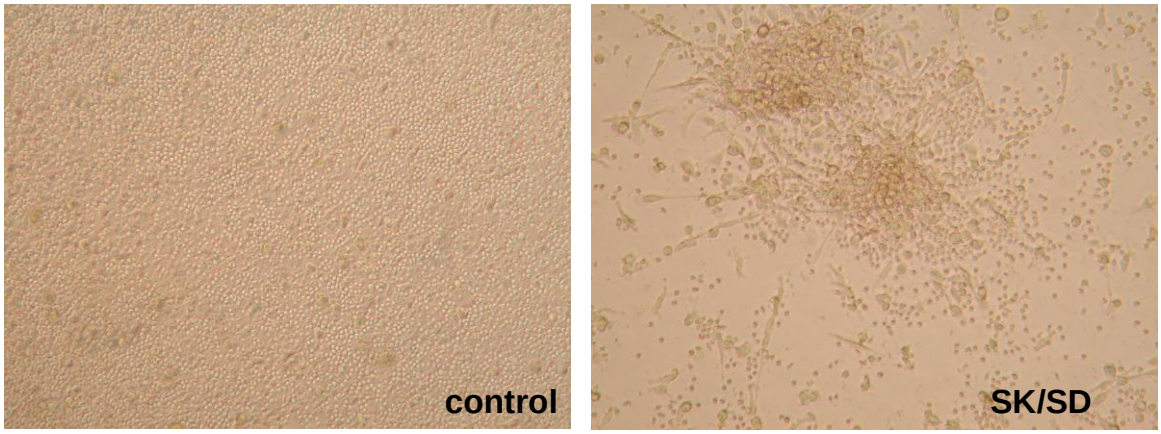


Figure 3.03: Addition of the antigen SK/SD to PBMCs stimulated the cells in culture and caused them to form clumps of cells (thought to result from a combination of antigen presentation and T-cell proliferation).

In all subjects the addition of either PHA or SK/SD resulted in a stimulation index (SI) of greater than 2 (which is used as a threshold level to label a subject allergic to an antigen when assessing allergic responses). Alpha-MSH, NDP-alpha MSH, MTII, SHU 9119 and KPV each suppressed mean SK/SD and PHA-induced PBMC proliferation to a similar extent, however there were differences in the degree of suppression by the compounds for each subject. Repeat experiments using PBMCs from certain individuals suggested that this variation was not operator dependent and that the experiments were generally reproducible. Overall, alpha-MSH suppressed

SK/SD-induced lymphocyte proliferation by 22% (at 10^{-13}M), which was to a similar degree as previously reported (Cooper et al., 2005), and decreased PHA-induced lymphocyte proliferation by 23% (at 10^{-13}M). The suppression varied between subjects ranging from 0% to 56% with alpha-MSH when PBMCs were stimulated with SK/SD and 0% to 69% when stimulated with PHA, figure 3.04 a-d. NDP-alpha-MSH reduced SK/SD-induced lymphocyte proliferation by a mean of 16% (at 10^{-13}M) and lowered PHA-induced lymphocyte proliferation by 23% (at 10^{-13}M), with NDP-alpha-MSH mediated suppression ranging from 0% to 64% in cultures with SK/SD and 0% to 59% in those with PHA, figure 3.05 a-d. MTII reduced SK/SD-induced lymphocyte proliferation by 22% (seen at 10^{-7}M) and PHA-induced lymphocyte proliferation by 26% (at 10^{-7}M), but similarly this suppression varied from 0% to 51% (SK/SD) and 0% to 69% (PHA), figure 3.06 a-d. The mean suppression by SHU 9119 was 24% (at 10^{-13}M), range 0% to 51%, and 33% (at 10^{-13}M), range 0% to 55% for the SK/SD and PHA cultures respectively, figure 3.07 a-d. KPV lowered SK/SD-induced lymphocyte proliferation by 22% (at 10^{-11}M) and PHA-induced lymphocyte proliferation by 21% (at 10^{-11}M); varying from 0% to 67% with KPV when stimulated with SK/SD and 0% to 60% when stimulated with PHA, figure 3.08 a-d.

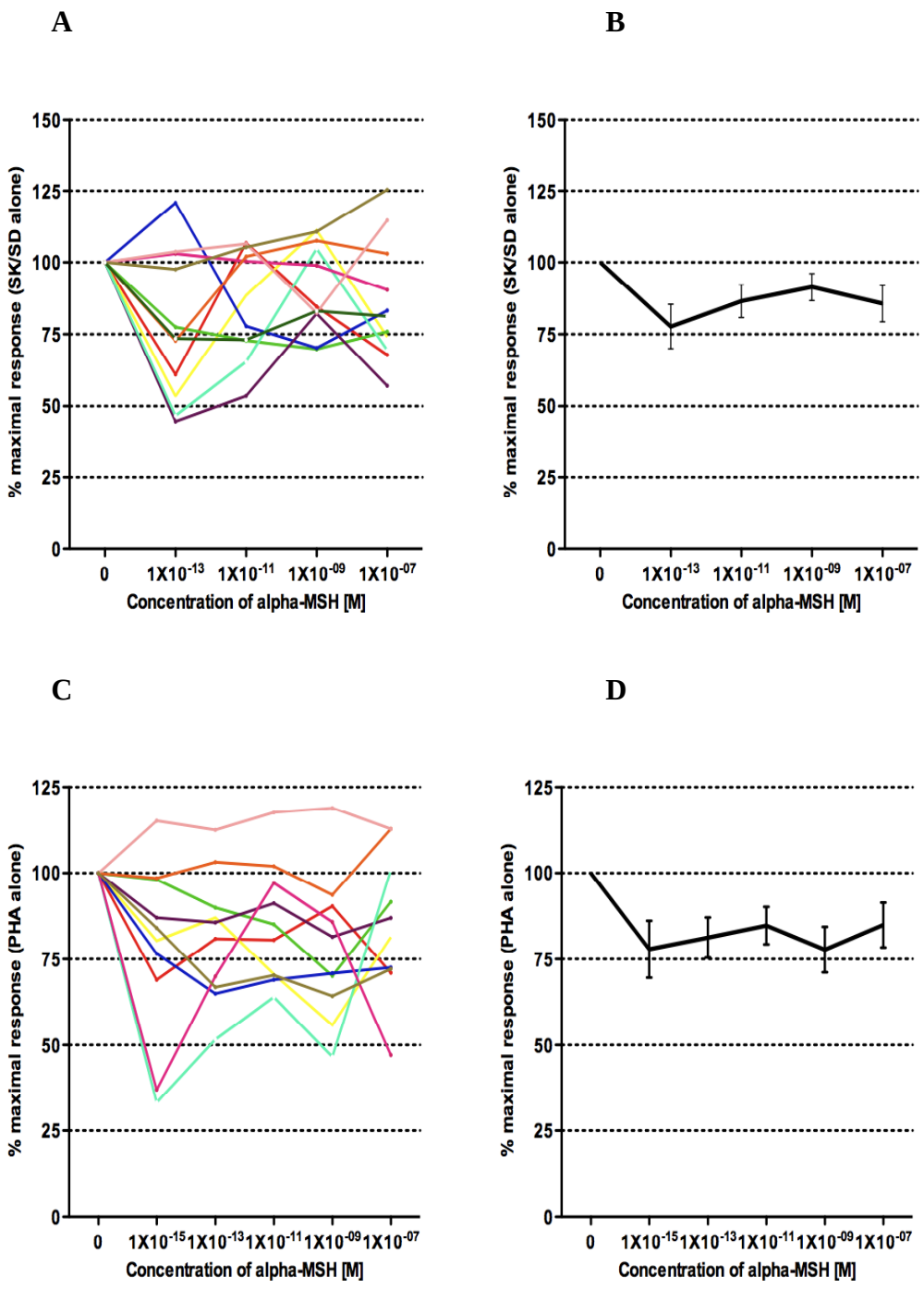


Figure 3.04: Effect of alpha-MSH on SK/SD (A and B) and PHA (C and D) induced lymphocyte proliferation in 11 subjects (SK/SD) and 10 subjects (PHA), showing each individual subject's response. Each line in (A) and (C) represents a single individual subject, error bars have been omitted for clarity, (B) and (D) show the mean suppression and the standard error of the mean (SEM). The effects of alpha-MSH at each concentration are compared to control cultures without addition of alpha-MSH, which is recorded as 100% proliferation.

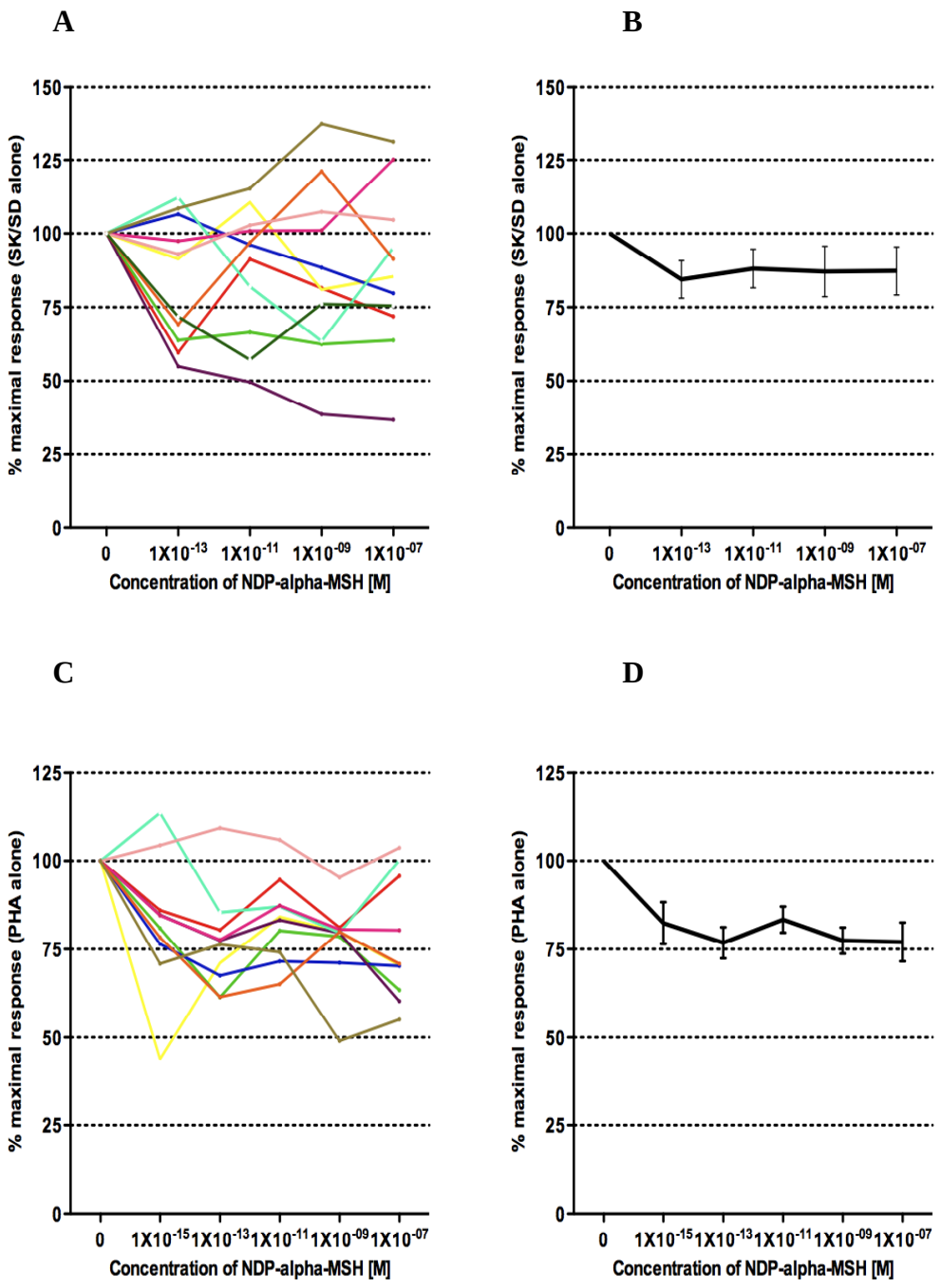


Figure 3.05: Suppression of SK/SD (A and B) and PHA (C and D) induced lymphocyte proliferation by NDP-alpha-MSH on SK/SD (A and B) and PHA (C and D) induced lymphocyte proliferation in 11 subjects (SK/SD) and 10 subjects (PHA), showing each individual subject's response (A and C). Figures (B) and (D) show the mean suppression and the SEM. The effects of NDP-alpha-MSH at each concentration are compared to control cultures without addition of NDP-alpha-MSH, which is recorded as 100% proliferation.

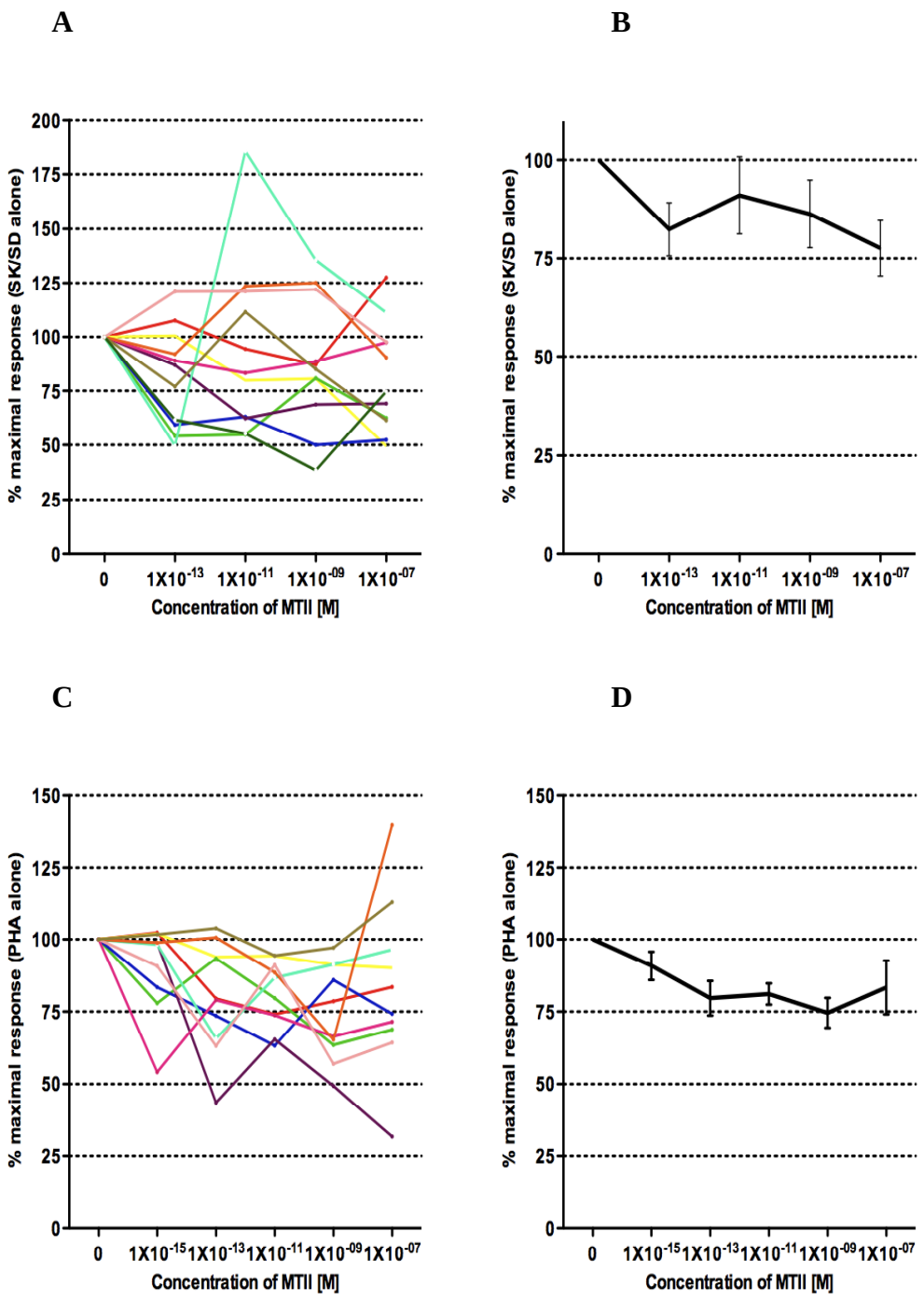


Figure 3.06: MTII induced suppression of SK/SD (A and B) and PHA (C and D) mediated lymphocyte proliferation in 11 subjects (SK/SD) and 10 subjects (PHA), showing each individual subject's response (A) and (C), error bars have been emitted for clarity. Figures (B) and (D) show the mean suppression and the SEM. The effects of MTII (at each concentration) are compared to no addition of MTII, which is taken as 100% proliferation.

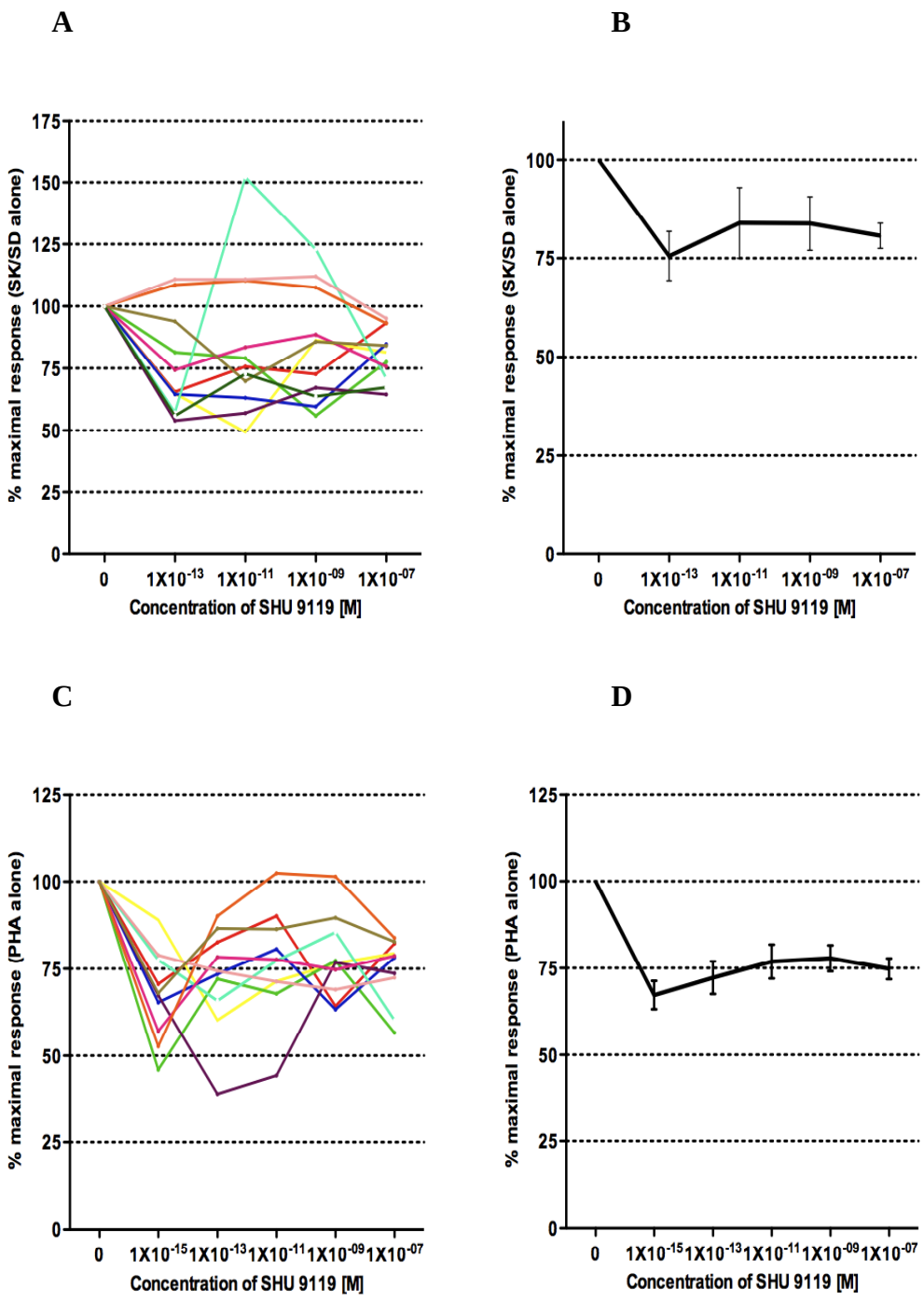


Figure 3.07: Effect of SHU 9119 on SK/SD (A and B) and PHA (C and D) induced lymphocyte proliferation in 11 subjects (SK/SD) and 10 subjects (PHA), showing each individual subject's response. Each line in (A) and (C) represents a single individual subject, error bars have been emitted for clarity, (B) and (D) show the mean suppression and the SEM. The effects of SHU 9119 (at each concentration) are compared to no addition of SHU 9119which is taken as 100% proliferation.

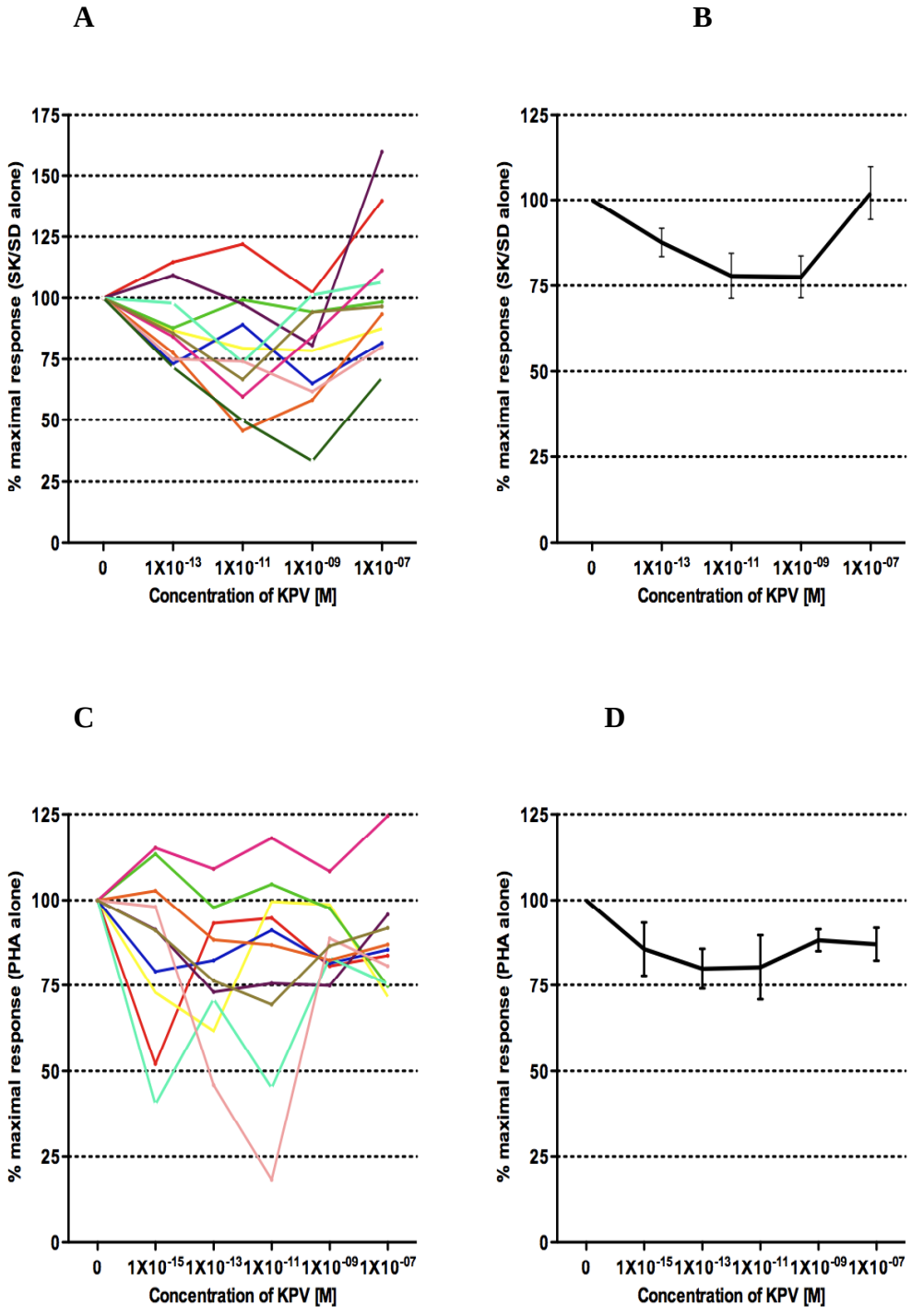


Figure 3.08: KPV induced suppression of lymphocyte cultures, stimulated with either SK/SD (A and B) or PHA (C and D). Figures (A) and (C) show each subjects response and figures (B) and (D) show the mean response and the SEM. 100% proliferation is with no addition of KPV.

Although alpha-MSH and its analogues which were used in this study seemed to suppress SK/SD-induced lymphocyte proliferation to a similar degree (mean suppression for each compound shown in figure 3.09), statistical analysis using ANOVA showed that only alpha-MSH, SHU 9119 and KPV significantly suppressed SK/SD-induced lymphocyte proliferation (alpha-MSH $p = 0.041$, SHU 9119 $p = 0.032$ and KPV $p = 0.008$), whereas the p-values for NDP-alpha-MSH and MTII were 0.132 and 0.394 respectively. All of the compounds except KPV significantly suppressed PHA-induced lymphocyte proliferation; alpha-MSH $p = 0.012$, NDP-alpha-MSH $p < 0.001$, MTII $p = 0.003$, SHU 9119 $p < 0.001$ and KPV $p = 0.126$.

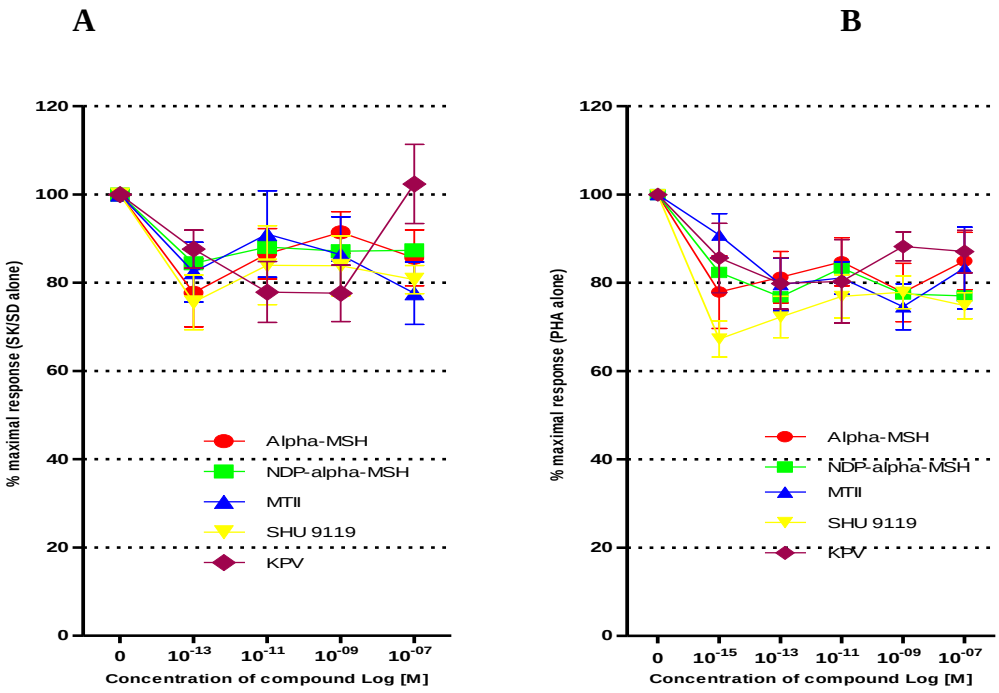


Figure 3.09: Mean suppression of (A) SK/SD- and (B) PHA induced lymphocyte proliferation by alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 and KPV (taken from figures 3.04 to 3.08).

3.3.3 Re-addition of alpha-MSH and analogues during culture.

Although suppression of SK/SD- and PHA-induced lymphocyte proliferation was observed with most of the compounds investigated, the degree of suppression was not impressive. Therefore the experimental method was subsequently modified to see whether repeated addition of the melanocortin compounds would reduce lymphocyte proliferation further; the rationale for this was because alpha-MSH and its analogues might have degraded during cell culture. Thus, in an attempt to replenish peptide concentrations, alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 and KPV were repeatedly added a total of 10 times over 48 hours to separate cultures with/without PHA; this was done by taking 50µl of the well supernatant out and adding 50µl of medium containing one of the analogues to the culture wells (50µl was also removed from the control wells and replaced with 50µl of fresh medium). Overall, re-addition of alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 or KPV did not suppress PHA-induced lymphocyte proliferation to a greater degree compared to only one addition of these peptides (figure 3.10). The suppression in the "repeated addition" experiments ranged from 0% to 44% with alpha-MSH, 0% to 54% with NDP-alpha-MSH, 0% to 48% with MTII, 0% to 51% with SHU 9119 and 0% to 52% with KPV. In the "repeated-addition" experiments only alpha-MSH, NDP-alpha-MSH and KPV significantly suppressed PHA-induced lymphocyte proliferation when the melanocortin peptide was added on a single occasion (alpha-MSH $p = 0.019$, NDP-alpha-MSH $p < 0.001$, MTII $p = 0.311$, SHU 9119 $p = 0.132$ and KPV $p = 0.003$). Interestingly, with the repeated addition of the compounds throughout the culture period, each of the compounds significantly suppressed PHA-

induced lymphocyte proliferation (alpha-MSH $p = 0.005$, NDP-alpha-MSH $p < 0.001$, MTII $p = 0.035$, SHU 9119 $p = 0.017$, KPV $p < 0.001$).

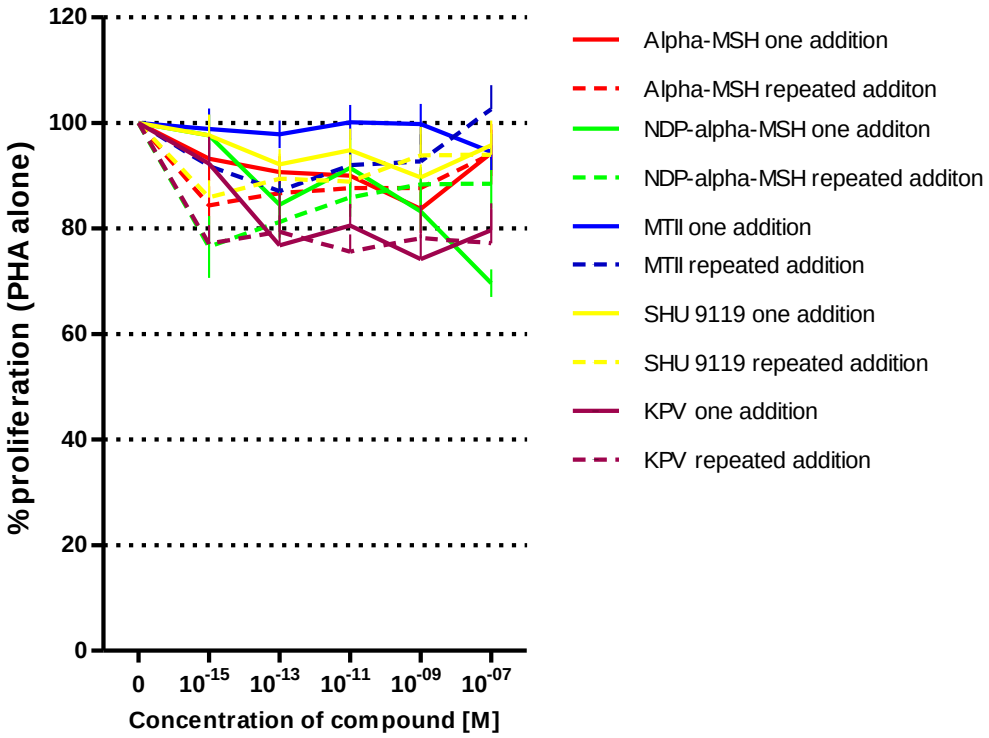


Figure 3.10: Mean effect of single addition of alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 or KPV and repeated additions of these peptides on PHA induced lymphocyte proliferation in 10 subjects. In repeated additions experiments the peptide was added 10 times during the culture period at 0, 2, 18, 20, 42, 24, 26 42, 44 and 46 hours.

3.3.4 MC1R genotype of subjects.

The degree of suppression in the above experiments with alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 and KPV varied between subjects. Alpha-MSH acts via MC1R to stimulate pigmentation and MC1R is also expressed by PBMCs (monocytes and some T lymphocytes), therefore the *MC1R* genotype of an individual might affect the degree of suppression by alpha-MSH, NDP-alpha-MSH,

MTII and SHU 9119, each of which are agonists at MC1R. Previous work has shown that there is no correlation between people with different MC1R genotypes and the degree of suppression of SK/SD-induced lymphocyte proliferation (Cooper et al., 2005), but it was thought important to investigate this further and to see if the same case existed for PHA-induced lymphocyte proliferation. In the current project, MC1R genotype was determined using PCR and DNA sequencing of DNA extracted from PBMCs taken from the subjects who had been investigated in the first set of experiments using SK/SD and PHA (figures 3.04 – 3.08), and showed that 6 subjects had a wild type MC1R genotype, 4 subjects had a heterozygous MC1R genotype and one subject had a homozygous MC1R genotype. Suppression of PHA-induced lymphocyte proliferation by alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 and KPV was observed in wild type *MC1R* subjects and in *MC1R* variant individuals with a similar degree of suppression between both groups of subjects, see figure 3.11 and tables 3.01 and 3.02.

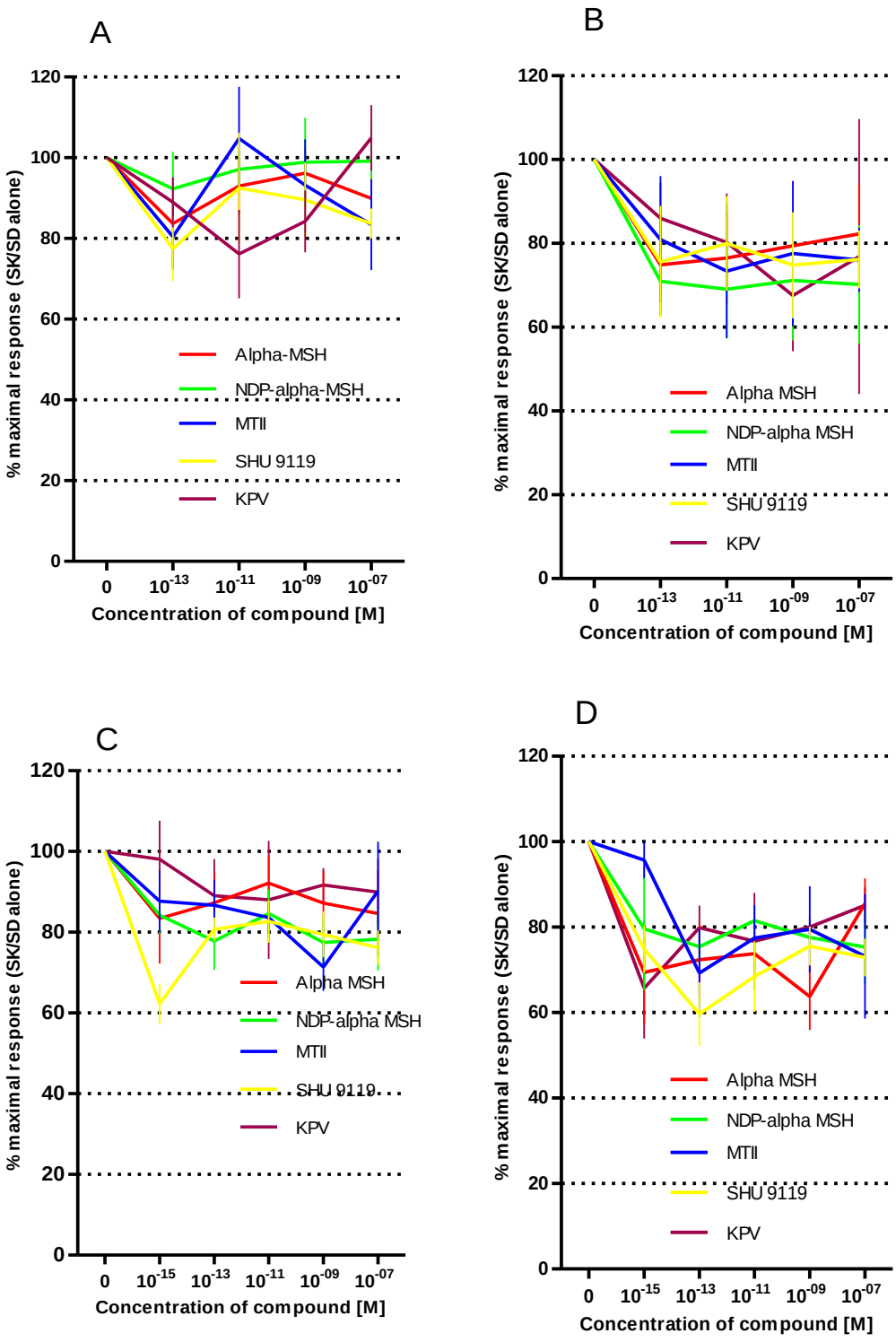


Figure 3.11: Suppression of lymphocyte proliferation assay in response to SK/SD (**A** and **B**) and to PHA (**C** and **D**), in subjects with a wild-type *MC1R* genotype (**A** and **C,** n= 6) or in subjects with a variant *MC1R* genotype (**B** and **D,** n=4).

Subject's MC1R Genotype	SI to SK/SD	Maximum % inhibition of SI by alpha-MSH	Maximum % inhibition of SI by NDP-alpha-MSH	Maximum % inhibition of SI by MTII	Maximum % inhibition of SI by SHU 9119	Maximum % inhibition of SI by KPV
WT/WT	59	39 (10^{-13}M)	41 (10^{-13}M)	15 (10^{-09}M)	35 (10^{-13}M)	0
WT/WT	5	40 (10^{-09}M)	20 (10^{-07}M)	56 (10^{-09}M)	40 (10^{-109}M)	40 (10^{-09}M)
WT/WT	50	8 (10^{-07}M)	2 (10^{-13}M)	18 (10^{-11}M)	27 (10^{-13}M)	41 (10^{-11}M)
WT/WT	30	27 (10^{-13}M)	33 (10^{-13}M)	10 (10^{-07}M)	10 (10^{-107}M)	75 (10^{-11}M)
WT/WT	7	57 (10^{-13}M)	43 (10^{-09}M)	50 (10^{-13}M)	50 (10^{-13}M)	25 (10^{-11}M)
WT/WT	30	0	0	40 (10^{-07}M)	31 (10^{-11}M)	34 (10^{-11}M)
WT Mean n=6	**30.2**	**28.5**	**27.8**	**31.5**	**32.0**	**43.0**
SEM	**8.9**	**8.8**	**6.9**	**7.9**	**5.5**	**7.7**
163ArgGln/WT	3	67 (10^{-13}M)	67 (10^{-07}M)	43 (10^{-11}M)	57 (10^{-13}M)	17 (10^{-09}M)
Val92Leu/WT	81	17 (10^{-09}M)	7 (10^{-13}M)	3 (10^{-11}M)	5 (10^{-07}M)	38 (10^{-09}M)
Arg163Gln/WT	14	50 (10^{-13}M)	21 (10^{-09}M)	56 (10^{-07}M)	56 (10^{-11}M)	22 (10^{-09}M)
Arg160Trp/WT	14	36 (10^{-09}M)	43 (10^{-09}M)	67 (10^{-11}M)	50 (10^{-09}M)	18 (10^{-13}M)
Single variant allele mean n=4	**28**	**42.4**	**34.6**	**41.9**	**41.9**	**23.9**
SEM	**17.9**	**10.5**	**12.9**	**13.9**	**12.4**	**4.9**
Phe65Tyr/His92Gln	15	27 (10^{-13}M)	57 (10^{-11}M)	71 (10^{-09}M)	57 (10^{-13}M)	73 (10^{-09}M)

Table 3.01: Maximum percentage inhibition of SK/SD-induced lymphocyte proliferation by melanocortin peptides, showing each subjects MC1R status, SI = stimulation index, WT = wild type.

Subject's MC1R Genotype	SI to PHA	Maximum % inhibition of SI by alpha-MSH	Maximum % inhibition of SI by NDP-alpha-MSH	Maximum % inhibition of SI by MTII	Maximum % inhibition of SI by SHU 9119	Maximum % inhibition of SI by KPV
WT/WT	224	31 (10^{-15}M)	20 (10^{-13}M)	26 (10^{-11}M)	36 (10^{-09}M)	49 (10^{-15}M)
WT/WT	69	30 (10^{-09}M)	39 (10^{-13}M)	32 (10^{-09}M)	55 (10^{-15}M)	27 (10^{-07}M)
WT/WT	182	65 (10^{-15}M)	22 (10^{-13}M)	46 (10^{-15}M)	43 (10^{-15}M)	0
WT/WT	102	5 (10^{-09}M)	38 (10^{-13}M)	38 (10^{-09}M)	52 (10^{-15}M)	18 (10^{-09}M)
WT/WT	28	0	7 (10^{-09}M)	43 (10^{-09}M)	33 (10^{-09}M)	82 (10^{-11}M)
WT/WT	24	38 (10^{-09}M)	33 (10^{-09}M)	4 (10^{-11}M)	32 (10^{-15}M)	32 (10^{-11}M)
Mean n=6	**104.8**	**26.4**	**26.6**	**31.6**	**42.0**	**33.4**
SEM	**33.6**	**10.9**	**5.1**	**6.3**	**3.9**	**12.3**
Val92Ile/WT	32	47 (10^{-09}M)	59 (10^{-15}M)	10 (10^{-07}M)	40 (10^{-13}M)	38 (10^{-13}M)
Arg160Trp/WT	113	35 (10^{-13}M)	41(10^{-13}M)	38 (10^{-11}M)	38 (10^{-09}M)	0
Arg160Trp/WT	224	19 (10^{-09}M)	40 (10^{-07}M)	69 (10^{-07}M)	55 (10^{-13}M)	27 (10^{-13}M)
Arg160Trp/WT	8	69 (10^{-15}M)	13 (10^{-09}M)	38 (10^{-13}M)	0	60 (10^{-15}M)
Single variant allele mean n=4	**94.3**	**42.3**	**38.2**	**38.8**	**29.9**	**27.3**
SEM	**48.7**	**10.5**	**9.7**	**12.1**	**14.9**	**16.0**

Table 3.02: Maximum percentage inhibition of PHA-induced lymphocyte proliferation by melanocortin peptides, showing each subjects MC1R status, SI = stimulation index, WT = wild type

3.3.5 Forskolin suppressed PHA and SK/SD induced lymphocyte proliferation

Alpha-MSH binds to melanocortin receptors and causes an increase in intracellular cAMP levels. To investigate if forskolin (which, as an agonist at adenylyl cyclase, also activates cAMP) (Seamon et al., 1981) was more potent an immunosuppressive agent than alpha-MSH, the effect of forskolin on PHA and SK-SD induced lymphocyte proliferation was investigated. Forskolin, added as a single dose, caused a significant suppression of lymphocyte proliferation, $p < 0.0001$ when cultures were stimulated with PHA or SK/SD, see figure 3.12. However, the potency of the immunosuppressive effect of forskolin seemed similar to that of alpha-MSH (compare figure 3.12 with figure 3.04).

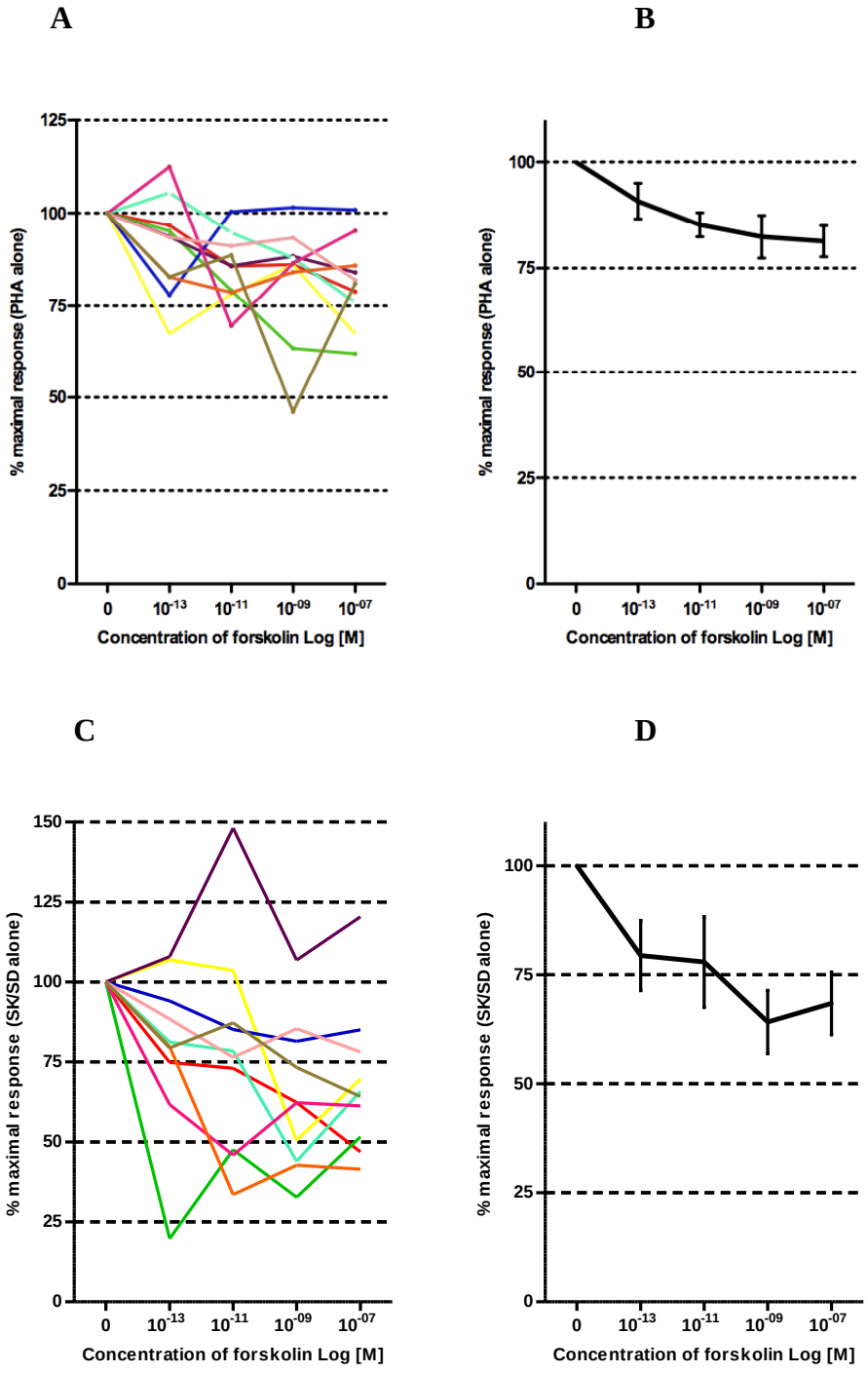


Figure 3.12: Effect of forskolin on PHA (A and B) and SK/SD (C and D) induced lymphocyte proliferation in 10 subjects. (A) and (C) show data from individual subjects and (B) and (D) show the mean (+/-SEM) suppression. Forskolin caused significant suppression of PHA induced lymphocyte proliferation; $p < 0.0001$ when stimulated with PHA and $p < 0.0001$ when stimulated with SK/SD.

3.3.6 Leptin did not have a significant effect on PHA induced lymphocyte proliferation.

Leptin is known to have effects on metabolic pathways which involve/interact with alpha-MSH, so the effects of leptin were also investigated in this system. Overall leptin did not have any significant effect on PHA-induced lymphocyte proliferation, p = 0.4682, figure 3.13. In some individuals leptin suppressed lymphocyte proliferation whereas in some subjects PHA- induced lymphocyte proliferation was augmented by leptin.

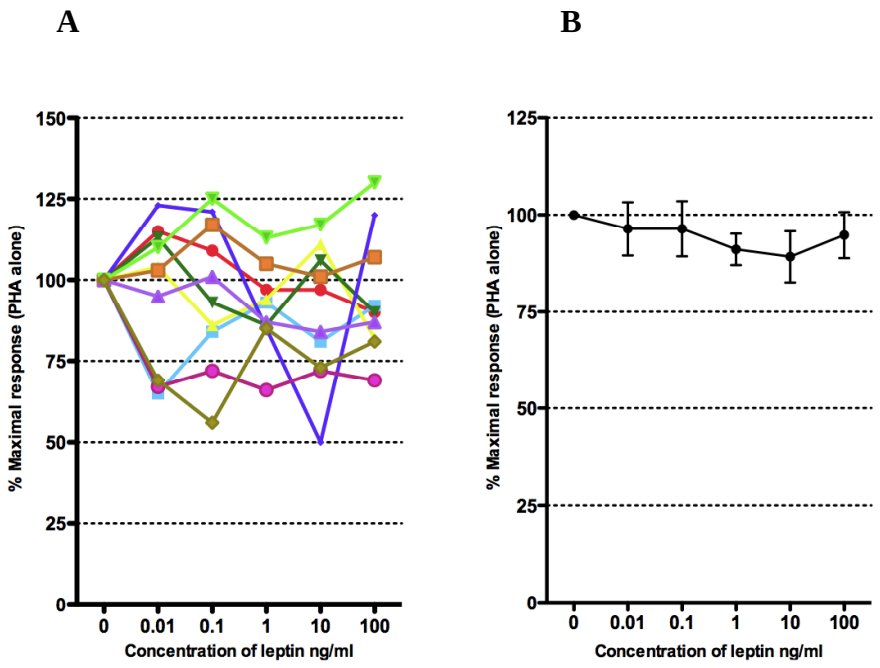
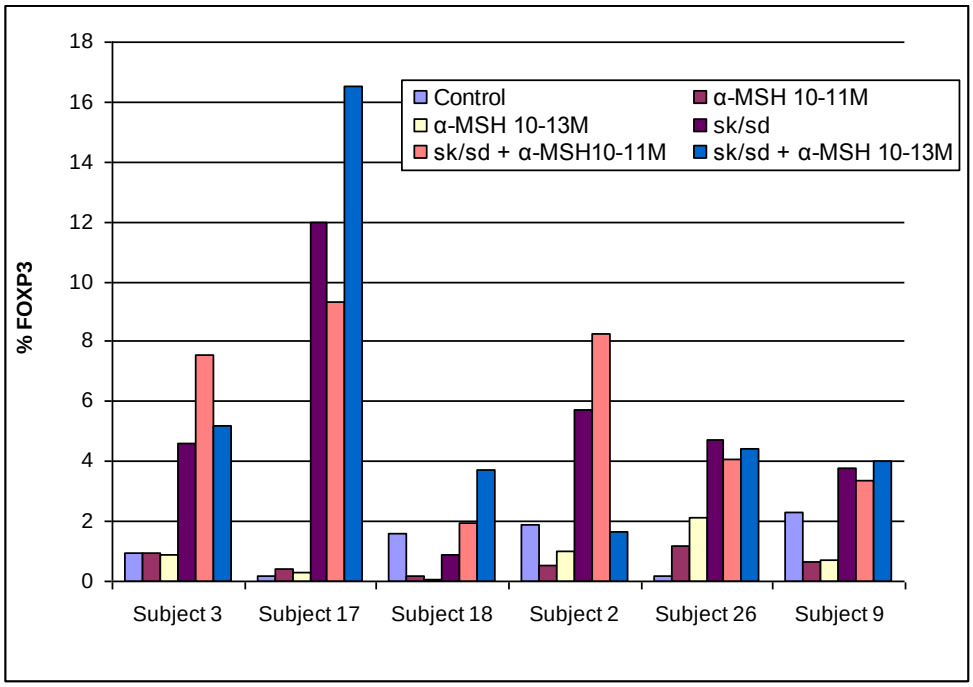


Figure 3.13: Effect of leptin on PHA-induced lymphocyte proliferation. (A) Individual data from 10 subjects. (B) Mean effect of leptin. Statistical analysis (ANOVA) indicated that leptin did not have any significant effect on PHA induced lymphocyte proliferation, p=0.4682.

3.3.7 T-regulatory cells.

None of the analogues of alpha-MSH (nor of the other agents investigated) seemed to be more potent than alpha-MSH in suppressing lymphocyte proliferation. Although there were no plans to investigate the mechanisms involved in melanocortin-induced immunosuppression in detail, other work in the laboratory at the same time had involved studying regulatory T cells, so it was decided to examine whether alpha-MSH induced the formation of regulatory T cells *in vitro* (which could be one possible mechanism which might be responsible for immunosuppression by melanocortin peptides *in vivo*). Therefore, using flow cytometry, the expression of FOXP3 (a transcription factor involved in the development and function of regulatory T cells) and CD25 by T-lymphocytes was investigated following culture with SK/SD (n=6), and separately with PHA (n=3), in the presence and absence of alpha-MSH (10^{-13}M and 10^{-11}M). Although there was an increase in the number of cells expressing FOXP3 and CD25 in cultures containing SK/SD and in those containing PHA, alpha-MSH did not consistently increase or decrease the expression of FOXP3 and/or CD25 in this system, figures 3.14 - 3.16.

A



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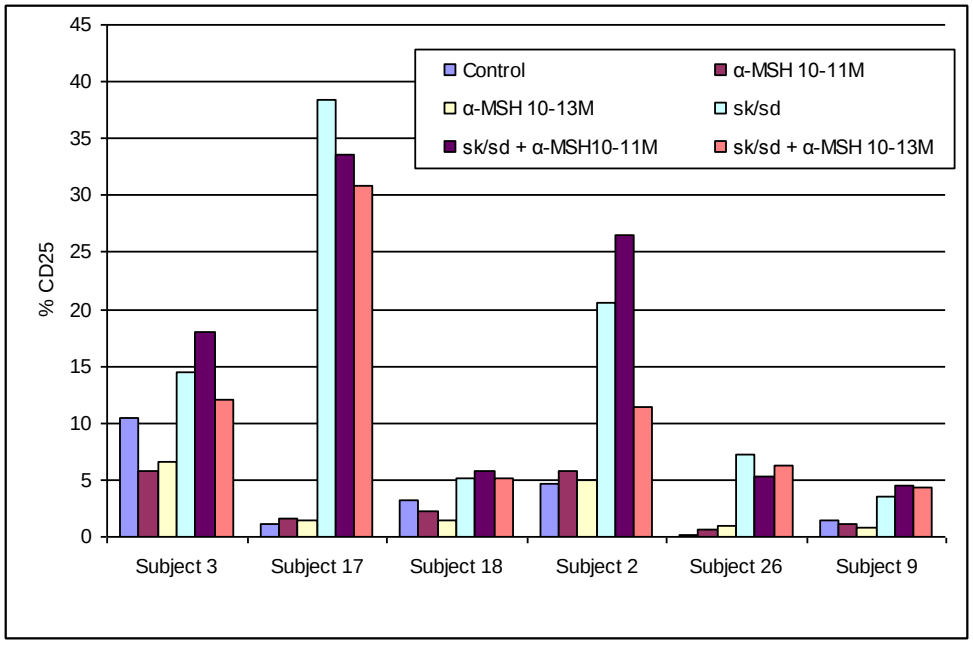
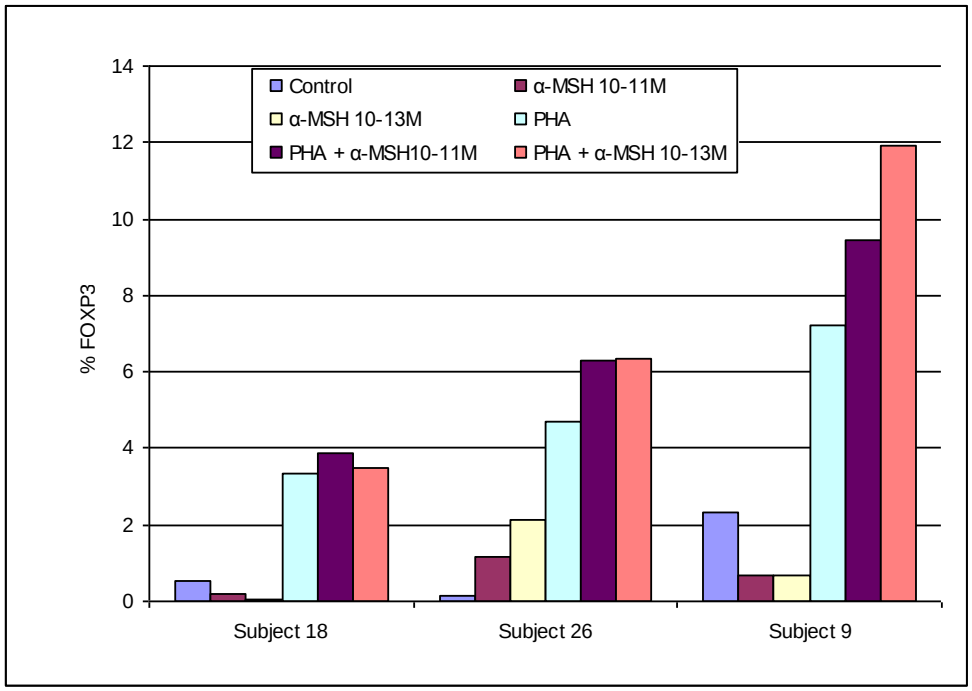


Figure 3.14: Expression of FOXP3 (**A**) and CD25 (**B**) by lymphocytes cultured for 48 hours with alpha-MSH in the presence/absence of SK/SD in 6 subjects.

A



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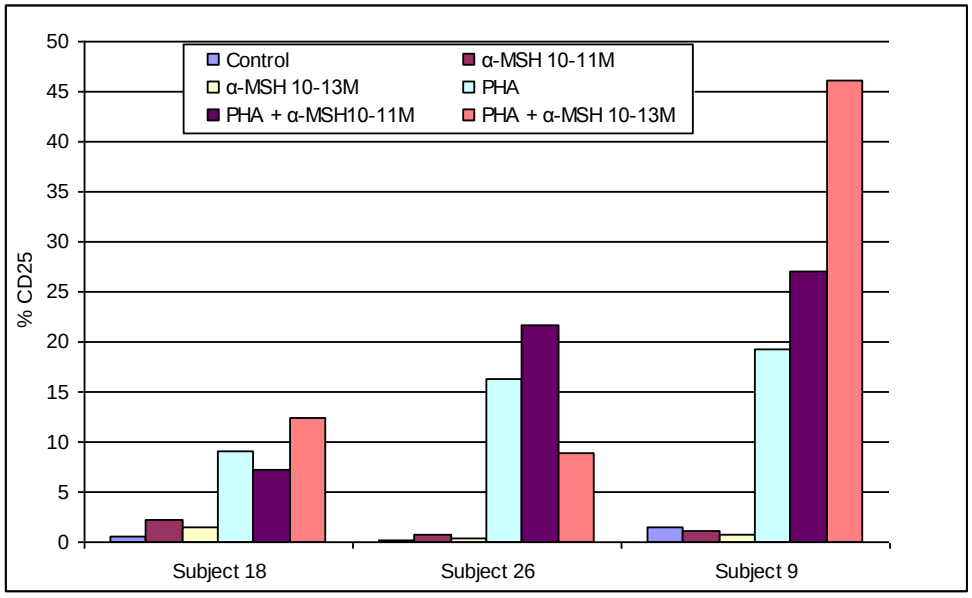
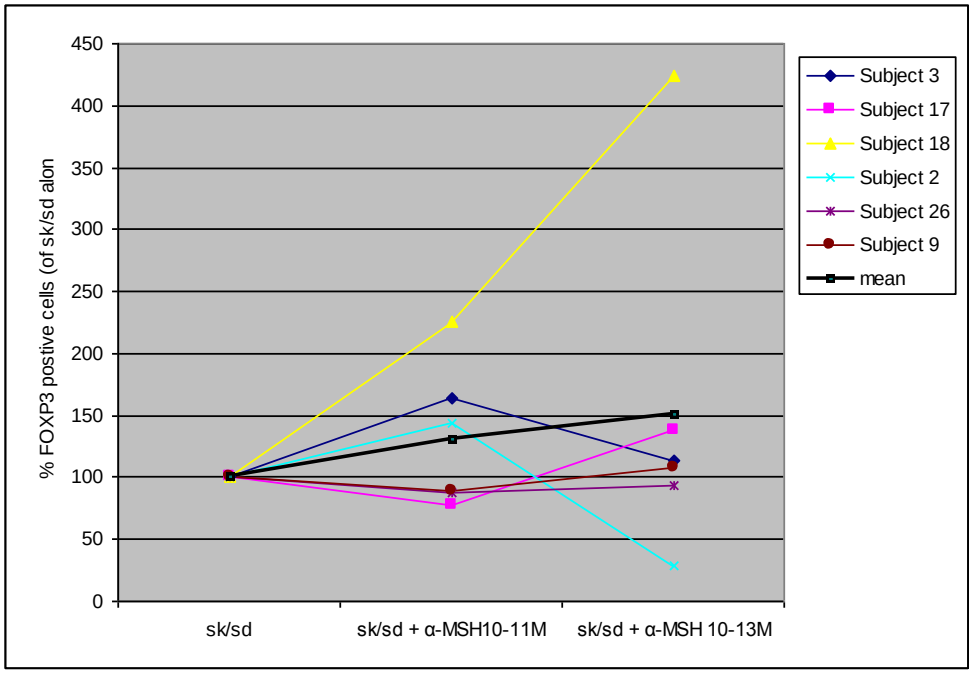


Figure 3.15: Expression of FOXP3 (**A**) and CD25 (**B**) by lymphocytes cultured for 48 hours with alpha-MSH in the presence/absence of PHA, n=3 subjects.

A



B

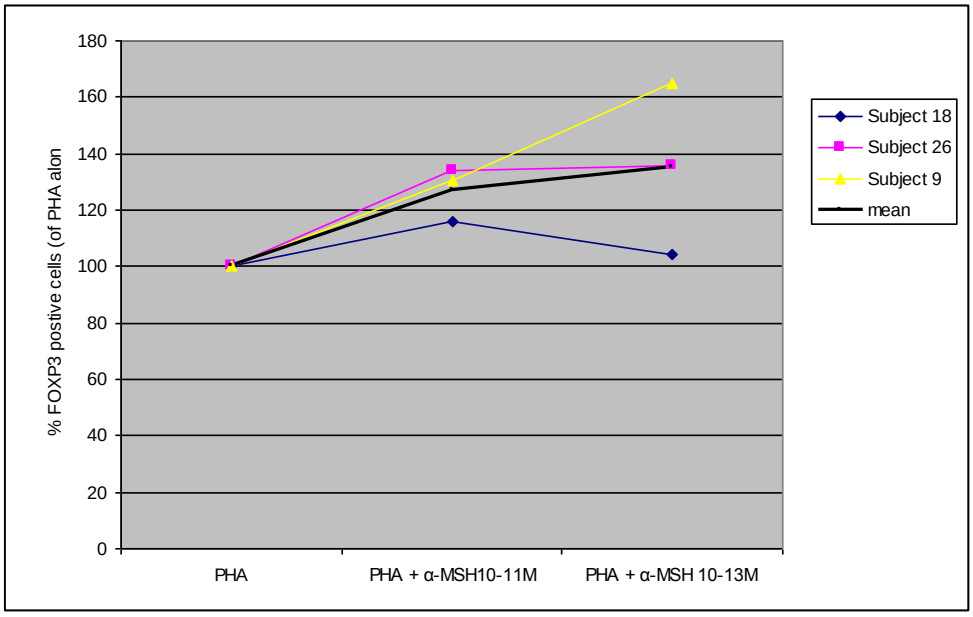


Figure 3.16: Percentage FOXP3 expression in lymphocytes stimulated with either SK/SD (**A**) or PHA (**B**) cultured for either 48 hours (PHA) or 6 days (SK/SD) in the presence/absence of alpha-MSH. There was no significant increase in FOXP3 expression in lymphocytes cultured in the presence of alpha-MSH in cultures stimulated with either SK/SD $p > 0.005$ or cultures stimulated with PHA $p > 0.05$ compared to stimulation with PHA or SK/SD alone.

3.4 Discussion

The main emphasis of the work presented in this chapter relates to investigating whether analogues of alpha-MSH (NDP-alpha-MSH, MTII, SHU 9119 and KPV) are more potent than alpha-MSH in their ability to suppress antigen-induced lymphocyte proliferation. Most of studies on the immunosuppressive effects of alpha-MSH have been carried out in animal models, and there are limited studies in humans to date. Cooper et al demonstrated that alpha-MSH suppressed SK/SD-induced human lymphocyte proliferation *in vitro,* but also showed that MTII and SHU 9119, two cyclic analogues of alpha-MSH, suppressed SK/SD induced lymphocyte proliferation to a greater degree than alpha-MSH. However, in that study the effects of MTII and SHU 9119 were only examined in two subjects, therefore in this study the effects of these and other analogues of alpha-MSH on PHA- and SK/SD-induced lymphocyte proliferation was investigated in greater detail.

In this study, alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 and KPV suppressed PHA- and antigen-induced lymphocyte proliferation to a similar extent. Although the SI did vary between subjects, something in the case of SK/SD could be due to recent streptococcal infections in people, there was no obvious relation between the SI and degree of suppression by these compounds. The suppression of lymphocyte proliferation is unlikely to be due to melanocortin-induced cell toxicity, as suppression was often greatest at the lower concentrations of the compound. This is similar to previous studies in which lower concentrations of alpha-MSH (e.g. 10^{-}

^{13}M) was more potent at stimulating IL-10 production by monocytes and suppressing SK/SD-induced lymphocyte proliferatin (Bhardwaj et al., 1996; Cooper et al., 2005). In contrast, higher concentrations of alpha-MSH have been demonstrated to stimulate cAMP production and melanin synthesis in melanocytes and melanoma cells (Hunt et al., 1995; Robinson & Healy, 2002). This study provides further evidence that alpha-MSH and related peptides have immunosuppressive activity, but the potency of the effects *in vitro* are limited and further *in vivo* work would be required to determine whether these agents would be useful in the treatment of immune diseases in humans, for example psoriasis, rheumatoid arthritis and asthma.

The mechanism of action of melanocortin-induced immuno-suppression is unclear. The melanocortin receptors expressed on cells of the immune system include MC1R, MC3R and MC5R. Because alpha-MSH, NDP-alpha-MSH, MTII and SHU 9119 are MC1R agonists, this gene was sequenced in a number of the subjects, but there were no differences between subjects with a wild type *MC1R* genotype and subjects with a variant *MC1R* genotype; this was similar to the study by Cooper et al (2005) which demonstrated that alpha-MSH suppressed SK/SD- induced lymphocyte proliferation in humans independently of MC1R gene status. *MC1R* variants reduce the ability of alpha-MSH to signal via cAMP, but Elliott et al (2004) have demonstrated an alternative MC1R intracellular signalling pathway that does not involve cAMP but involves increases in intracellular calcium (Elliott et al., 2004). It is possible that the MC1R variants could still signal through this calcium pathway, but the fact that KPV does not have the core sequence of alpha-MSH (i.e. HFRW) required to bind to melanocortin receptors suggests that the immunosuppression may

not be occurring via MC1R or any of the melanocortin receptors. Haddad et al (2001) have demonstrated that KPV is an IL-1β receptor antagonist, inhibiting nuclear accumulation of NFκB. It is possible that KPV suppresses immune responses by a different mechanism than that by the other four agents. However, many studies have demonstrated that alpha-MSH can also inhibit NFκB (Manna *et al* 1998; Kalden *et al* 1999) therefore it is not inconceivable that all the melanocortin peptides, including the C-terminal peptide, may have immunosuppressive effects by acting as antagonist at NFκB.

Activation of melanocortin receptors by alpha-MSH causes an increase in intracellular cAMP (Gantz *et al* 1993a; Penhoat *et al*, 1989; Konda *et al* 1994; Gantz *et al* 1993b; Hoogduijn *et al* 2002). Forskolin is a cell permeable compound which activates intracellular cAMP levels via adenylate cyclase (Seamon et al., 1981). The effect of forskolin on PHA-induced lymphocyte proliferation was similar to the suppression by alpha-MSH, suggesting that forskolin would not offer any advantages over the melanocortin peptides as an immunosuppressant and/or for treatment of immune-mediated diseases. Similarly, the data on leptin suggests that this would not be a useful immunosuppressive agent. In the overall group, leptin did not suppress the immune response, but some subjects showed suppression with this hormone. One confounding factor is that as leptin is a hormone that affects appetite and energy homeostasis, the time of day that the blood was taken from the individuals (for example before or after a meal) might have affected the degree of suppression due to the amount of leptin the PBMC population had been exposed to *in vivo.*

In conclusion alpha-MSH and related compounds suppress antigen- and mitogen-induced lymphocyte proliferation in vitro, but none of the compounds examined in this study showed a consistently greater immunosuppressive response than alpha-MSH alone.

Chapter 4

4.1 Introduction.

Ultraviolet radiation is used to treat skin diseases that are due to overactive immune responses, for example psoriasis and atopic eczema. Studies in mice and humans have demonstrated that UVA and UVB cause immunosuppression, for example UV-irradiated mice have reduced contact hypersensitivity responses to haptens (Greene *et al*., 1979; Toews *et al*., 1980; Bestak & Halliday, 1996) and in humans UVB suppresses contact hypersensitivity responses to sensitisers such as dinitrochlorobenzne (DNCB) (Yoshikawa *et al*., 1990). The mechanisms of UVB induced immunosuppression are not entirely clear. It is known that UVB modulates the function of the antigen presenting cells (APCs) as observed by APCs derived from the spleen of UVB irradiated mice having a reduced capacity to induce proliferation of T cells (Letvin *et al*., 1980). Work has also suggested that UVB-induced immunosuppression is due to UVB causing alterations in Langerhans cells function because UVB-irradiated Langerhans cells have a distorted morphology with reduced dendritic processes (Toews *et al*., 1980). However, one problem with many of the studies (those mentioned above and below) is that it is unclear whether the effect is a direct one on the APC itself or an indirect effect via another cell through the production of cytokines which alter the function of the APC.

The studies to date have suggested that many factors may be involved in the production of UV-induced immunosuppression. UVB-induced DNA damage

(cyclobutane pyrimidine dimer formation, CPD) triggers UVB-mediated immunosuppression and reducing CPDs in UVB-irradiated mice prevents UVB-induced immunosuppression (Applegate *et al*., 1989; Kripke *et al*., 1992). UVB irradiation of human skin causes an increase in prostaglandin E_2 (PGE_2) (Rhodes *et al* 1995) and murine monocytes can release PGE_2 after UV, which can then inhibit ICAM-1 expression (Black *et al*., 1978; de Waal Malefyt *et al*., 1991; Grewe *et al*., 2000). Cis-UCA is also released in skin (by the stratum corneum) after UV and, based on the ability of anti-cis-UCA antibodies to inhibit cis-UCA mediated immunosuppression, is thought to be involved in the suppression of immune responses following UV (Moodycliffe *et al* 1996). IL-10 is another factor contributing to the UVB-induced immunosuppression (Kang *et al*., 1994; Beissert *et al*., 1995) but the evidence suggests that this cytokine may come from more than one source. For example, injection of supernatants from UVB-irradiated keratinocytes into mice inhibited CHS and injection of anti-IL-10 antibodies reversed this immunosuppression, indicating that one source of the IL-10 was keratinocytes (Rivas & Ullrich, 1992). Kang *et al* suggested that the most potent source of IL-10 in the epidermis was from UVB-irradiated macrophages (Kang *et al*., 1994), but Enk *et al*. (1995) showed an increase in IL-10 expression in human skin and by cultured human keratinocytes after UV. Similarly, Kock *et al*. (1990) found increased levels of TNF-α after UV irradiation of human keratinocytes, and anti-TNF-α antibodies have been shown to reverse UV-induced immunosuppression in mice (Kurimoto & Streilein, 1992). IL-12 is a cytokine which, in contrast to the above agents, is reduced by UVB and administration of this cytokine permits the development of a

CHS response in UVB-irradiated mice (Schwarz *et al*., 1996; Boonstra *et al*., 2000). In addition, Schmitt *et al*. (1995) demonstrated that IL-12 is able to prevent the induction of suppressor T cells, as shown by a CHS response in mice that had adoptively transferred spleen cells from UVB-irradiated mice treated with IL-12. Schwarz *et al*. (2005) more recently demonstrated that the prevention of UVB radiation-induced immunosuppression by IL-12 may be dependant on DNA repair. In contrast to many of the UVB-altered cytokines, transforming growth factor-beta (TGF-beta) increases in human skin after exposure to UVA and may be relevant in UVA-immunosuppression, but does not seem to increase after UVB (Gambichler *et al*., 2007). In fact, UVB irradiation of human fibroblasts has been reported to decrease the expression of TGF-beta (Choi *et al*., 2007).

Most of the studies on UV induced immunosuppression have been carried out on whole tissue (often in animal models) and it can be difficult to interpret direct from indirect effects on certain cell types in those situations. In addition, less work seems to have been done to look at direct effects on APCs, especially on human APCs. Therefore, the aim of this chapter was to develop and use a simplified human *in vitro* model culture system to look for direct and indirect effects of UV on human APCs.

4.2 Methods

4.2.1 Isolation of monocytes and T cells from PBMCs.

PBMCs were isolated from human blood as described in chapter 2. Monocytes were isolated from the PBMCs using CD14 MACS beads (Miltenyi Biotec) by positive selection and T lymphocytes were isolated from the CD14 negative cell population by negative selection using the autoMACS system (Miltenyi Biotec). All cells were cultured in RPMI medium supplemented with 1% sodium pyruvate (Invitrogen Paisley, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen Paisley, UK) and 5% human serum (Sigma) at 37^{o}C in 5% CO_2.

4.2.2 UV irradiation of cells.

$CD14^{+}$ cells (monocytes) (1 million/well; 3 wells per sample) were irradiated with a UV lamp (TL12, Philips, emitting both UVA and UVB) at a range of doses; 0, 5, 10, 20, 40 and 80 mJ/cm^2. UV output was monitored using a UV radiometer as per the manufacturer's instructions. The control dishes were sham irradiated, by placing the Petri dishes into a black box and leaving the box on the bench top for the duration of the longest irradiation time (the time for the highest dose of UV). Following irradiation (and sham irradiation), the cells were centrifuged in PBS and made up to 1 million cells per ml (counted using a haemocytometer) in complete RPMI medium.

PHA was added to each of the wells except the "no-PHA" control wells, and the cells were cultured for 48 hours at 37^{o}C in 5% CO_2. At 42 hours of culture, the cells were pulsed with tritiated thymidine and cultured for a further 6 hours at 37^{o}C in 5%

CO_2. The cells were transferred to a 96 well plate (the cells from each culture dish were put into 4 wells of a 96 well plate, 200µl per 96 well), for counting using a Top Count scintillation counter (as per Chapter 2).

In the supernatant experiments (to investigate what the immunosuppressive factor was) either indomethacin (Sigma), anti-IL-10 (Bachem), TGF-beta receptor antagonist (Invitrogen) or 1-methyl-tryptophan (Sigma) was added and in the control wells the same volume of complete medium was added to the culture system (UV-irradiated monocytes cultured with T lymphocytes in the presence of PHA).

4.3 Results.

4.3.1 UV irradiation of PBMCs.

In order to look at the effects of UV on APCs, the ability of UV to suppress mitogen-induced proliferation of PBMCs was studied initially. Therefore, PBMCs (1 X 10^6 per ml) were irradiated in PBS with UV doses ranging from 0 – 80mJ/cm^2, following which the PBMCs were transferred to medium containing 2μg/ml PHA. Following 48 hours of culture, it was noted that UV had caused significant suppression of proliferation in PBMCs from each of three separate subjects, $p < 0.0001$ (ANOVA), figure 4.01, with a mean reduction of 88% at 10mJ/cm^2. This demonstrated that UV exposure of PBMCs had an inhibitory effect on the mitogen-induced proliferation, but the irradiation of entire PBMC cultures did not allow one to distinguish whether this effect was mediated through APCs, another cell type or a combination of both.

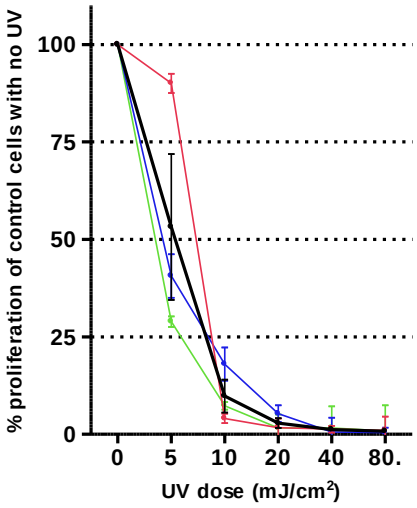


Figure 4.01: Suppression of mitogen-induced proliferation of PBMCs by UV. The graph shows a UV-dose dependant decrease in proliferation of PHA-stimulated PBMCs *in vitro* from each of three separate volunteers (blue, green, red lines) and the mean suppression (black line), $p < 0.0001$.

4.3.2 UV irradiation of monocytes.

In subsequent experiments, $CD14^+$ cells (monocytes) were isolated from PBMCs using MACS bead separation. These $CD14^+$ cells were irradiated in PBS with UV at doses ranging from 0 – 160mJ/cm^2 and then cultured with $CD14^-$ PBMCs and PHA for 48 hours. A UV-dose dependant decrease in PHA-induced proliferation was observed in all 10 subjects (figure 4.02). This showed that UV irradiation of monocytes (i.e. APCs) could suppress the proliferative response of the PBMCs, indicating that UV could suppress the immune response through a direct action on APCs.

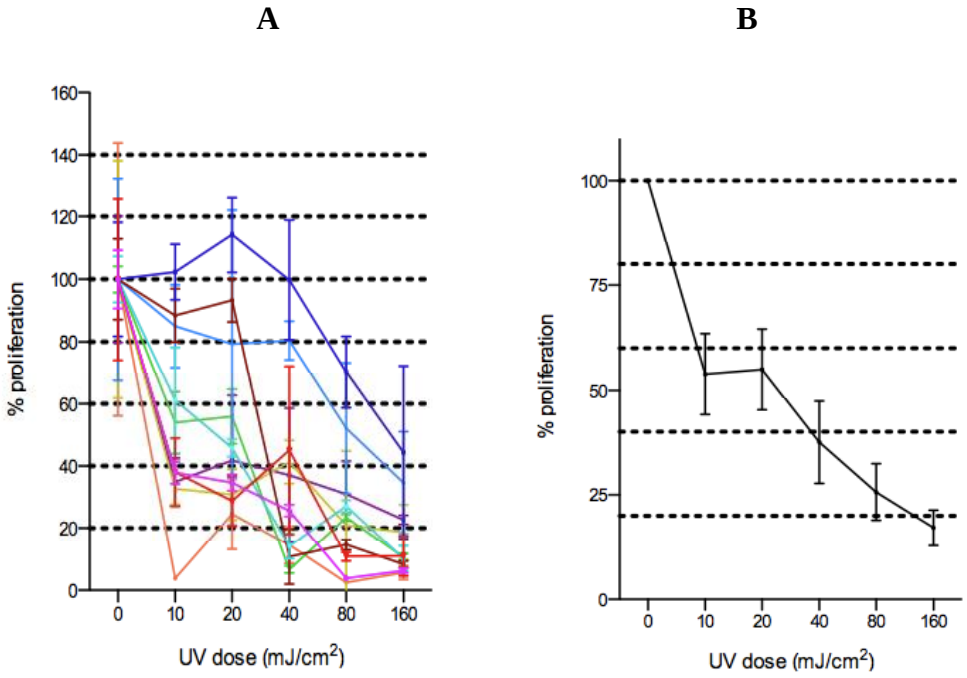


Figure 4.02: Effect of culturing UV irradiated $CD14^+$ PBMCs (monocytes) with CD14 negative PBMCs in the presence of PHA (UV dose range 10mJ/cm^2 to 160mJ/cm^2, n=10); showing percentage proliferation in comparism with CD14 negative PBMCs cultured with unirradiated monocytes. A: individual subjects response, B: mean response, with SEM. There was a significant UV-dose dependant decrease in proliferation of PHA-stimulated lymphocytes, $p < 0.0001$. Statistical test conducted using ANOVA.

However, there was quite an amount of variation in the degree of suppression by UV between the different individuals. There was a concern that some of the variation might be due to the presence of other APCs in the $CD14^-$ PBMC population, for example B cells, and that these additional APCs might compensate for the immunosuppressive effect of UV on the monocytes, i.e. although the UV-irradiated monocytes may have had an impaired ability to present to T cells, other APCs in the $CD14^-$ PBMC population may have been able to do so. Therefore, T lymphocytes were negatively separated from the $CD14^-$ PBMC population using MACS beads separation. Fluorescence activated cell-sorting (FACS) analysis indicated that the $CD14^+$ isolated fraction had a purity of 99% monocytes and that the T lymphocytes were 98% pure after the relevant MACS bead separations, figure 4.03.

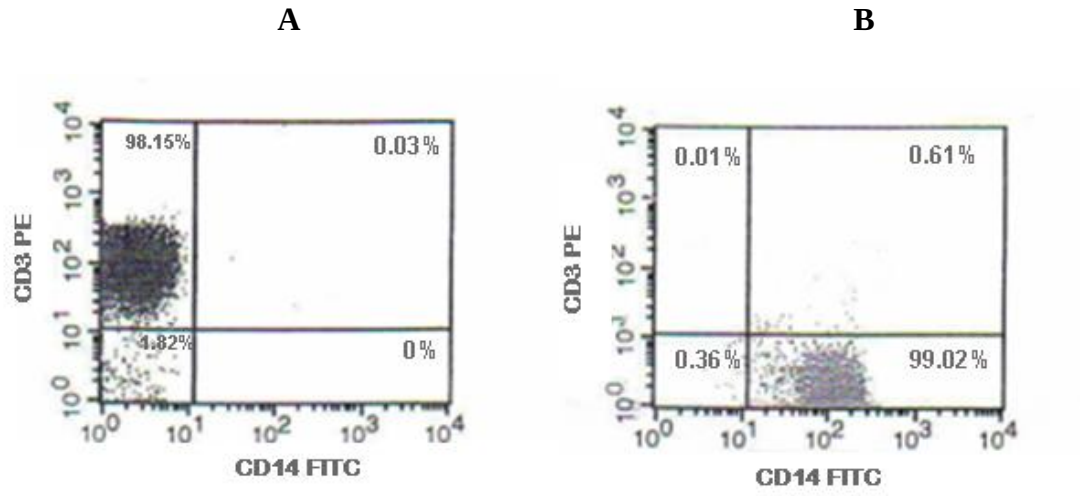


Figure 4.03: FACS plot of negatively selected T lymphocytes from PBMCs (A) and positively selected $CD14^+$ monocytes from PBMCs (B) using MACS bead separation, showed 98% and 99% purity respectively in each cell population. This FACS plot is a representative example from 10 separate subjects.

$CD14^+$ monocytes isolated from PBMCs using MACS bead separation were UV irradiated at doses ranging from 0 – 320mJ/cm^2 and then cultured with purified T lymphocytes and PHA for 48 hours. UV irradiation of $CD14^+$monocytes significantly inhibited PHA-induced purified T lymphocyte proliferation in a UV dose dependant manner, n=15, $p < 0.0001$, using ANOVA (figure 4.04). This demonstrated that UV-irradiated monocytes stimulate T lymphocyte proliferation to a lesser extent than unirradiated monocytes.

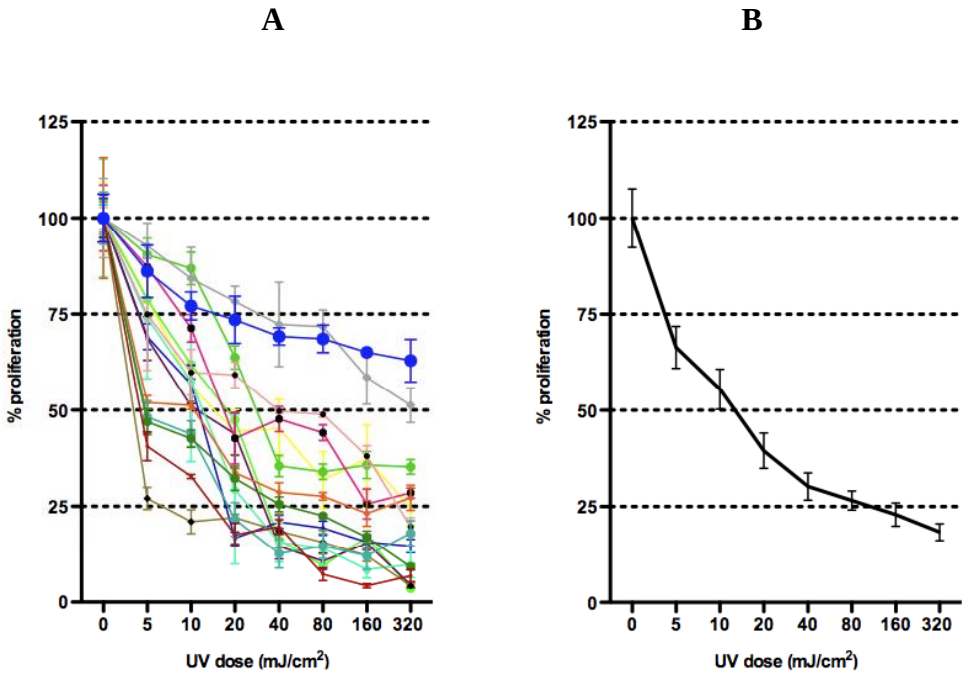


Figure 4.04: Culture of UV-irradiated $CD14^+$ monocytes and $CD3^+$ T cells in the presence of PHA (UV dose range 0mJ/cm^2 to 320mJ/cm^2). A: individual subjects response B: mean response, with SEM; showing % proliferation in comparism with $CD3^+$ T cells cultured with unirradiated monocytes. PBMCS cultured with PHA-induced lymphocyte proliferation was significantly decreased following UV irradiation of monocytes, n=15, $p < 0.0001$ (ANOVA).

It was considered possible that the reduction in lymphocyte proliferation in the above cultures was due to UV causing harm to or death of the monocytes, such that the

monocytes could no longer present antigen / stimulate T cell proliferation. Therefore, to investigate whether the decrease in lymphocyte proliferation after culture with UV-irradiated monocytes was due to reduced healthy numbers of antigen presenting cells, PBMCs were cultured with UV-irradiated monocytes, i.e. the cultures now contained UV-irradiated monocytes, T lymphocytes (in the PBMCS) and non UV-irradiated monocytes (in the PBMCs). Figure 4.05 shows that PHA-induced PBMC proliferation was also suppressed by the presence of UV-irradiated monocytes.

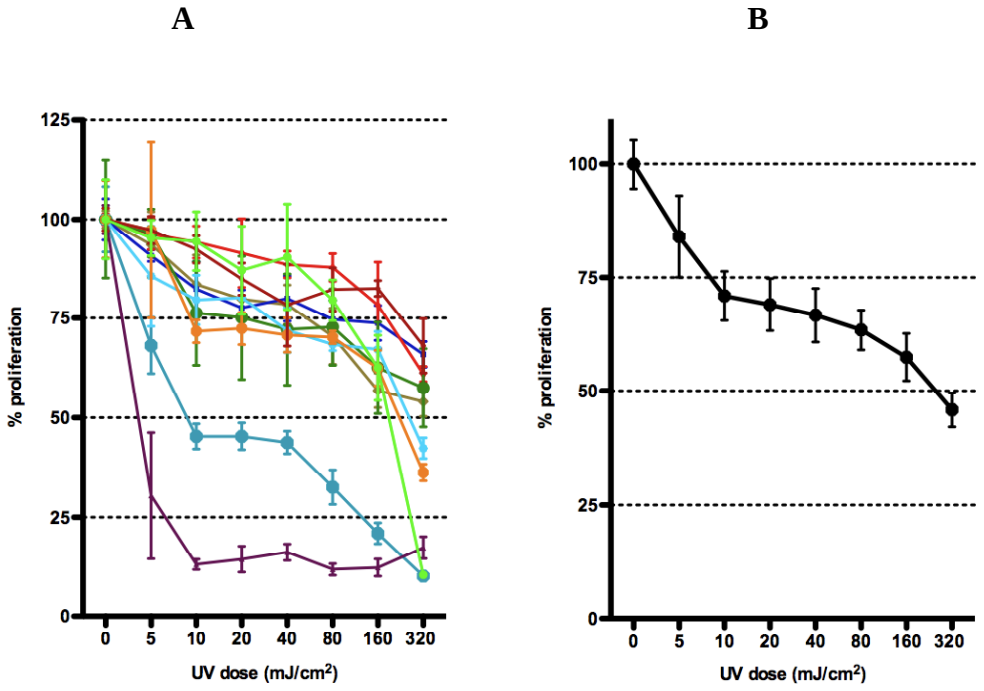


Figure 4.05: Effect of culturing UV-irradiated $CD14^+$ monocytes with PBMCs (which include T cells and non-irradiated monocytes) in the presence of PHA. A: individual subjects response, B: mean response, with SEM; showing percentage proliferation in comparism with PBMCs cultured with unirradiated monocytes.
PHA-induced PBMC proliferation was suppressed by UV-irradiated monocytes, suggesting that the immunosuppression was not simply due to reduced capacity of the irradiated antigen presenting cells to present antigen, n=10, $p < 0.0001$ (ANOVA).

Therefore, because suppression of PHA-induced lymphocyte proliferation was observed in cultures containing both UV-irradiated monocytes and non-UV irradiated monocytes, it suggested that the suppression of T cell proliferation in these cultures and in the earlier monocyte/T cell cultures was unlikely to be due to the UV irradiation reducing the antigen presenting capacity of the irradiated monocytes.

4.3.3 Determination of cell death by trypan blue.

To ensure that cell death of antigen presenting cells / monocytes was not a factor in the immunosuppression following UV, cell death was examined by trypan blue uptake. Dying cells should take up the dye and therefore appear blue. UV-irradiated monocytes were investigated for trypan blue uptake at 24 hours and 48 hours after UV irradiation; the same number of high power fields in each well were counted under the microscope to ensure fair testing. There was some uptake at both time points, however the percentage of cells that were trypan blue positive was less than 10%, figure 4.06.

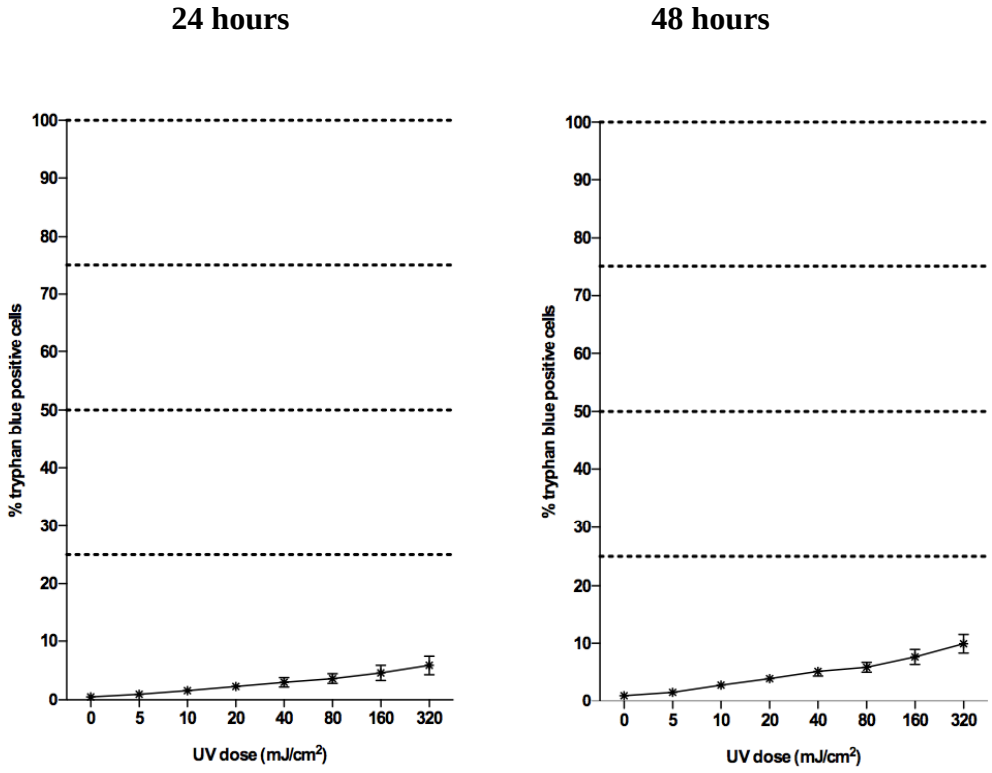


Figure 4.06: Trypan blue uptake by monocytes at 24 and 48 hours after UV irradiation. Trypan blue uptake increased in a UV-dose dependant manner. The overall proportion of dead cells was low at each time point, suggesting that the immunosuppressive effects of UV were not predominantly due to UV-induced cell death, n=7, values are mean (and SEM).

4.3.4 Supernatant of UV irradiated monocytes.

To determine whether an immunosuppressive factor is released by the UV-irradiated monocytes, supernatants collected after 24 hours from UV-irradiated monocytes were added to T cells and stimulated with anti-CD3/anti-CD28 antibodies; anti-CD3/anti-CD28 were used because PHA is not able to induce T cell proliferation in the absence of APCs. The supernatants from UV-irradiated monocytes significantly suppressed anti-CD3/anti-CD28-induced T cell proliferation, figure 4.07.

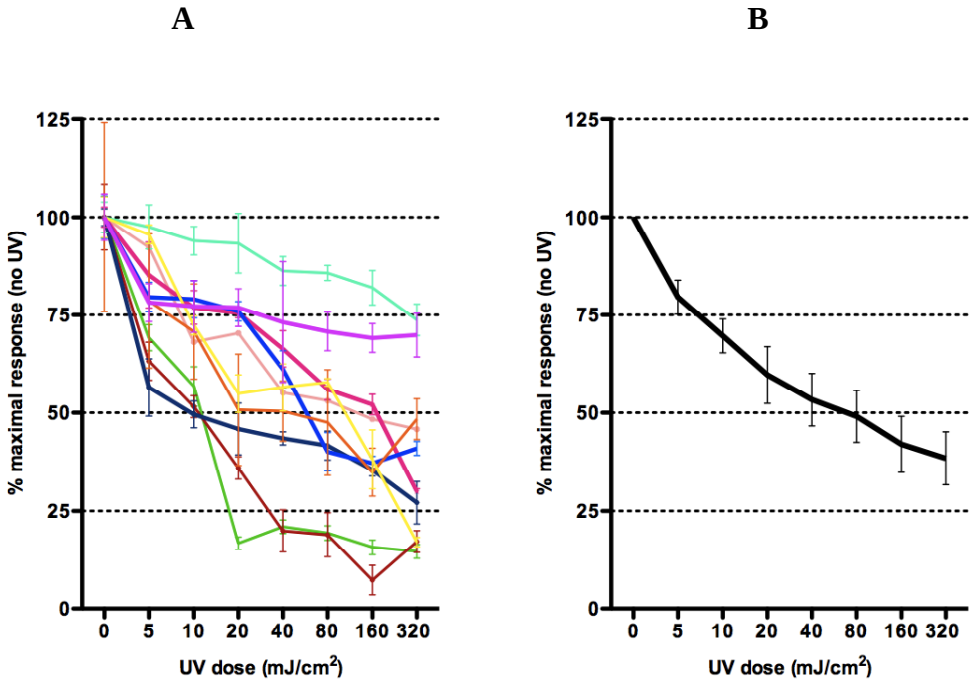


Figure 4.07: Supernatants from UV-irradiated monocytes decreased anti-CD3/anti-CD28-induced T cell proliferation, n=10, $p < 0.0001$ (ANOVA). A: individual subjects response, B: mean subjects response, with SEM. Percentage proliferation in comparism with T cells cultured with the supernatant of unirradiated monocytes. Supernatants were taken from wells at 24 hours after UV irradiation of monocytes and added to anti-CD3/anti-CD28 T cell cultures. UV doses represent the dose of UV that the original monocytes received.

These results suggested that an immunosuppressive soluble factor is released from the UV-irradiated monocytes. To obtain further support for this interpretation, the UV-irradiated monocyte supernatant was cultured with PBMCs in the presence of PHA. Addition of the supernatant from UV-irradiated monocytes caused significant suppression of PHA induced PBMC proliferation, figure 4.08, $p < 0.0001$, ANOVA.

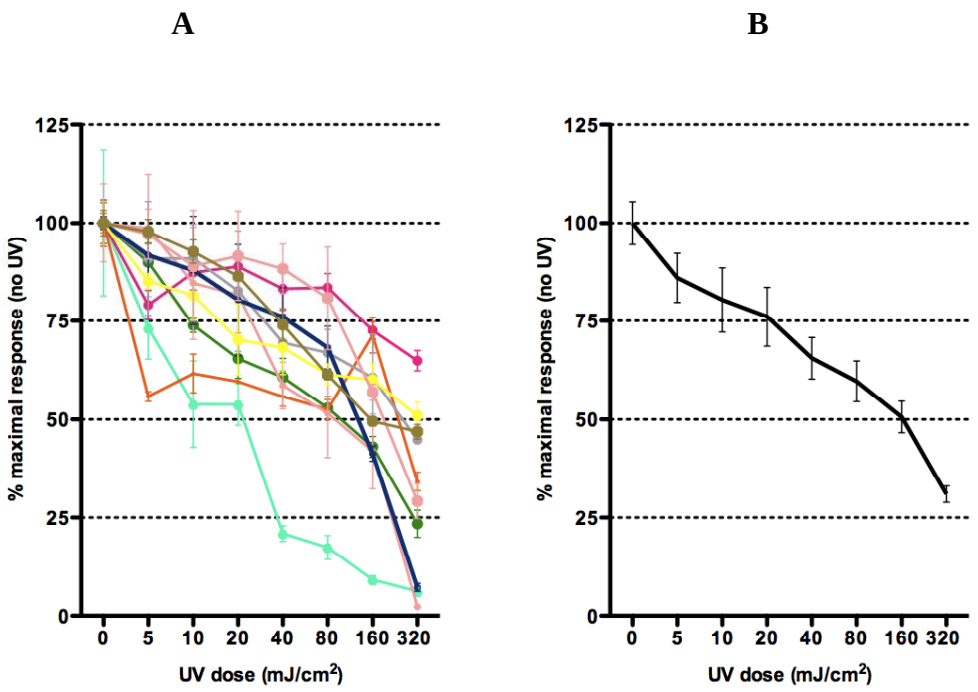


Figure 4.08: Supernatants from UV-irradiated monocytes reduced PHA-induced PBMC proliferation, n=10, $p < 0.0001$ (ANOVA). Percentage proliferation in comparism with PBMCs cultured with the supernatant of unirradiated monocytes. A: Individual subjects response, B; mean response, with SEM.

4.3.5 Investigation of the immunosuppressive factor: Prostaglandins.

It is known that prostaglandins released in response to UV can lead to immunosuppression (de Waal Malefyt *et al* 1991; Hart *et al* 1993). Indomethacin is a COX-1 and COX-2 inhibitor, therefore UV-irradiated monocytes and PBMCs were cultured with PHA in the presence/absence of indomethacin to identify whether the immunosuppression by UV was due to prostaglandin release, figure 4.09.

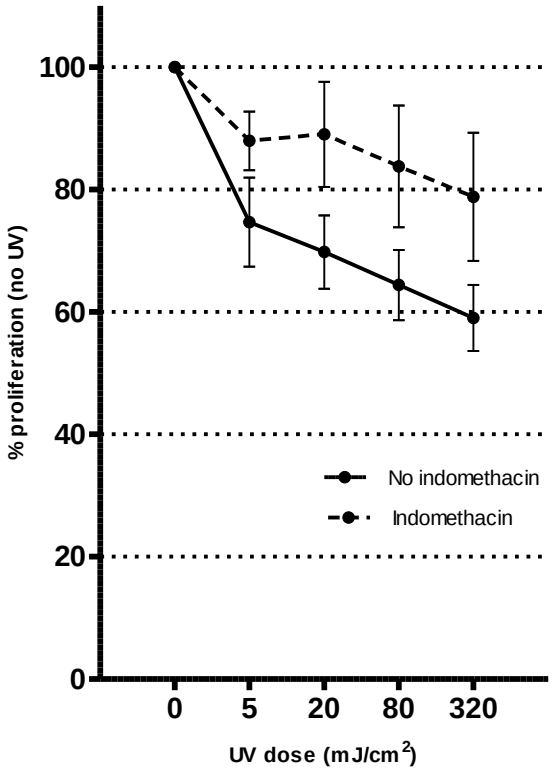


Figure 4.09: Effects of the addition of indomethacin to UV-irradiated monocytes cultured with PBMCs in the presence of PHA, n=10. % proliferation in comparism with PBMCs cultured with unirradiated monocytes, values are meant (and SEM). There was no significant difference in PBMC proliferation with the addition of indomethacin, $p > 0.05$ (ANOVA).

Although the lymphocyte proliferation in cultures that had indomethacin added seemed to have a higher level of proliferation, there was no significant difference in proliferation between cultures with and those without indomethacin ($p > 0.05$, n=10) (ANOVA). Therefore indomethacin was added to cultures of purified T cells and UV-irradiated monocytes which were then incubated for 48 hours in the presence of PHA. The addition of indomethacin significantly reduced the degree of immunosuppression by UV in these cultures, n=10, $p = 0.0196$ (ANOVA), figure 4.10.

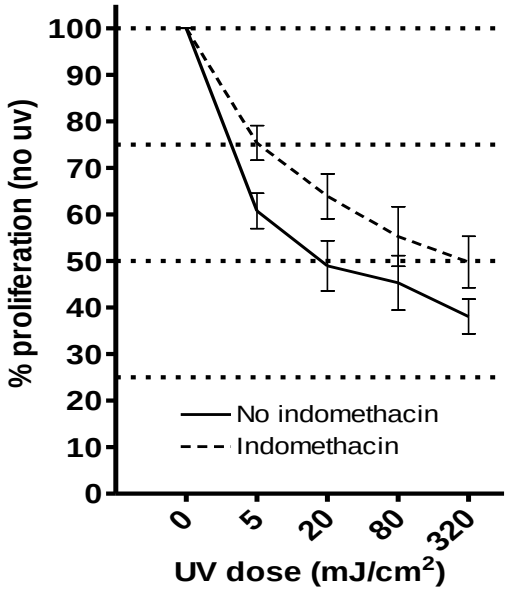


Figure 4.10: Indomethacin significantly inhibited the suppression of anti-CD3/anti-CD28 induced T lymphocyte proliferation by UV-irradiated monocytes, n=10, p = 0.0196 (ANOVA). Percentage proliferation in comparism with T lymphocytes cultured with unirradiated monocytes, values are mean (and SEM).

Therefore, although the addition of indomethacin to the cultures resulted in less suppression by the UV-irradiated monocytes, indomethacin did not completely remove the suppressive effect of UV irradiated monocytes. This suggested that prostaglandins (possibly PGE_2) or other arachidonic acid metabolite(s) were responsible for part of the immunosuppression, but that some other soluble factor was also involved in the UV-mediated suppression of lymphocyte proliferation in the above experiments. Therefore further investigation was required to attempt to identify what this soluble immunosuppressive factor was.

4.3.6 Investigation of the immunosuppressive factor: IL-10.

Many studies have demonstrated that IL-10 is an important immunosuppressive factor released after UV irradiation of skin. For example, injection of IL-10 into mice results in an absence or reduction of a CHS response after hapten application, which is similar to the suppression of CHS as seen with UV irradiation (Rivas & Ullrich, 1992). In addition IL-10 mRNA is enhanced in the skin of mice after UV irradiation (Kang *et al*., 1994). This suggests that IL-10 plays a key role in UV-induced immunosuppression. To investigate whether IL-10 was the immunosuppressive factor released in our culture system, IL-10 was assayed in supernatants of various cultures (as below) using ELISA. Firstly, the supernatant of UV-irradiated monocytes cultured with T lymphocytes and PHA was sampled for IL-10 after 48 hours of culture, figure 4.11.

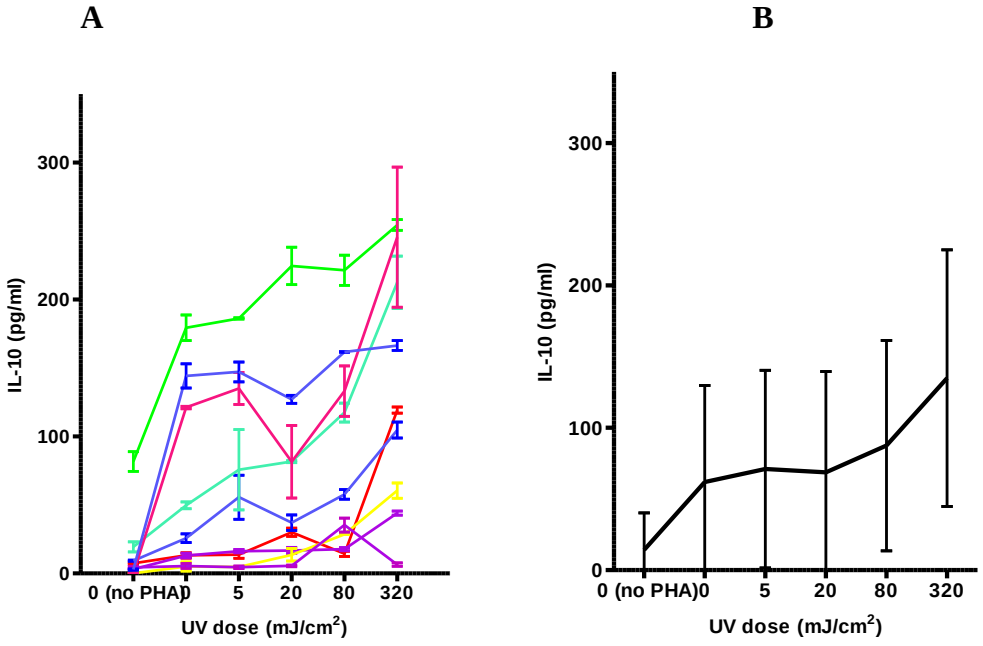


Figure 4.11: The supernatants of UV-irradiated monocytes cultured with T lymphocytes and PHA were collected after 48 hours and IL-10 (pg/ml) detected by ELISA. A: individual subjects response, B: mean response, with SEM. There was an increase in IL-10 release into the culture supernatants only in the presence of PHA (p= 0.0017, ANOVA).

There was a UV dose dependant increase in IL-10 release in UV irradiated monocyte/T cell cultures, however only in the presence of PHA; p= 0.0017 in the presence of PHA and p= 0.0578 with no PHA, ANOVA. Therefore in the next experiment UV-induced IL-10 release was assayed in a range of cultures, including (i) UV-irradiated monocytes alone, (ii) T cells and UV-irradiated monocytes, (iii) PBMCs and UV-irradiated monocytes, (iv) T cells and PHA, (v) UV-irradiated monocytes and PHA, (vi) T cells and UV-irradiated monocytes and PHA; in addition IL-10 release was examined in unirradiated (vii) monocytes and PBMCs and PHA and (viii) PBMCs in the presence of PHA, figure 4.12.

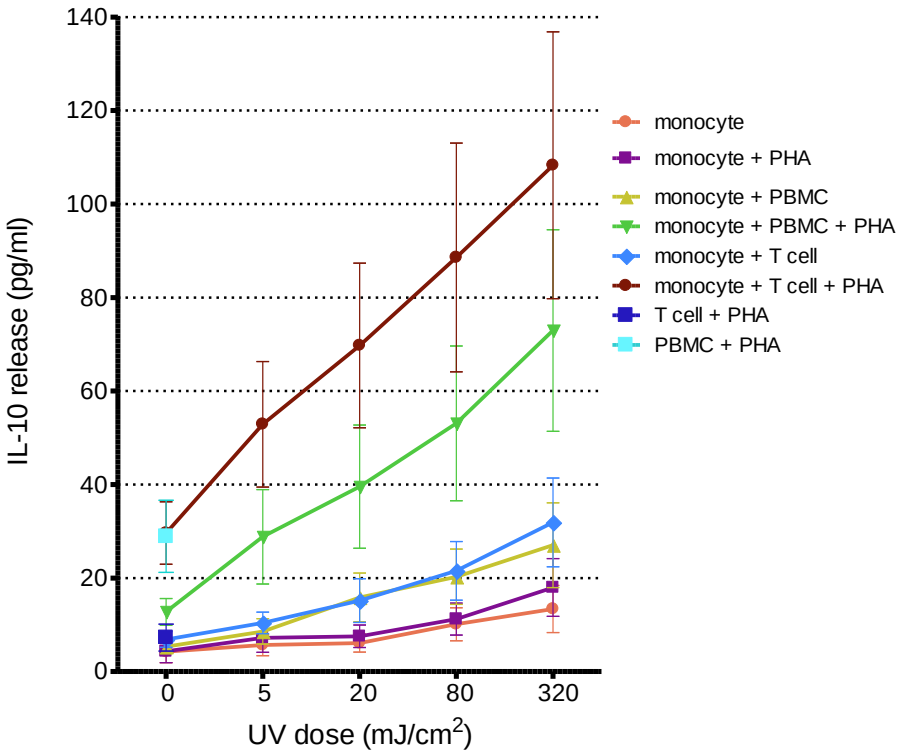


Figure 4.12: IL-10 release after 48 hour culture of various cell types (as listed in graph) in the presence or absence of PHA, n=10. Showing IL-10 (pg/ml) release in each cell type, values are means (and SEM). There was a significant UV dose-dependant increase in the release of IL-10 in the presence of PHA $p < 0.05$ (ANOVA). UV-irradiated monocytes alone, UV-irradiated monocytes and T cells and UV-irradiated monocytes and PBMCS, did not have a significant increase in IL-10 release $p > 0.05$), ANOVA.

Therefore IL-10 is released in a UV dose dependant manner in the presence of PHA, as there was a significant increase in IL-10 in the cultures that had PHA in them (T-cells and PHA; UV-irradiated monocytes and PHA; UV-irradiated monocytes, T-cells and PHA; UV-irradiated monocytes, PBMCs and PHA; PBMC and PHA, $p < 0.05$, but not in the cultures that did not have PHA (UV-irradiated monocytes; UV-irradiated monocytes and T cells; UV-irradiated monocytes and PBMCs ($p > 0.05$). The earlier supernatant experiments (figures 4.07 and 4.08) used supernatant from UV-irradiated monocytes; therefore IL-10 was unlikely to be the immunosuppressive factor in those experiments because IL-10 was not released by UV-irradiated monocytes in large amounts in the current experiment. It is possible that low levels of IL-10 in the supernatant might have caused immunosuppression in the earlier investigations (or possibly that IL-10 could have been released but not detected in the ELISA process (e.g. as a result of degradation), therefore anti-IL10 antibodies were added to UV-irradiated monocytes/PBMC cultures in the presence of PHA and suppression of lymphocyte proliferation investigated, figure 4.13.

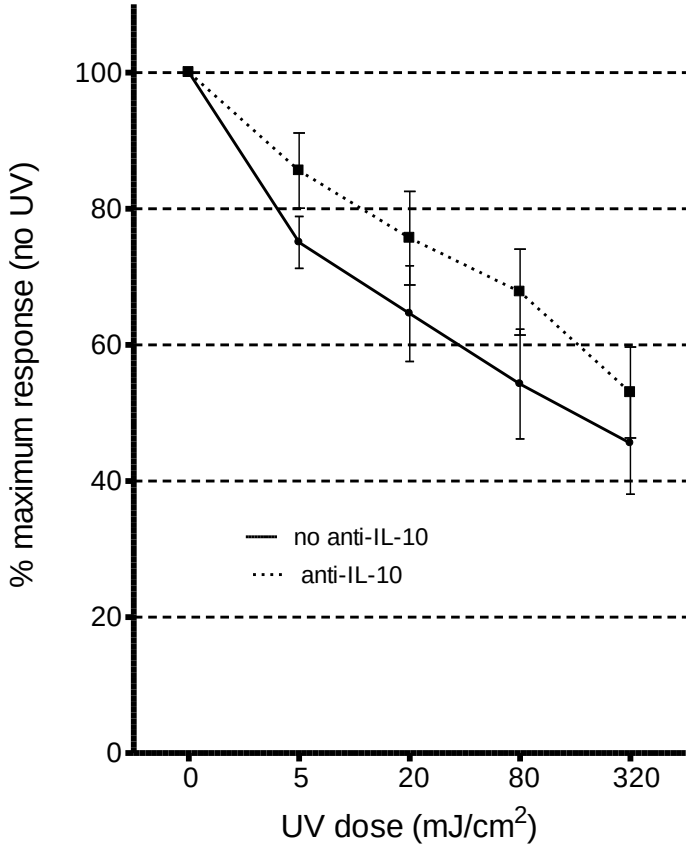


Figure 4.13: PHA-induced lymphocyte proliferation in the presence and absence of anti-IL-10 antibodies when UV-irradiated monocytes were cultured with PBMCs in the presence of PHA, n=5. Percentage proliferation (maximal response) in comparism with lymphocytes cultured with unirradiated monocytes, values are mean (with SEM). There was no significant difference between cultures with the addition of anti-IL-10, $p > 0.05$ (ANOVA).

Anti-IL-10 antibodies did not significantly effect UV induced suppression of lymphocyte proliferation (in response to PHA). This could be due the the range of cell types in the PBMCs. Therefore to examine the effect of anti-IL-10 antibodies in a more "pure" system, i.e. in cultures of monocytes and T cells, the same antibodies were added to UV-irradiated monocytes/T lymphocyte cultures in the presence of PHA, figure 4.14.

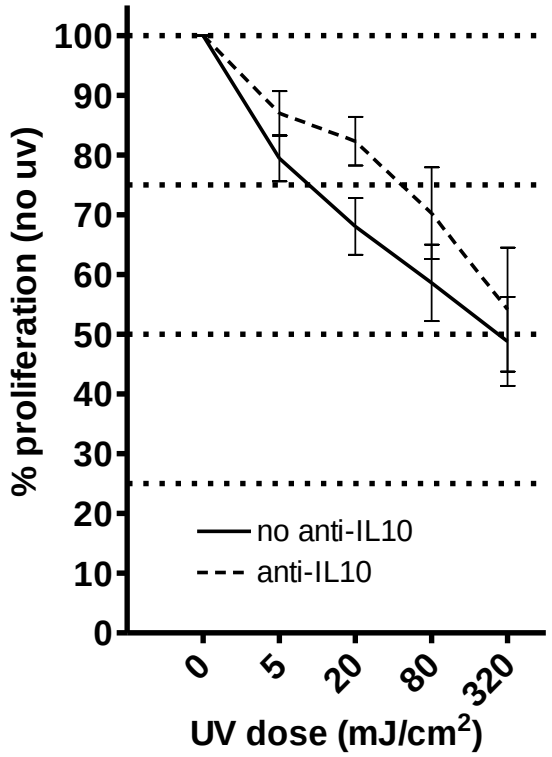


Figure 4.14: PHA-induced T-lymphocyte proliferation in the presence and absence of anti-IL-10 antibodies when UV-irradiated monocytes were cultured with lymphocytes in the presence of PHA; showing percentage proliferation in comparism with T lymphocytes cultured with unirradiated monocytes. Values are mean (SEM). Anti-IL-10 antibodies inhibited the suppression of T lymphocyte proliferation by UV-irradiated monocytes, n=6, p = 0.0012 (ANOVA) at 20mJ/cm^2.

There was a significant inhibition of T lymphocyte proliferation by UV-irradiated monocytes at at 20mJ/cm^2 p = 0.0012. However IL-10 is unlikely to be the main immunosuppressive factor in this system, as although a significant suppression was seen there was not a complete reduction of the UV-induced immunosuppressive response.

In addition, the effect of anti-IL-10 treated supernatant from UV-irradiated monocytes on anti-CD3/anti-CD28-induced T lymphocyte proliferation was investigated, figure 4.15.

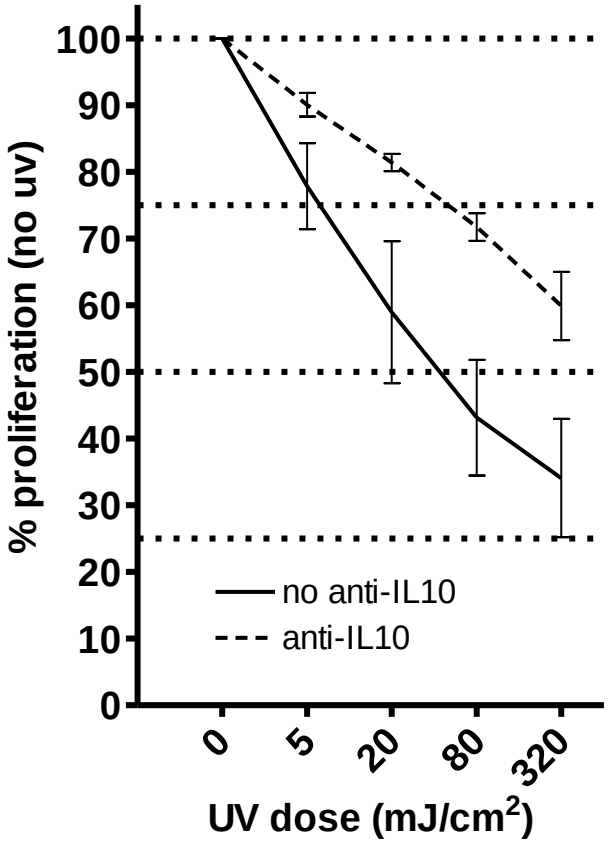


Figure 4.15: Anti-CD3/anti-CD28-induced T lymphocyte proliferation in the presence and absence of anti-IL-10 treated supernatant taken from UV irradiated monocytes. Showing percentage proliferation in comparism to T lymphocytes cultured with unirradiated monocyte supernatant, values are mean (and SEM). There was significantly less immunosuppression by the supernatant from UV-irradiated monocytes when this was treated with anti-IL-10 antibodies $p < 0.005$ (ANOVA).

Anti-IL-10 treatment of the supernatants from UV-irradiated monocytes resulted in significantly less suppression of anti-CD3/anti-CD28-induced T cell proliferation when compared with untreated supernatant, n=6, $p < 0.05$ (ANOVA). Therefore, the results of the anti-IL-10 experiments in the purer monocyte/T cell cultures differed according to the stimulus for lymphocyte proliferation, suggesting that IL-10 might account for part of the immunosuppressive activity of UV-irradiated monocytes.

However, because the anti-IL-10 antibodies did not completely negate the suppressive effects following UV, IL-10 is unlikely to be the sole immunosuppressive factor involved.

4.3.7 Investigation of the immunosuppressive factor: IL-12.

It has been previously reported that the cytokine interleukin-12 (IL-12) is able to prevent the suppression of the CHS response by UVR in mice (Schwarz *et al*., 1996). In addition IL-12 can break UVB-induced hapten specific tolerance in mice (i.e. in mice that have been initially tolerized by UVB prior to application of a hapten, there is a CHS response upon reapplication of the same hapten in the presence of IL-12 (Schwarz *et al*., 1996). Therefore, release of IL-12 was investigated, by ELISA, in the supernatant of UV-irradiated monocytes cultured with T lymphocytes in the presence of PHA, figure 4.16. Results from two individuals suggested that there was less IL-12 in the supernatant following irradiation of monocytes with UV.

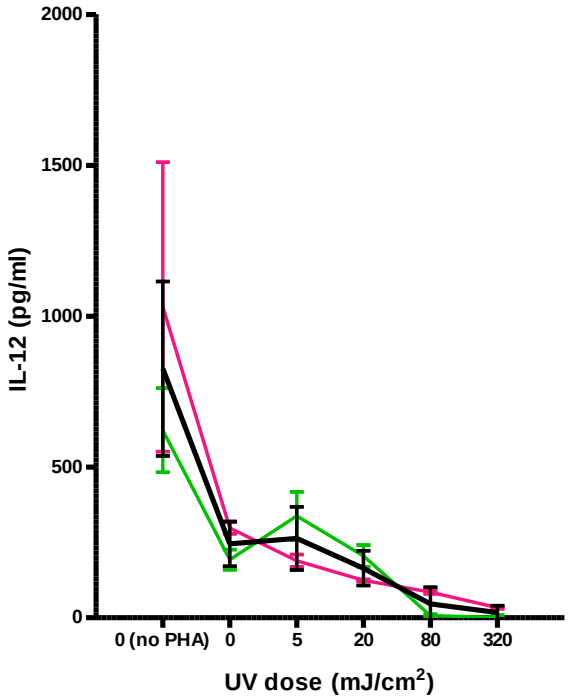


Figure 4.16: Examination for IL-12 release by UV-irradiated monocytes/T cell cultures in the presence of PHA, mean values (with SEM). The results from two individuals suggested that IL-12 release was reduced when the monocytes had been exposed to UV.

Admittedly, the possible reduction in IL-12 release would not be consistent with IL-12 being the immunosuppressive factor in the UV-irradiated monocyte supernatants, but some preliminary investigations were carried out on IL-12 release by various cultures, including (i) monocytes, (ii) monocytes and T cells, (iii) monocytes and PBMCs, (iv) T cells and PHA, (v) monocytes and PHA, (vi) monocytes and T cells and PHA, (vii) monocytes and PBMCs and PHA, and (viii) PBMCs in the presence of PHA, figure 4.17.

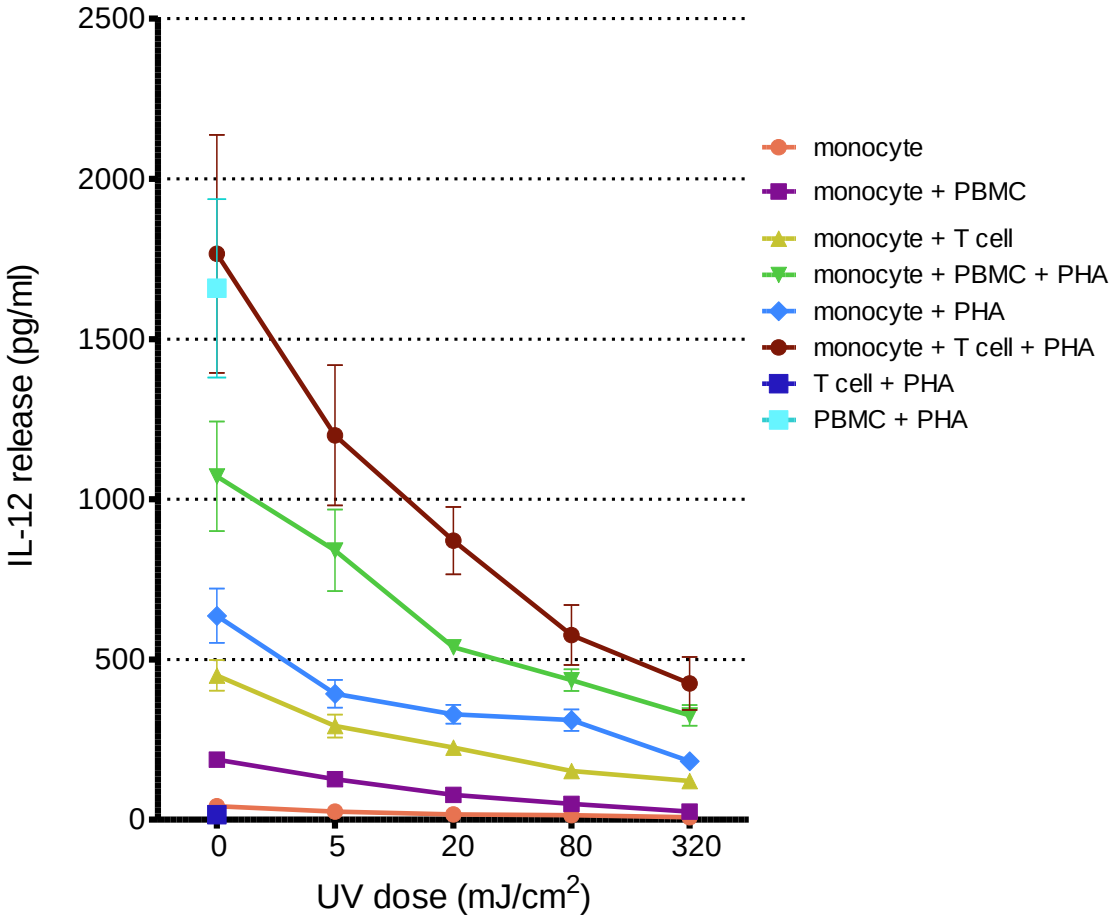


Figure 4.17: IL-12 release after 48 hours in cultures as delineated in graph in the presence or absence of PHA, n=3. UV-irradiated monocytes caused a dose-dependant decrease in IL-12 release in all cultures ($p < 0.05$, ANOVA) except UV-irradiated monocytes cultured alone ($p = 0.0147$, ANOVA). Values are mean (and SEM).

IL-12 release was decreased UV-dose dependently for all cultures except UV irradiated monocytes cultured alone. As a control IL-12 release was also detected for PBMCs and PHA and separately T cells and PHA, as expected as there were no monocytes in the T cells cultured with PHA alone therefore there was no activation of the T cells and so little IL-12 was detected, in comparism to PBMCs cultured with PHA.

4.3.8 Investigation of the immunosuppressive factor: TGF-beta.

TGF-beta is a cytokine that has a range of functions including regulating cell proliferation, differentiation, tissue remodelling and immunity (Massague J 1998). Therefore to investigate whether TGF-beta was involved in the UV-induced immunosuppression observed in the above system, a TGF-beta receptor I blocker (which inhibits TGF-beta signalling) was added to the cultures (UV irradiated monocytes and T lymphocytes in the presence of PHA), figure 4.18.

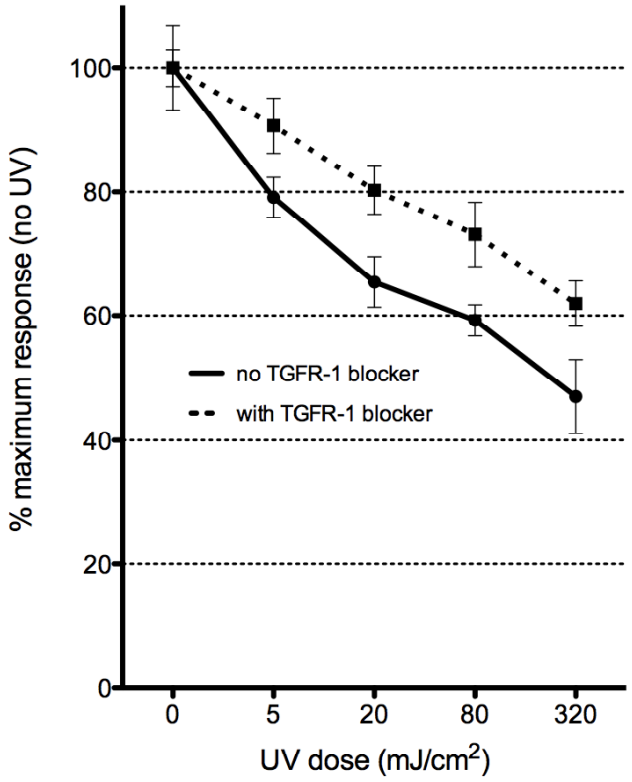


Figure 4.18: Effect of a TGF-beta receptor 1 (TGFR-1) blocker on the suppression of PHA induced lymphocyte proliferation by UV irradiated monocytes, n=10. The TGFR-1 blocker significantly reduced UV-induced suppression of lymphocyte proliferation, p= 0.0013 (ANOVA). Showing percentage proliferation in comparism to T lymphocytes cultured with unirradiated monocytes, values are mean (and SEM).

Addition of the TGF-beta-receptor 1 blocker significantly reduced the suppression by UV of PHA-induced lymphocyte proliferation, p = 0.0013 (ANOVA) figure 4.18. However the TGF-beta blockade did not completely abolish UV induced immunosuppression, suggesting that TGF-beta may be involved but not as the single immunosuppressive agent released from UV-irradiated monocytes.

4.3.9 Investigation of the immunosuppressive factor: IDO.

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that breaks down the essential amino acid tryptophan (Taylor et al 1991). IDO is able to suppress T cell responses; metabolites of tryptophan are toxic and may be the mechanism by which IDO suppresses T cell activity (Taylor *et al* 1991). Therefore the IDO inhibitor, 1-methyl-tryptophan, was added to UV irradiated monocytes/T lymphocyte cultures in the presence of PHA for 48 hours and the effect on lymphocyte proliferation was noted, figure 4.19. 1-methyl-tryptophan significantly reduced the suppression of T lymphocyte proliferation induced by UV irradiated monocytes.

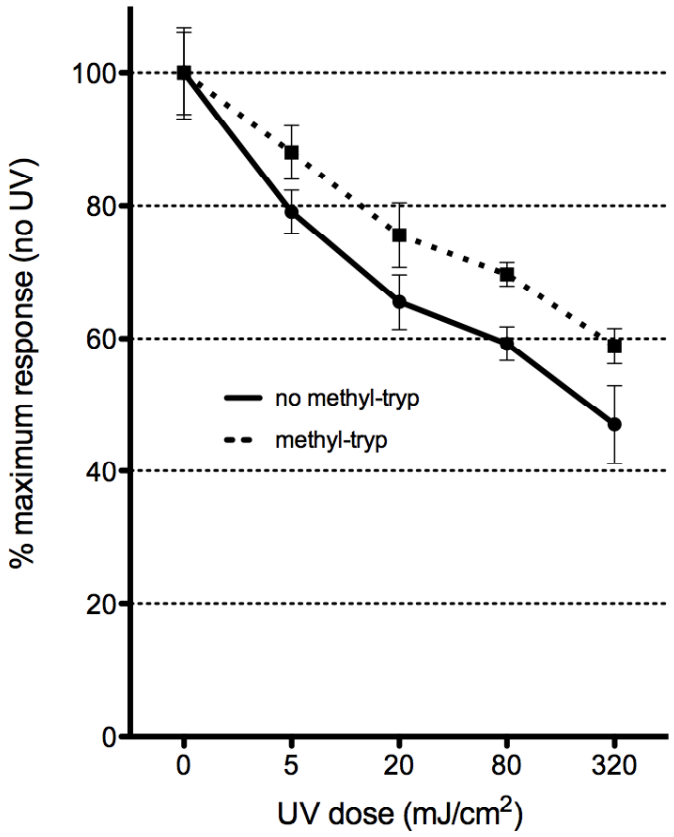


Figure 4.19: Effect of 1-methyl-tryptophan (methyl-tyrp) on the suppression of PHA-induced lymphocyte proliferation by UV-irradiated monocytes. Showing percentage proliferation in comparism to T lymphocytes cultured with unirradiated monocytes, values are mean (and SEM). 1-methyl-tryptophan significantly decreased the suppression of T cell proliferation by UV irradiated monocytes, n=10, p= 0.0021 (ANOVA).

Although 1-methyl-tryptophan significantly decreased the suppression of T cell proliferation by UV irradiated monocytes, p= 0.0021 (ANOVA) there was not a complete reduction in the UV induced suppression of PHA induced proliferation. Therefore IDO is unlikely to be the immunosuppressive factor.

4.4 Discussion

The exact mechanism(s) responsible for UV-induced immunosuppression is/are unclear. Furthermore, much of the work on this topic has been performed in mice and less is known about these mechanisms in humans. In addition, it is not clear whether the immunosuppressive effects of UV in humans result from direct effects on antigen presenting cells or indirectly due to irradiation of other cell types (e.g. keratinocytes) within the skin. One aim of the current study was to examine whether UVR has immunosuppressive effects via direct actions on human antigen presenting cells. In order to do this, an initial aim was to develop an *in vitro* culture system that allowed detailed investigations to be carried out on individual cell types. Although Langerhans cells are perhaps more ideal than monocytes, it is more difficult to obtain sufficient Langerhans cells from human skin to conduct multiple experiments, therefore monocytes were used as antigen-presenting cells. However, Langerhans cell cultures may be used in the future after the monocyte/T cell system has helped to suggest which are the more appropriate Langerhans cell experiments to perform.

The use of highly purified monocytes and lymphocytes in the current study demonstrated that PHA-induced lymphocyte proliferation was significantly suppressed in a dose dependant manner by UV irradiated monocytes. The limited uptake of trypan blue (a dye which is excluded from the cell if the cell is viable) indicated that UV had not simply killed the monocytes and that cell death was unlikely to be responsible for the UV-induced suppression. The addition of PBMCs (containing unirradiated and "normal" monocytes) to irradiated monocyte cultures

demonstrated that the immunosuppression was not due to an insufficient amount of antigen presentation (e.g. due to irradiated monocytes becoming "unhealthy" after UV exposure). Overall, these and the supernatant experiments (in which a soluble factor released from the UV-irradiated monocytes suppressed lymphocyte proliferation in both anti-CD3/anti-CD28 and PHA stimulated PBMCs) indicated that the irradiated antigen presenting cells either released, or stimulated the release from T cells, of an immunosuppressive factor.

The results of the subsequent investigations, which attempted to identify what the soluble immunosuppressive factor was, did not find a single agent which was responsible for the UV-induced immunosuppression in this system. Experiments with various inhibitors, and the measurement of certain cytokines, suggested that prostaglandins (possibly PGE_2), IL-10, TGF-beta and IDO and probably some other factor(s) are involved. The results indicating that prostaglandins and IL-10 are relevant mediators which are partially responsible for UV-induced immunosuppression by antigen presenting cells are supported by certain previous publications. For example, there is an increase in PGE_2 in human skin after UVB irradiation (Black AK *et al* ., 1978) and PGE_2 is also released by monocytes following UVB irradiation and causes a decrease in pro-inflammatory cytokines including TNF-alpha (Hart *et al* ., 1993). In addition, IL-10 has been demonstrated in many studies to be an important cytokine linked with the immunosuppressive effects of UV irradiation. IL-10 is elevated in the serum of mice after UV exposure (Rivas & Ullrich, 1994) and injection of anti-IL-10 antibodies into mice following

UV irradiation resulted in no suppression of CHS (Shreedhar *et al* ., 1998a). The fact that the experiments with anti-IL-10 antibodies in the current thesis was *in vitro* and on human antigen presenting cells/T cells might account for the difference between the partial effect of blocking IL-10 in this study and the more complete effect in the mice *in vivo* projects.

Although a reduction in IL-12 was seen following UV in this study, and it is known that IL-12 can reverse the immunosuppressive effects of UV (Schwarz *et al* 2005), it was clear that IL-12 was not the immunosuppressive factor that was released by the monocytes. However, the development of this UV-irradiated purified *in vitro* human antigen presenting cells / T cell system and the results so far indicate that this system may provide a useful way of identifying which factors / cytokines mediate the direct and indirect immunosuppressive effects of UV (for example in the system used in this study or following modification with the addition of supernatant from irradiated keratinocytes). Overall, the results also suggest that more than one mediator is released when antigen presenting cells are exposed to UV, but it is not clear at present whether a few or multiple mediators are involved in the consequent immunosuppression.

Chapter 5

Alpha-MSH has been demonstrated by previous studies to modulate inflammation and to suppress immune responses, with many of these studies performed using animal models and/or cell culture systems (Robertson *et al*., 1986; Vulliemoz *et al*., 2006; Grabbe *et al*., 1996). For example, alpha-MSH has been shown to suppress contact hypersensitivity response in mice (Grabbe *et al*., 1996; Rheins *et al*., 1989) and to reduce CD14 expression on macrophages (Bhardwaj *et al*., 1997). High levels of alpha-MSH have also been identified at sites of inflammatory disorders, including bronchial asthma, inflammatory bowel disease and rheumatoid arthritis (Catania *et al* ., 2004; Catania *et al*., 1994; Ceriani *et al* ., 1994),). An inverse correlation between the severity of inflammation and amount of alpha-MSH has been observed with higher levels of alpha-MSH associated with lower degrees of inflammation (Catania *et al.*, 2004). Furthermore, a previous study looking at the *in vitro* effects of alpha-MSH on lymphocyte activity demonstrated that there was significant suppression of antigen-induced lymphocyte proliferation in the presence of alpha-MSH, but that this suppression ranged from 0% to 80% between individuals (Cooper *et al*, 2006).

Based on the anti-inflammatory / immunosuppressive effects of alpha-MSH in the literature, it might be possible to use this agent as a treatment for inflammatory skin diseases or inflammation in other organs. For example, alpha-MSH has been demonstrated to be as potent as corticosteroids in a rabbit model of corneal trauma and inflammation (Naveh *et al*., 2001). Furthermore in a rat model of adjuvant arthritis, administration of alpha-MSH prevented the development of arthritis

(clinical and histological signs of arthritis was inhibited) to a similar extent as rats administered with the corticosteroid Prednisolone (compared with saline as control, which did not prevent the development of arthritis) (Ceriani *et al* 1994). However, limited work has been carried out on the anti-inflammatory and immunomodulatory effects of alpha-MSH in humans (both *in vivo* and in *vitro*). In addition, based on the variability in immunosuppression seen by Cooper *et al* (2006), it is possible that many people might not respond adequately to alpha-MSH. Interestingly, in the same study it was noted (in a very limited experiment) that SHU 9119 and MTII seemed to suppress more potently than alpha-MSH *in vitro*, therefore the central question of the work in first part of the thesis was to see whether analogues of alpha-MSH have more potent immunosuppressive effects than alpha-MSH alone in an *in vitro* model. Indeed, other work that suggested that SHU 9119 and MTII should be investigated in this manner, comes from observations on anti-inflammatory effects of both agents, albeit in a limited number of studies. For example, MTII suppressed monosodium urate-induced neutrophil influx by suppressing the chemokine KC (a neutrophil attractant chemokine) in a murine model of gout (Getting *et al* ., 1999). Similarly, SHU 9119 has been reported to reduce certain cytokine responses, but it has also been shown to decrease IL-10 responses after injection into LPS-treated rhesus moneys (Vulliemoz *et al*., 2006), so it was not clear prior to the current study whether the net effect of SHU 9119 would have been that of a potent immunosuppressive agent.

However, rather than limit the current study to just MTII and SHU 9119, it seemed sensible to include NDP-alpha-MSH and KPV (as well as alpha-MSH to allow comparison with this melanocortin). This is because NDP-alpha-MSH is a potent agonist at all the melanocortin receptors except MC2R (Schiöth HB *et al* ., 1995) and 26 times more potent than alpha-MSH in terms of stimulating pigmentation (Sawyer *et al* ., 1980). Additionally, NDP-alpha MSH has been shown to decrease leukocyte infiltration into, and inflammatory mediators in, the allograft following heart transplantation in rats, thus reducing the chance of rejection (Gatti *et al* ., 2002; Colombo *et al* ., 2005). KPV has also been demonstrated to have immunosuppressive activity, for example KPV inhibited the production of NO and the pro-inflammatory cytokines TNF-α and IL-6 in a microglial cell line after stimulation with LPS (Delgado *et al*., 1998).

The data in this thesis shows that each of the analogues of alpha-MSH (including NDP-alpha-MSH, MTII, SHU 9119 and KPV) as well as alpha-MSH suppressed PHA- and antigen-induced lymphocyte proliferation but that none of the synthetic analogues of alpha-MSH are more potent than alpha-MSH. If any of the analogues had been more potent, it might have formed the basis for future studies to be carried out *in vivo* to examine the efficacy of that agent in inflammation. However, although none of the analogues were more potent in the assays, the fact that alpha-MSH, NDP-alpha-MSH, MTII, SHU 9119 and KPV were previously shown to have immunosuppressive or anti-inflammatory effects in other studies, it would still seem reasonable to test these compounds further in future *in vivo* studies. Despite the lack

of potent effects in most people in the studies in this thesis, it is possible that the analogues act more potently *in vivo*. For example, alpha-MSH might act indirectly as well as directly on inflammation, because previous studies have demonstrated that alpha-MSH acts centrally to inhibit peripheral inflammation (Lipton *et al*., 1991). Indeed, the reduced inflammation secondary to centrally administered alpha-MSH could be prevented by spinal cord transection (Lipton *et al*., 1991), and in mice with spinal transection, intraperitoneal administration of alpha-MSH had a smaller anti-inflammatory effect (Macaluso A., *et al* 1994). Therefore it is likely that the *in vitro* work does not measure the same thing as an *in vivo* study, but the *in vitro* experiments in this thesis are helpful in that they indicate that none of the melanocortin analogues are likely to be more effective than alpha-MSH alone. Furthermore, the fact that there were no differences in suppression of lymphocyte proliferation between subjects with a wild type *MC1R* genotype and subjects with a variant *MC1R* genotype suggests that any future potential therapy with one of these melanocortins may be equally useful in red-headed people with *MC1R* variants as well as those with wild type *MC1R*. Admittedly, the results in the thesis show that immune responses of some individuals were more suppressed by the melanocortins compared to other subjects, so it may be useful to investigate this in more detail in the future as there may be a subgroup where alpha-MSH or its analogues have potent anti-inflammatory actions, and thus a group in whom these agents would be more effective as therapies.

It is known that injection of alpha-MSH causes skin-darkening (via MC1R) (Lerner & McGuire, 1961; Abdel-Malek *et al* ., 1995) and has effects on appetite (via MC3R and MC4R) (Huszar *et al* ., 1997; Chen *et al* ., 2000) and on erectile function (via MC4R) (Van der Ploeg *et al* ., 2002). Therefore, it would be good to identify an analogue of alpha-MSH that immunosuppressed but causes minimal side effects in these other areas. KPV may be a useful analogue for this reason because it seemed similarly effective in the studies in chapter 3, but does not cause skin darkening or have effects on appetite (Elliott *et al* ., 2004) and has been shown in other studies to causes a suppression of immune responses (Hill *et al* ., 2006; Getting *et al* ., 2003; Delgado *et al* ., 1998). On the other hand, it may be acceptable to have some adverse effects such as skin darkening (as would be expected from NDP-alpha-MSH, SHU9119 and MTII), because UV is used to treat psoriasis and some other skin diseases and this has the adverse effect of tanning (which some patients seems to like).

Another agent that causes immunosuppression, UVR, was also investigated in this thesis. It is thought that UVR-induced immunosuppression aids the development of cutaneous squamous cell carcinoma, but conversely, the immunosuppressive activity of UVR is employed by dermatologists for the treatment of inflammatory skin diseases such as psoriasis. Many studies have demonstrated that UVB induces immunosuppression but the exact mechanism(s) responsible for UVB-induced immunosuppression is/are unclear. DNA-damaged Langerhans cells can be identified in the skin-draining lymph nodes of mice after UV exposure (Toews *et al*

1980) and UVB-irradiated Langerhans cells have a distorted morphology with reduced dendritic processes (Toews *et al*., 1980), so one important question is whether a significant amount of the UVB-induced immunosuppression occurs as a result of effects on Langerhans cells / APCs. In addition, little data is available on humans and there are potential problems in extrapolating from mice to humans. For example, many of the mouse studies have used UVR doses which cause significant systemic (as well as cutaneous) immuno-suppression. Furthermore, many of the investigations have not addressed the consequences of UVR on individual cell types within the skin, for example whether the effects on antigen presenting cells are directly in response to UVR exposure or indirectly due to irradiation of the surrounding keratinocytes.

In this thesis, one aim was to set up an *in vitro* model system to examine the effects of UVR on APCs and on immune cells in general. The monocyte, which is a precursor of Langerhans cells, was used as a model APC, with the aim that if the evidence suggested that UVR had significant direct effects on APCs, then some future *in vitro* work could be carried out using Langerhans cells. The limitations of using Langerhans cells from the outset is that this requires samples of skin rather than blood and that one would have to work with fewer cells because of the more limited number of cells that can be obtained from skin. Although other research groups have looked at UVR effects on APC (eg IL-10 release and prostaglandin production), the experiments in this thesis were the first time that the effects of UV-irradiation of APCs on T lymphocyte proliferation was investigated. Interestingly, UV-irradiated monocytes significantly suppressed PHA induced T lymphocyte

proliferation. Furthermore, the supernatant of monocytes exposed to UVR significantly suppressed T lymphocyte proliferation suggesting that a potent immunosuppressive soluble factor is produced by the irradiated monocytes.

The addition of various inhibitors and examination of the supernatant for cytokines, including IL-10, suggested that prostaglandins and IL-10 may be released by the UV irradiated monocytes, however it seems likely that the supernatant from the irradiated monocytes contains some other immunosuppressive factor(s). Thus, the results in this thesis indicate that APCs can be directly involved in UVR-induced immunosuppression and that their involvement is at least partially through the release of a soluble factor. There are potential benefits to using the same model system with adaptations (such as the addition of irradiated and, separately, unirradiated keratinocytes) to investigate the cellular components of UVR-induced immunosuppression in humans. In addition, there are potential benefits of identifying the immunosuppressive factor in the irradiated-monocyte supernatant because one may be able to use this agent as a therapy for inflammatory diseases in the skin (e.g. for psoriasis) or for inflammation in other organs.

It is possible that the UVR immunosuppressive factor which is released from APCs is a cytokine (protein) so it may be feasible to use 2D gels to identify the protein by looking for proteins that are present or increased in the supernatant from monocytes that have been exposed to UVR as compared with unirradiated monocytes. Alternatively, the immunosuppressive factor may be a lipid, which might be

identified using mass spectrometry. If the immunosuppressive factor is identified, and can be purchased or produced synthetically in sufficient amounts, it could be added to unirradiated APC and T cell co-cultures to see if it has the same effect as UV irradiation of monocytes on this system. Subsequently, it may be possible to perform investigations to see whether the same factor is immunosuppressive *in vivo*, for example by using a mouse model of contact allergic dermatitis. Admittedly, there is a possibility that the immunosuppressive effects following UV-irradiation of APCs arise from a combination of soluble factors in the supernatant, and this could make it more difficult to identify the relevant agents.

The source of UVR in this thesis was a TL12 lamp, which emits UVB and some UVA, so future work could also investigate which wavelengths of UVR / part of UVR spectrum (UVA or UVB or both) are required to cause the APC-mediated immunosuppression. This data, and the findings on the effects of UVR on APCs in this thesis, might be helpful in other aspects of UVR-related biology, for example in understanding skin cancer susceptibility and development and in understanding how UVR works as a therapy for the treatment of skin disease. Furthermore, this information could influence / form part of future health messages on the importance of suitable protection against UVR by sunscreens and regarding behavior to reduce the detrimental effects of sunshine.

Appendix

A. 1.1 Cell culture reagents.

A. 1.1.1 RPMI

To 500ml of RPMI 1640 (Gibco-Invitrogen, Paisley, Catalogue number 21875-034) containing L-glutamine add:

1% penicillin/streptomycin (100U/ml penicillin and 100µg/ml streptomycin, Invitrogen Life Technologies)

1% sodium pyruvate (Invitrogen Life Technologies).

A. 1.1.2 Dulbecco's Modified Eagle Medium (DMEM).

To 500ml of DMEM (Gibco-Invitrogen, Paisley, Catalogue number 21969-035) containing sodium pyruvate and without L-Glutamine add:

10% EU approved heat inactivated FBS (Gibco-Invitrogen, Paisley, UK)

2mM L-Glutamine (Gibco-Invitrogen, Paisley, UK)

1% Penicillin/streptomycin (Gibco-Invitrogen, Paisley, UK)

A. 1.1.3 Cell storage medium.

10% DMSO (Sigma, Poole, UK)

20% fetal bovine serum (Gibco-Invitrogen, Paisley, UK)

In RPMI or DMSO (Gibco-Invitrogen, Paisley, UK)

1.1.4 Cell dissociation solution (Sigma, Poole, UK).

1x non-enzymatic sterile filtered cell dissociation solution, prepared on HBSS without calcium and without magnesium.

A. 1.2 Buffers used in DNA extraction/purification Qiagen kit (W. Sussex, UK).

A. 1.2.1 Buffers of unspecified content (Qiagen, W. Sussex, UK)

Qiagen protease QP

Lysis buffer AL

Wash buffer PE

Buffer PB

Wash buffer AW1 (wash buffer made up as manufacturers instructions)

Wash buffer AW2 (ethanol based wash buffer, made up as manufacturers instructions)

Elution buffer AE

Buffer QG (for DNA extraction from gel)

A. 1.2.2 DNA gel electrophoresis

50x TAE stock solution:

2M Tris (Sigma, Poole, UK)

50mM EDTA (Sigma, Poole, UK)

Prepared in dH_20, pH8.5

57% (v/v) glacial acetic acid (Sigma, Poole, UK).

Orange G:

0.05g Orange G salt

12ml 50x TAE

3.2ml UHQ H_20

6g glycerol (Sigma, Poole, UK).

A. 1.2.3 Polymerase chain reaction

Reaction buffer

160mM $(NH_4)_2S0_4$

670mM Tris HCL

Prepared in UHQ dH_20, pH 8.8 at 25^oC

0.1% Tween 20

A. 1.2.4 Qiagen solutions used for DNA extraction and purification kits Qiagen. W. Sussex, UK).

Buffer P1

5omM Tris base

3,2g $Na_2EDTA\ 2H_2O$

Prepared into 1 L dH_20, pH 8.0

Add 100 mg RNAase A per litre (supplied)

Buffer P2

200mM NaOH

Prepared into 950ml dH_20

50ml 20% SDS solution

Buffer EB

10mM Tris CL

Prepared in dH_20, pH 8.5

Buffer QBT

750mM NaCl

50mM MOPS (free acid)

Prepared in 800ml dH_20, pH 7.0

Add 150ml isopropanol

15nk 10% Triton X-100

Make up to 1 L with dH_20

A. 1.3 DNA sequencing

Sequencing gel

25ml 4.8% Acrylamide. 6M Urea Gene PAGE plus gel solution (Amresco, Anachem, London, UK)

Add the following and invert to mix:

150µl 10% APS (ammonium persulphate, Sigma, Poole, UK, prepared in UHQ dH_20)

15µl TEMED (Sigma, Poole, UK)

A. 1.4 Flow cytometry.

FACS buffer

0.1% Sodium azide (Sigma, Poole, UK)

1% BSA (Sigma, Poole, UK)

Prepared in 500ml PBS

A. 1.5 Cell separation using the autoMACS separator.

A. 1.5.1 Buffer

Sterile filtered

PBS pH 7.2

0.5% BSA

2mM EDTA

A. 1.5.2 Solutions required for operation of autoMACS

Running buffer

Sterile filtered

PBS pH 7.2

0.5% BSA

2mM EDTA

Rinsing butter

Sterile filtered

PBS pH 7.2

2mM EDTA

Storage solution

70% ethanol in distilled water

A. 1.5.3 Preparation/priming of the autoMACS.

The autoMACS solutions are made up as 1.7.02. The solution bottles are connected to the corresponding tube of the autoMACS. The autoMACS is switched on and the clean programme selected. The autoMACS is then ready for cell preparation.

It is important that all solutions remain cold (4^{o}C) therefore it is best to prepare the autoMACS separator close to when the cells are ready to be separated.

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