

The Delayed Type
Hypersensitivity Response:
a model for studying the
defects in skin immunity in
the old

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I, Elaine Agius confirm that the work presented in this thesis is my own.
Where information has been derived from other sources I confirm that
this has been indicated in the thesis.

Signed:

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Dedication

This work is dedicated to my parents Herbert and Therese Agius.

Abstract

Immunity declines during ageing, however the mechanisms involved are not known. In this study we show that the cutaneous delayed type hypersensitivity response (DTH) to recall antigens is significantly decreased in old individuals and that this was unrelated to CCR4, CLA or CD11a expression or physical capacity for migration of CD4⁺ T cells. Instead, there was defective activation of dermal blood vessels of these subjects that resulted from decreased TNF- α secretion by macrophages after antigen-challenge *in vivo*. However, isolated skin macrophages from these subjects could be induced to secrete TNF- α after stimulation with TLR 1/2 or TLR 4 ligands *in vitro*, indicating that the defect is reversible. The decreased conditioning of tissue microenvironments by macrophage-derived cytokines may therefore lead to defective immunosurveillance by memory T cells. This may be a predisposing factor for the development of malignancy and infection in the skin during ageing.

Abbreviations

APC	Antigen presenting cell
APC	Allophycocyanin
BCG	Bacillus of Calmette and Guérin
BSA	Bovine serum albumin
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte antigen
CMV	Cytomegalovirus
CNS	Central nervous system
Cpm	Counts per minute
Cy5	Cyanin 5
DAB	3,3'-diaminobenzidine
DC	Dendritic cell
DDC	Dermal dendritic cell
DMEC	Dermal microvascular endothelial cells

DTH	Delayed type hypersensitivity
EI	Erythema-index
E-selectin	Endothelial cell selectin
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPEX	Immune dyregulation, polyendocrinopathy, enteropathy, X-linked) syndrome
JAM	Junctional adhesion molecule
LC	Langerhans cell
LFA	Leucocyte function-associated antigen
LPS	Lipopolysaccharide
MACS	Magnetic activated cell sorting

MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MT	Mantoux test
mRNA	Messenger ribonucleic acid
OSM	Oncostatin M
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PECy5.5	Phycoerythrin-cyanin 5.5
PerCP	Peridinin chlorophyll protein
PHA	Phytohemagglutinin
PKC	Protein kinase C
PPD	Tuberculin purified protein derivative
P-selectin	Platelet selectin
PRRs	Pattern recognition receptors

RBC	Red blood cell
RPMI	Roselyn Park Memorial Institute
SB	Skin suction blister
SCC	Squamous cell carcinoma
SD	Standard deviation
SEM	Standard error of the mean
SSC	Side scatter
Tregs	Regulatory T cells
TARC	Thymus- and activation regulated chemokine
TB	Tuberculosis
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
VZV	Varicella zoster virus

WBC

White blood cell

Publications, abstracts and awards

PUBLICATIONS

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ABSTRACTS

E Agius, K. Lacy, M Vukmanovic-Stejic, AP Papageorgiou, J Curnow, J Greenwood, N Klein, MH Rustin and AN Akbar. Decreased endothelial activation results in defective secondary T cell immunity in aging skin. *Journal of Investigative Dermatology*. April 2008, Volume 128: S1-S226

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Table of Contents

Chapter 1. Introduction.....	1
1.1. The Skin Immune System.....	1
1.2. T cell mediated immune responses	2
1.2.1. Primary T cell response	2
1.2.2. Memory T cell response	3
1.2.3. Memory T cell phenotype.....	4
1.2.4. Delayed Type Hypersensitivity Responses and the Skin	5
1.2.5. Initiation of DTH responses in the skin.....	8
1.2.6. T cell migration.....	12
1.2.7. T cell proliferation and clonal expansion during DTH responses in the skin.....	14
1.2.8. Regulatory T cells and their role in cutaneous memory responses	15
1.3. Innate immune cells in human skin and their blood precursors	17
1.3.1. Blood Monocytes and their relationship to tissue macrophages and DCs.....	17
1.3.2. Blood Monocyte Subsets	19
1.3.3. Macrophages	21
1.3.4. Dendritic cells	23
1.4. Pattern recognition receptors.....	26
1.4.1. Toll-like receptors	27

1.5. Chemokines and cytokines and their role in memory responses in the skin	27
1.6. Ageing and the immune system	29
1.6.1. Studying ageing and the immune system	29
1.6.2. Ageing and its effect on T cells	32
1.6.3. Ageing and its effect on Innate Immunity	33
1.6.4. Ageing and its effect on skin structure and cellular constituents	34
1.7. Aims and Objectives	34
Chapter 2. Materials and Methods	37
2.1. Volunteer recruitment	37
2.2. Skin testing	38
2.2.1. Candida skin testing	38
2.2.2. Mantoux test	38
2.2.3. Assessment of skin test responses	38
2.3. Skin sampling	39
2.3.1. Suction blisters	39
2.3.2. Skin biopsy	44
2.4. Blood sample	45
2.4.1. PBMC isolation	45

2.4.2.	CD4 isolation for migration experiments	45
2.5.	Immunohistology	48
2.5.1.	Antibodies used in the study	48
2.5.2.	Indirect Immunofluorescence	50
2.5.3.	Indirect Immunohistochemistry	53
2.5.4.	Imaging and Quantification/Assessment of Immunofluorescence micrographs	56
2.5.5.	Quantification of Immunohistochemistry	58
2.6.	Skin Explant Culture	59
2.7.	Flow cytometry.....	59
2.7.1.	Surface staining by direct Immunofluorescence	59
2.7.2.	Intracellular staining	60
2.8.	T Lymphocyte transendothelial migration.....	62
2.8.1.	Endothelial culture	62
2.8.2.	Activation of endothelial cells for migration studies	63
2.8.3.	Migration experiments.....	63
2.9.	<i>In Vitro</i> Cell Culture	64
2.9.1.	Measurement of cellular proliferation by [3H] thymidine incorporation	64
2.9.2.	Monocyte cell culture with TLR ligands	64
2.10.	Cytokine assays	64

2.11. Statistics	65
Chapter 3. <i>Decreased clinical and cellular response to injected recall antigen in old skin</i>	67
3.1. Introduction.....	67
3.2. Candida Skin testing.....	69
3.2.1. Clinical response to the Candida skin test in young and old individuals	69
3.2.2. Clinical response to the Candida skin test correlated to peripheral blood response in young and old individuals.....	71
3.2.3. Differences in T cell number accumulation during the candida skin test in young and old subjects	72
3.2.4. Differences in numbers of candida-specific memory T cell accumulation in young and old	77
3.2.5. Clinical response to the candida skin test correlated to numbers of T cells <i>in situ</i>	77
3.3. Discussion	78
Chapter 4. <i>Impaired memory T cell-endothelial interaction affects transmigration into ageing skin</i>.....	83
4.1. Introduction.....	83
4.2. Expression of skin homing receptors on CD4 ⁺ T cells is not reduced during ageing	84
4.2.1. CLA expression on T lymphocytes in peripheral blood.....	84
4.2.2. CCR4 expression.....	85

4.2.3.	CD11a (LFA-1) expression on peripheral blood T lymphocytes.....	86
4.3.	Capacity of CD4⁺ T cells to migrate is not reduced during ageing.....	87
4.3.1.	Endothelial monolayer culture and activation and control studies using pre-activated T cells for migration	87
4.3.2.	CD4 ⁺ T cell migration	89
4.4.	Reduced expression of adhesion molecules by dermal endothelium during a DTH response in old subjects.....	90
4.4.1.	E-selectin expression	91
4.4.2.	VCAM-1 expression.....	92
4.4.3.	ICAM-1 expression	93
4.5.	Endothelial activation using TNF-α and IFN-γ in young and old skin explant culture	94
4.6.	Surface area of endothelial cells in young and old dermis	96
4.7.	Discussion	97
 <i>Chapter 5. The innate immunity and its effects on secondary memory cutaneous immune responses in ageing skin.....</i>		 <i>101</i>
5.1.	Introduction.....	101
5.2.	Early inflammatory cytokine levels during cutaneous secondary memory immune responses.....	102
5.3.	Distribution of TNF-α expression in skin tissue.....	103

5.4. Numbers of macrophages and dendritic cells in young and old skin by immunohistochemistry	105
5.5. TNF-α expression by monocyte derived cells by immunohistochemical analysis of skin tissue.....	108
5.6. Dermal macrophages from old individuals can be induced to secrete TNF-α <i>in vitro</i>	111
5.7. Monocyte and DC transmigration into skin.....	113
5.7.1. DC-SIGN expression on monocytes and DCs	113
5.7.2. TLR activation leads to upregulation of DC-SIGN expression by peripheral monocytes and DCs	114
5.8. Discussion	118
<i>Chapter 6. Regulatory T cells and their role in secondary cutaneous immune responses</i>	<i>123</i>
6.1. Introduction.....	123
6.2. Accumulation of CD4⁺ Foxp3⁺ T cells during a secondary response to antigen <i>in vivo</i>	124
6.3. Accumulation and proliferation of CD4⁺ T cells during a secondary response to antigen <i>in vivo</i>	127
6.4. Transient expression of Foxp3 expression induced by activation of CD4⁺ T cells <i>in vitro</i>	129
6.5. Foxp3⁺ T cells at the site of MT proliferate in parallel to effector T cells and have a regulatory T cell phenotype.....	130

6.6.	Foxp3⁺ T cells at the site of MT have regulatory T cell function.....	132
6.7.	Foxp3⁺ T cells at the site of MT proliferate in parallel to effector T cells....	133
6.8.	Increased representation of CD4⁺Foxp3⁺ regulatory T cells (Tregs) in the skin of old humans.....	135
6.9.	Discussion	136
Chapter 7.	<i>Summary and future directions</i>	<i>141</i>
	<i>Reference List.....</i>	<i>148</i>
	<i>Published papers</i>	<i>187</i>

List of Figures

Figure 1-1: The role of DCs, macrophages and memory T cells in secondary cutaneous immune responses.....	11
Figure 1-2: Cellular components of the innate immune system in human skin during steady state and inflammation.....	18
Figure 2-1: The clinical DTH response in the skin and skin sampling.....	42
Figure 2-2: Skin suction blister induction.....	43
Figure 2-3: Explanation of bleed-through illustrated with the fluorochrome couple fluorescein iso-thiocyanate/AlexaFluor546.....	52
Figure 3-1: Cutaneous clinical responses to the Candida skin test are reduced in old individuals.....	70
Figure 3-2: PBMC proliferation in response to Candida antigen <i>in vitro</i> plotted against clinical scores to Candida skin test antigen.....	72
Figure 3-3: Cellular infiltrate at the site of cutaneous DTH response is reduced in the old.....	73
Figure 3-4: CD3 ⁺ , CD4 ⁺ T cell numbers at the site of the Candida antigen skin test	74
Figure 3-5: Candida-specific T cell infiltration in the skin following the Candida skin test in young and old.....	74
Figure 3-6: Kinetics of T cell proliferation during a cutaneous response to Candida skin test antigen in young and old.....	76

Figure 3-7: T cell number found on histology of Candida skin test injected skin correlates to the clinical score in both young and old.	78
Figure 4-1: Expression of CLA on circulating CD3 ⁺ and CD4 ⁺ T lymphocytes in the young and old.	85
Figure 4-2: Expression of CCR4 on CLA ⁺ and CLA ⁻ CD4 ⁺ T lymphocytes in the young and old.	86
Figure 4-3: Expression of CD11a on CLA ⁺ and CLA ⁻ CD4 ⁺ T lymphocytes in the young and old.	87
Figure 4-4: CD4 ⁺ T cell migration across dermal microvascular endothelium assays.	88
Figure 4-5: Comparison of young and old subject peripheral CD4 ⁺ T cell migration across dermal endothelial monolayer <i>in vitro</i>	89
Figure 4-6: Effect of adding blocking antibodies on CD4 ⁺ T lymphocyte migration.	90
Figure 4-7: Dermal endothelium in old individuals shows reduced expression of adhesion molecule E-selectin.	91
Figure 4-8: Dermal endothelium in old individuals shows reduced expression of adhesion molecule VCAM-1.	92
Figure 4-9: Dermal endothelium in old individuals shows reduced expression of adhesion molecule ICAM-1.	93
Figure 4-10: Old endothelium can upregulate adhesion molecules <i>in vitro</i>	95

Figure 4-11: Dermal CD31 ⁺ endothelial loop surface area and number is similar in young and old skin.	97
Figure 5-1: Early pro-inflammatory cytokines are reduced in old skin blisters.	103
Figure 5-2: Lack of TNF- α expression in old skin compared to young.....	104
Figure 5-3: Similar number of CD163 ⁺ macrophages in skin from young and old subjects.....	106
Figure 5-4: Similar number of CD11c ⁺ dendritic cells in skin from young and old subjects.....	107
Figure 5-5: TNF- α expression is reduced in CD163 ⁺ macrophages in old skin	109
Figure 5-6: Perivascular cell cluster autofluorescence obscures detection of possible TNF- α expression by CD11c ⁺ cells.	110
Figure 5-7: Macrophages isolated from the skin of old donors secrete TNF- α in response to TLR signals <i>in vitro</i>	112
Figure 5-8: DC-SIGN expression by CD163 ⁺ macrophages and CD11c ⁺ dendritic cells in skin not altered in old subjects.....	114
Figure 5-9: TLR stimulation with LPS upregulates DC-SIGN but not CLA expression on circulating monocytes from young and old subjects <i>in vitro</i>	115
Figure 5-10: Monocytes from young and old subjects upregulate DC-SIGN and CLA expression similarly upon TLR stimulation.....	116
Figure 5-11: Old circulating myeloid DCs show reduced upregulation of DC-SIGN on TLR stimulation.	117

Figure 5-12: Old peripheral blood DCs show reduced capacity to upregulate DC-SIGN expression after 72 hours of LPS stimulation <i>in vitro</i>	118
Figure 6-1: Treg gating and phenotype.	124
Figure 6-2: Human CD4 ⁺ CD25 ^{hi} regulatory cells turnover rapidly <i>in vivo</i>	125
Figure 6-3: Accumulation of CD4 ⁺ Foxp3 ⁺ T cells during a secondary response to antigen <i>in vivo</i>	126
Figure 6-4: CD4 ⁺ T cell proliferation at the site of the MT	128
Figure 6-5: Memory T cells induced to express Foxp3 transiently in humans by stimulation.	130
Figure 6-6: Foxp3 ⁺ T cells isolated from the MT have a Treg phenotype.	131
Figure 6-7: Foxp3 ⁺ T cells isolated from MT show Treg function.	133
Figure 6-8: Foxp3 ⁺ T cells have a Treg phenotype and proliferate at the site of an immune response.	134
Figure 6-9: Proliferating Foxp3 ⁺ T cells in the skin express low levels of CD127.	135
Figure 6-10: Numbers of CD4 ⁺ Foxp3 ⁺ Tregs are increased in the skin of old subjects.	136

List of Tables

Table 1-1: Cells of the Skin Immune system	1
Table 1-2: Adhesion molecules involved in T cell transendothelial migration into skin	12
Table 1-3: Human and mouse monocyte subsets	21
Table 2-1: Exclusion Criteria	37
Table 2-2: Clinical Scoring System	39
Table 2-3: Antibodies used for Indirect Immunohistochemistry	48
Table 2-4: Antibodies used for indirect immunofluorescence	49
Table 2-5: Antibodies used for flow cytometry	61

Chapter 1. Introduction

1.1. The Skin Immune System

The skin is the largest organ of the human body and its principal function is that of a physical barrier. It protects the organism by being impermeable to a multitude of harmful exogenous substances and maintains internal homeostasis by preventing excessive water and heat loss (Kalinin et al., 2002; Candi et al., 2005). In addition there is a highly specialised immune system consisting of immune cells which are resident, recruited or recirculating within the skin (Table 1-1) (Bos Jan D, 2004; Nestle and Nickoloff, 2007; Zaba et al., 2009). These cells are distributed in the epidermal and dermal layers of the skin and are capable of participating in both adaptive and innate immune responses. They are also responsible for distinguishing self from non self which is of fundamental importance since the skin comes into daily contact with exogenous substances. In particular, skin immune cells play an important role in the initiation and amplification of immune responses, with close interlinking between innate and adaptive pathways (Bos Jan D, 2004).

Table 1-1: Cells of the Skin Immune system

	Resident	Recruited	Recirculating
Innate	Keratinocytes	Monocytes	Natural Killer Cells
	Endothelial cells -Vascular -Lymphatic	Granulocytes -Basophilic -Eosinophilic -Neutrophilic	Dendritic cells
	Mast cells	Mast cells	?Promonocytes
	Tissue macrophages	Epitheloid cells	
Adaptive	T lymphocytes	T lymphocytes	T lymphocytes
	Dendritic cells	B lymphocytes	

1.2. T cell mediated immune responses

1.2.1. Primary T cell response

Primary T cell responses are mediated by naïve T cells that, after leaving the thymus, home continuously from the blood to lymph nodes and other secondary lymphoid tissue (Butcher and Picker, 1996; Mackay et al., 1990). Homing to lymph nodes occurs in high endothelial venules (HEV) which express molecules for the constitutive recruitment of lymphocytes. The initiation of a primary T cell response depends on dendritic cells (DCs) picking up antigens at peripheral sites and migrating to regional lymph nodes, where they present antigen to the naive T cells. Naive T lymphocytes only become activated and proliferate when their antigen receptors (TCRs) bind to cognate peptides presented on major histocompatibility complex (MHC) molecules (Rudolph et al., 2006; Stockinger et al., 2006). Recognition of antigen results in the triggering of the T cell receptor (TCR)/ CD3 complex as well as CD4 or CD8 co-receptors with the formation of an immunological synapse between the T cell and APC. Cell triggering is also dependent on the ligation of additional co-stimulatory, accessory and adhesion molecules such as CD28, LFA-1, CD40L, OX40 that serve to augment T cell activation and expansion by stabilising the immunological synapse and inducing intracellular signalling molecules (Jenkins et al., 2001; Croft, 2003; Watts and DeBenedette, 1999; Watts, 2005). The number of T cells specific for a particular antigen is naturally very low. It was recently shown that specific naive CD4⁺ T cell populations vary in frequency from 20 to 200 cells in mice (Moon et al., 2007). Moreover, the size of the specific naive population size predicted the size and TCR diversity of the primary CD4⁺ T cell response after immunisation with relevant peptide. Therefore, variation in naive T cell frequencies can explain why some peptides are stronger immunogens than others (Moon et al., 2007).

An encounter with an antigen induces the proliferation of T-cell clones, yielding approximately 1000 times more descendants with identical antigenic specificity (Blattman et al., 2002). Eventually, these activated lymphocytes acquire effector

functions and home to sites of inflammation. Some effector cells orchestrate humoral responses by interacting with activated B cells in lymphoid organs (King et al., 2008). Activation of effector CD4⁺ cells at the site of inflammation results in cellular differentiation with resulting polarised patterns of cytokine production. Cytokines are key determinants of whether an uncommitted T cell develops into a mature Th1 or Th2 cell. IL-12 promotes the development of Th1 polarised cells that secrete IFN- γ and TNF- α whereas IL-4 promotes the generation of Th2 polarised cells that secrete IL-4, IL-5, IL-10 and IL-13 (Bird et al., 1998; Murphy and Reiner, 2002). Some CD4⁺ T cells secrete both types of effector cytokines and are known as Th0 cells. Th17 cells that produce interleukin 17 (IL-17) have also been identified (Harrington et al., 2005). These are highly proinflammatory and induce severe autoimmunity. IL-6 and transforming growth factor-beta (TGF-beta) induce the differentiation of Th17 cells from naive precursors, whereas IL-23 serves to expand previously differentiated Th17 cell populations, (Bettelli et al., 2007). Also it was shown that IFN- γ and IL-4, inducers of Th1 and Th2 development, respectively, suppressed development of Th17 cells (Harrington et al., 2005). In general, Th1 responses are thought to be protective against intracellular infections whereas Th2 and Th17 responses are effective against extracellular infections. Most effector cells die after antigen is cleared, but a few antigen-experienced memory cells remain for long-term protection. Different subgroups of memory cells patrol lymphoid organs and peripheral tissues to mount rapid responses whenever the antigen returns (Sallusto et al., 1999).

1.2.2. Memory T cell response

Memory or recall responses occur more rapidly and are more pronounced than primary responses (Ahmed and Gray, 1996; Hu et al., 2001). This may be due to an increase in the frequency of antigen specific memory T cells within the T cell pool compared with naïve cells. However, it is also thought that memory T cells have a reduced requirement for activation in that they respond to lower concentrations of antigen, need less co-stimulatory signals, need a shorter duration of antigenic stimulation (Berard and Tough, 2002). They also enter into cell cycle more rapidly

following TCR stimulation (Veiga-Fernandes et al., 2000). Memory T cells also express different patterns of adhesion molecules and chemotactic receptors, enabling them to interact with APCs more effectively and also to migrate into inflamed non-lymphoid tissues. Memory T cells differ from naïve T cells in their tissue distribution and circulation pattern. They can be found in both lymphoid and non-lymphoid sites (Masopust et al., 2001; Reinhardt et al., 2001) and are readily activated by antigen in all tissues (Chalasanani et al., 2002) (Klonowski et al., 2004; Ely et al., 2003). Tissues such as the liver, lung and gut are major reservoirs of antigen experienced T cells (Chalasanani et al., 2002).

1.2.3. Memory T cell phenotype

CD45, the leukocyte common antigen, is used to differentiate naïve from effector and memory T cells. The relative expression of the CD45 isoform can be used to distinguish the state of T cell differentiation (Trowbridge and Thomas, 1994). CD45 is an integral membrane protein tyrosine phosphatase (Charbonneau et al., 1988) and is expressed on all nucleated haematopoietic cells including leucocytes (Hermiston et al., 2003). The precise role and ligand for the receptor have yet to be fully determined (Holmes, 2006) however CD45 appears to play an important role in T cell signal transduction (Kung et al., 2000; Mustelin et al., 2003). Naïve T cells express the CD45RA isoform, however following activation the expression of CD45RO is rapidly upregulated and CD45RA expression is lost over a few days (Akbar et al., 1988). All CD45RA⁺ naïve T cells also express high levels of CD45RB. CD45RO⁺ T cells can be divided into CD45RB high and low subsets depending on whether they have recently been primed or have undergone numerous rounds of stimulation and are highly differentiated respectively (Salmon et al., 1994). The loss of the co-stimulatory molecules CD27 and CD28 on memory T cells can also be used to identify cells with a more differentiated phenotype (Romero et al., 2007).

Recent studies have shown that human memory CD8⁺ T cells can re-express CD45RA and lose CD45RO expression in a process known as reversion (Dunne et

al., 2002; Faint et al., 2001; Wills et al., 1999). CD8⁺ revertant T cells, unlike naïve T cells, express low levels of L-selectin (CD62L) and CCR7 but high levels of LFA-1/CD11a. Limited evidence also suggests that CD4⁺ T cells may also revert to CD45RA⁺ phenotype (Bell et al., 2001). The precise role of revertant memory cells has yet to be identified but they may form a more stable, non-cycling memory population.

CD45RO⁺ memory T cells can be further divided on the basis of their migratory ability. The so called central memory cells express a repertoire of homing molecules similar to that of naïve T cells including CD62L and CCR7 and migrate preferentially to secondary lymphoid organs whilst effector memory cells lack expression of CD62L and CCR7 and migrate to peripheral tissues (Sallusto et al., 1999). Central memory cells lack immediate effector function, but they proliferate well, produce IL-2, efficiently stimulate dendritic cells and differentiate into CCR7⁺ effector cells upon secondary stimulation. In contrast, effector memory T cells do not express CD62L or CCR7 (Sallusto et al., 2004), express organ specific tissue homing receptors and can reside in both secondary lymphoid tissues as well as non-lymphoid tissues (Campbell et al., 2003). These cells have been found to rapidly secrete effector cytokines IFN- γ , IL-4 and IL-5 after restimulation (Sprent and Surh, 2002) but may not proliferate as efficiently as the central memory T cells. It has therefore been proposed that the effector memory subset is specialised for quickly entering inflamed tissues in order to provide immediate effector function whereas the central memory subset is responsible for a more sustained memory response (Sallusto et al., 2004).

1.2.4. Delayed Type Hypersensitivity Responses and the Skin

Delayed type hypersensitivity (DTH) responses in the skin can be induced by either the topical application of hapten or the intradermal injection of antigen into the skin and are representative of a cutaneous T cell mediated memory response (Turk, 1980). The delayed type hypersensitivity response to the intradermal injection of bacterial, viral and fungal antigens can therefore be used to assess individuals' cell-mediated

immune responses to particular antigens (Takahashi et al., 2003; Sadaoka et al., 2008; Ahmed and Blose, 1983). Moreover since the time of antigen application/injection is known the kinetics of the response can be effectively monitored. A positive skin test denotes prior antigenic exposure, T cell competency and an intact inflammatory response (Ahmed and Blose, 1983).

Responsiveness to DTH skin testing is assessed by the diameter of induration induced in the forearm skin in humans at 48 hours or by the amount of ear or footpad swelling at 24 hours in mice (Ahmed and Blose, 1983; Turk, 1980). In humans, following the injection of intradermal antigen, a clinical response is seen within a few hours with white or rose-coloured induration of the skin. The visible response in the skin peaks by 48-72 hours with a localized red, indurated area of skin. Rarely, vesicles and petechiae may also be seen. Ulceration at the site of injection has also been reported. The clinical response then reduces and resolves by 10-14 days (Turk, 1980; Ahmed and Blose, 1983).

DTH responses are used clinically to detect either infection with or previous exposure to various pathogens including *Mycobacterium tuberculosis* (Ahmed and Blose, 1983; Turk, 1980). The classical model of DTH is the Mantoux test where a sterile aqueous solution of tuberculin purified protein derivative, a complex mixture of peptides and carbohydrates derived from *M. tuberculosis*, is injected intradermally into the skin. It is used widely as an experimental model in that it produces a reproducible clinical response in the skin in young, healthy individuals previously exposed to *M. tuberculosis* and those immunized with the Bacillus of Calmette and Guerin (BCG) vaccine, prepared from live attenuated strains of *M. Bovis* (Turk, 1980; Ahmed and Blose, 1983). The likelihood of previous immunisation versus acute infection is determined by the diameter of the clinical response with a diameter of over 15mm regarded as suspicious of current infection (Tissot et al., 2005), warranting further investigation (NICE guidelines, March 2006).

DTH responses against common pathogens such as Candida or vaccine antigens (Tetanus) can also be used clinically as a measure of overall cell mediated immunity.

The Candin skin test, using a sterile solution produced from the culture filtrate and cells of two strains of *Candida albicans*, has been licenced for the evaluation of cellular immune responses in the skin in patients with HIV (Ahmed and Blose, 1983).

The DTH response in the skin is biphasic with an early non-specific infiltration of cells that occurs in all individuals and a second specific peak that is only seen in those who have previously been sensitized to the antigen (BOUGHTON and SPECTOR, 1963). First the presence of antigen and trauma to the skin induce non-specific “danger signals”, that recruit and activate cells of the innate immune system (Matzinger, 2002). These early signals are crucial for the conditioning of the inflammatory environment and to enable the recruitment of leucocytes from the blood (Kupper and Fuhlbrigge, 2004). Histological analysis shows that at around 4-6 hours there is an infiltration of neutrophils into the skin (Platt et al., 1983). Injection of latex beads or simple wounding to the skin induces a similar accumulation of neutrophils (John and Hunter, 2008). This is followed by macrophages that peak in numbers at around 24-48 hours. Around 12 hours after challenge T lymphocytes and dendritic cells have been shown to appear around dermal blood vessels (Platt et al., 1983). After 48 hours the majority of infiltrating cells are T lymphocytes with CD4⁺ lymphocytes exceeding CD8⁺ lymphocytes at all time points. Only a very small number of B lymphocytes have been observed at the site of DTH responses (Platt et al., 1983; Gibbs et al., 1984; Poulter et al., 1982). The majority of cells are seen to accumulate perivascularly, however T cells and macrophages are also found within the interstitium in the upper dermis and also around adnexal structures such as hair follicles and sweat glands. Lymphocytes may also be seen infiltrating the epidermis (Gibbs et al., 1984; Platt et al., 1983; Poulter et al., 1982).

It is very important that memory T cells migrating via the blood stream are able to enter the tissue at the site of the antigenic insult, via the endothelium that had been activated by the original danger signals to amplify the response (Kupper and Fuhlbrigge, 2004; Campbell and Butcher, 2002; Greening et al., 2003). The fact that non-immune individuals do not mount a DTH response to antigen, despite the fact

that equal levels of antigen as well as trauma to the skin occurs, highlights the importance of the memory T cell recruitment for the amplification step of this response to take place (Reed et al., 2004). There are therefore antigen-specific as well as antigen-non-specific events that have to be induced and co-ordinated to enable a response to take place and both are required for the clinical response (erythema and induration) in subjects who are immune (Turk JL., 1980).

1.2.5. Initiation of DTH responses in the skin

The conventional model of DTH responses in the skin suggests that lymphocytic recruitment to the skin occurs following secondary lymph node T cell proliferation after migration of antigen presenting cells from the skin to the draining lymph node. Although Langerhans cells are conventionally regarded as the main antigen presenting cells of the skin (Mathers and Larregina, 2006), their importance in initiating memory immune responses in the skin has been questioned (Grabbe et al., 1995) and it is unknown whether they are able to take up and process antigen that has been injected intradermally. Their contribution to the DTH response is therefore unclear. Another population of dendritic cells in the dermis, known as dermal dendritic cells has been shown to be found in close contact with resident T lymphocytes in normal skin suggesting that communication between these two cell types might occur early in the immune response to antigen (McLellan et al., 1998). Other cells within the skin also have antigen-presenting capability such as macrophages (Geppert and Lipsky, 1989) and keratinocytes (Albanesi et al., 1998; Barker et al., 1991; Roychowdhury and Svensson, 2005; Wittmann and Werfel, 2006) and may play a more prominent role in antigen presentation following secondary immune challenge. Macrophages have been shown to remain resident in skin however and therefore antigen presentation would have to occur locally in the skin perhaps to skin resident T cells, whereas the dermal dendritic cell is known to transmigrate across lymphatic channels to draining lymph nodes (Randolph et al., 1999). In addition human dermal microvascular endothelial cells (DMEC) may present antigen to circulating memory cells. DMEC express high levels of MHC class 1 and class 2 molecules at basal resting levels, can upregulate expression of

these molecules in the presence of IFN- γ and can induce human CD4⁺ and CD8⁺ memory but not naïve T cells to proliferate and secrete cytokines *in vitro* (Pober et al., 2001; Vora et al., 1994).

Normal skin contains T cells at a density of $\sim 1 \times 10^6/\text{cm}^2$. Moreover there is an estimated 20 billion T cells in the entire skin surface, nearly twice the number in the entire circulation (Clark et al., 2006). It is thought that this large number of T cells provides cutaneous immunosurveillance (Kupper and Fuhlbrigge, 2004; Clark, 2009). Skin resident T cells are predominantly CD4⁺ CD45RO⁺ memory T cells which are able to home to the skin because of their high level expression of cutaneous lymphocyte antigen (CLA), CCR4 and CCR6 (Clark et al., 2006; Kupper and Fuhlbrigge, 2004). The CCR8-CCL1 interaction is also important for homeostatic cutaneous T cell traffic (Schaerli et al., 2004). Skin resident T cells have a remarkably diverse TCR repertoire, and most have a Th1 effector memory cell phenotype, although Th2 cells, central memory cells and functional T regulatory cells are also present (Clark et al., 2006).

It is possible that interactions between resident T cells and antigen presenting cells are responsible for the initiation of DTH responses in the skin without the need for the initial migration of dendritic cells to the lymph nodes. Although T cell activation has been observed in draining lymph nodes following secondary challenge, this occurs at a reduced rate compared with the primary response (Jones et al., 2000). Based on calculations of circulating volumes and cardiac output it has been estimated that the time taken for antigen to reach the inguinal lymph nodes from the toe would take around 12 hours (Mestas and Hughes, 2004), however DTH responses are seen as early as 4 hours. In addition, recall immune responses in humans occur in spite of compromised afferent lymphatics (Mallon et al., 1997). All of these findings suggest that localised initiation of the secondary immune response may occur within the skin.

The close apposition of antigen presenting cells and T cells within the skin during recall responses (Katou et al., 2000; Willis et al., 1986) may indicate that the activation of resident antigen- specific T cells by antigen presenting cells within the

skin is possible. Activation of antigen specific cells within the skin could potentially result in the initiation of the immune response via the secretion of acute phase pro-inflammatory mediators such as TNF- α and IFN- γ resulting in the activation and recruitment of monocytes, neutrophils and memory T cells. Resident T cells may also be able to traffic to the lymph node where they are able to accelerate appropriate T and B cell responses. Figure 1-1 shows an overview of the roles of DCs, macrophages and T cells in the secondary cutaneous immune response.

One of the problems with this model however is the large diversity of antigen-specific cells required in order to accommodate the vast array of antigens that are encountered by the skin. Antigen specific cells may only be resident at the sites of previous antigenic challenge. This theory is supported by the observation that repeated allergen exposure to a site of previous allergic contact dermatitis in guinea pigs results in a more rapid clinical response, not seen at other non-exposed sites (Rustemeyer et al., 2002). In addition, in humans, IFN- γ producing CD8⁺ T cells have been found in the epidermis of fixed drug eruptions before and after secondary challenge (Mizukawa et al., 2002).

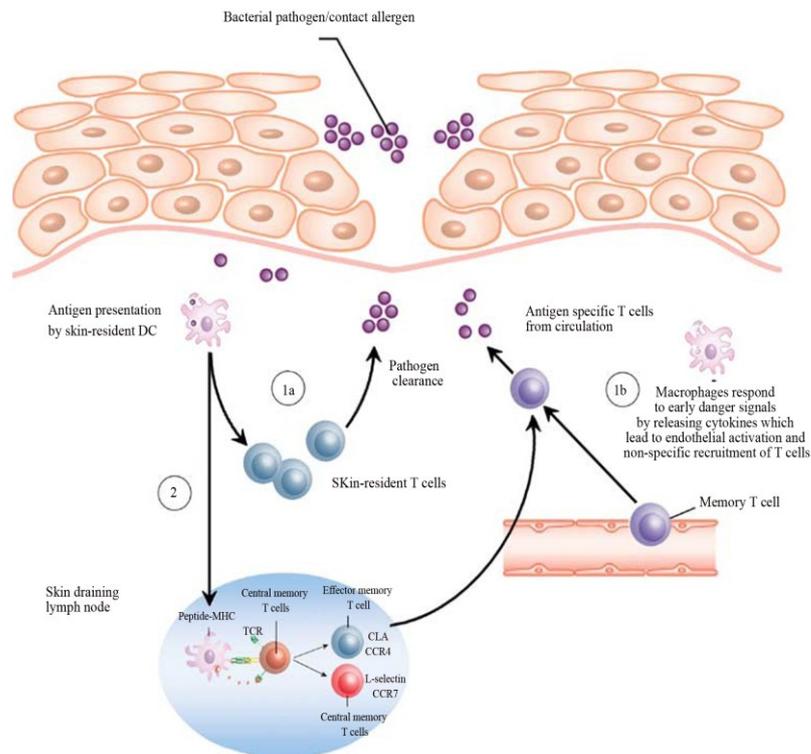


Figure 1-1: The role of DCs, macrophages and memory T cells in secondary cutaneous immune responses.

Memory immune responses can be divided into 2 stages. First, DCs take up antigen following pathogen/allergen re-exposure and present it to effector memory T cells resident within the skin. These cells proliferate and effect clearance of the pathogen (1a). Recognition of non-specific danger signals by macrophages leads to their activation and release of inflammatory cytokines which leads to endothelial activation and nonspecific recruitment of antigen-specific T cells from the blood (1b). The small numbers of antigen-specific T cells recruited into the skin in this way can also participate in clearance of the pathogen. Second, DC carry endocytosed antigen to the skin-draining lymph nodes where it is presented to central memory T cells. These central memory T cells then give rise to new populations of skin-homing effector memory T cells that migrate to the skin and clear the pathogen/allergen (Figure adapted from Clark, R.A., 2009).

1.2.6. T cell migration

For a secondary memory cutaneous immune response to develop, it is essential that circulating memory T cells are attracted to the area of inflammation and allowed to migrate into the skin by activated dermal post-capillary venule endothelium. For this to occur both the T cell and the endothelial cells must express corresponding adhesion molecules so that the T cell binds tightly to the endothelium before transmigration of the T cell into skin can occur (von Andrian and Mackay, 2000). Expression of different adhesion molecules on T lymphocytes allows homing to different organs, for example all T cells bound for the skin express the cutaneous lymphocyte antigen (CLA) (Berg et al., 1991). Gut-homing T cells are identified by their expression of CCR9 and $\alpha 4\beta 7$ integrin (Butcher et al., 1999). The leucocyte adhesion cascade consists of three main sequential steps: selectin-mediated rolling, chemokine-triggered activation and integrin-dependent arrest. Transmigration is then mediated mainly paracellularly or transcellularly (in the case of central nervous system) by CD31 (platelet/endothelial cell adhesion molecule, PECAM-1), CD99 and junctional adhesion molecule (JAM) (Ley et al., 2007). The adhesion molecules involved in migration of T cells into skin are shown in Table 1-2

Table 1-2: Adhesion molecules involved in T cell transendothelial migration into skin

Step in leucocyte migration	Lymphocyte adhesion molecule	Endothelial adhesion molecules
Rolling	CLA PSGL-1	E-selectin P-selectin
Arrest	<u>Integrins</u> CD11a (LFA-1) VLA-4	<u>Immunoglobulin superfamily</u> ICAM-1 VCAM-1
Transmigration	CD31(PECAM-1) ?	CD31(PECAM-1) Junctional adhesion molecule (JAM)

1.2.6.1. Leucocyte rolling

Initial tethers are formed between selectins expressed on the endothelium (E and P-selectin) which bind their ligands (oligosaccharides related to sialyl-Lewis^x) on circulating T cells. In the skin, the selectin expressed on the endothelium of greatest importance is E-selectin (ELAM-1) along with the less skin specific P-selectin (Picker et al., 1991; Yan et al., 1994). E-selectin expression is increased in response to exposure to the pro-inflammatory cytokines interferon-gamma (IFN- γ) and TNF- α whilst P-selectin is constitutively expressed by endothelial cells. Mice deficient in both E and P-selectins display defects in leucocyte extravasation as well as susceptibility to opportunistic bacterial infections (Frenette et al., 1996). Selectins bind their corresponding ligands on T cells which include L-selectin, which mediates migration to lymph nodes and spleen, P-selectin glycoprotein ligand-1 (PSGL-1) and cutaneous lymphocyte antigen (CLA), which is a modified PSGL-1 molecule and is expressed on memory but not naive T cells. E-selectin binds cutaneous lymphocyte antigen (CLA) on skin-homing memory T cells (Berg et al., 1991). Selectin mediated bonds are impermanent and as the flowing blood exerts pressure, adhesion bonds dissociate at the upstream end of the cell and new bonds form downstream. This results in a rolling motion (von Andrian and Mackay, 2000).

1.2.6.2. Leucocyte activation and arrest

To stop rolling cells must engage additional (secondary) receptors. All secondary adhesion molecules belong to the integrin family, specifically CD11a (also referred to as LFA-1/CD11aCD18/ $\alpha_1\beta_2$) and the two α_4 integrins: very late antigen-4, VLA-4 ($\alpha_4\beta_1$) and $\alpha_4\beta_7$ (von Andrian and Mackay, 2000). CD11a and VLA-4 bind ICAM-1 and VCAM-1 on endothelium respectively. $\alpha_4\beta_7$ binds mucosal-addressin cell adhesion molecule 1 on endothelium which enables homing of lymphocytes to gut (von Andrian and Mackay, 2000). Integrins must be activated to mediate adhesion. Rolling T cells activate integrins when they receive signals from chemokines on endothelial surfaces (von Andrian and Mackay, 2000). Chemokines are secreted polypeptides which can bind to heparin-like glycosaminoglycans on cell surfaces and

in the extracellular matrix. Chemokine receptors on T lymphocytes track down and bind to these immobilised chemokines (a process known as haptotaxis) (Carter, 1965). CCR4 is a crucial chemokine receptor for skin homing of T cells as it allows binding to its agonist thymus and activation-regulated chemokine, TARC, secreted within inflamed skin tissue (Imai et al., 1997). After the arrest phase leucocytes then need to penetrate the endothelial cells barrier and its associated basement membrane and the pericyte sheet (a discontinuous layer of smooth muscle cells wrapped around endothelial cells of almost all post-capillary venules) (Ley et al., 2007).

1.2.7. T cell proliferation and clonal expansion during DTH responses in the skin

During the course of the DTH response there is initial expansion and then subsequent contraction of T cell numbers at the site of inflammation (Poulter et al., 1982; Platt et al., 1983). Although studies in mice have demonstrated the persistence of memory T cells within non-lymphoid tissues that appear to convey protective immunity (Hogan et al., 2001; Masopust et al., 2001), other studies have shown that memory T cells resident within non-lymphoid tissues do not proliferate upon re-activation (Harris et al., 2002; Ostler et al., 2001). Blocking chemokine or integrin mediated recruitment of T lymphocytes during DTH responses in mice diminishes the response (Grabbe et al., 2002; Reiss et al., 2001) suggesting that recruitment of T cells within the circulation or resident within secondary lymphoid tissues is critical in mediating a recall response. In mice, therefore, the increase in the number of lymphocytes at the site of secondary immune responses is unlikely to be due to proliferation of antigen-specific cells within non-lymphoid tissues.

Published work from our laboratory, however, suggests that the expansion of PPD specific CD4⁺ T cells during the course of the Mantoux test response in humans is due in part to the *in situ* proliferation of T cell clones present in the skin at early time points (Reed et al., 2004). This conclusion is supported by the findings that there was a high level of the proliferation marker Ki67 present in the skin at Day 7 and that there was an increase in the number of antigen specific cells up to day 14. However,

there was no associated change in the number of circulating Ki67 or PPD-specific T cells in the peripheral blood that would be expected if the T cells were recruited to the skin following proliferation in the lymph node (Reed et al., 2004).

DTH responses may therefore be mediated by several mechanisms in different species, some of which may become redundant under certain conditions, including activation of resident cells and the recruitment of memory T cells from the peripheral blood some of which will have been pre-activated in the draining lymphoid tissue.

1.2.8. Regulatory T cells and their role in cutaneous memory responses

Certain T cells, referred to as suppressor or regulatory T cells (Tregs) actively suppress the actions of other T cells that can cause tissue damage. Tregs play an active part in both immunological self-tolerance and in the resolution of an immune response to non-self antigens (Sakaguchi, 2004).

They have been extensively studied over the past decade since the identification of suppressive CD4⁺CD25⁺ T cells in mice (Sakaguchi et al, 1995). Their importance in immune regulation was evident from the initial demonstration that the depletion of this subset (~10%) from a population of CD4⁺ T cells from a normal adult mouse resulted in the development of a spectrum of autoimmune diseases when the remaining CD4⁺CD25⁻ T cells were transferred to immunocompetent recipients. Foxp3 is a transcription factor selectively expressed by CD4⁺CD25⁺ T cells and is required for Treg development. Mice lacking expression of Foxp3 (scurfy mice) and humans with IPEX (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome are affected by a fatal autoimmune syndrome (Hori et al., 2003). These disorders have led many to suggest that Foxp3 is a definitive marker of regulatory activity (Fontenot & Rudensky, 2005). However, in humans, it has been shown in a number of studies that activated responder CD4⁺ T cells can transiently express Foxp3 (Walker et al, 2003; Morgan et al, 2005; Wang et al, 2006). The balance between responsive T cells and Tregs during an immune response is crucial

to maintain controlled immunity and both cell types need to be present for the lifetime of the organism (Akbar et al., 2007).

Various hypotheses have been proposed, covering how they function and where they are generated (Grossman et al, 2004; Asseman et al, 1999; Oida et al, 2006). Tregs are widely thought to be self-reactive cells, generated in the thymus (Hsieh et al, 2006). Whereas autoreactive T cells are generally deleted in the thymus (Hoffmann et al, 1995; Klein et al, 1998), Tregs are hypothesised to escape deletion and instead gain suppressive capacity (Jordan et al, 2001; Liu, 2006). However, there has been an accumulation of evidence suggesting that Tregs may be generated in the periphery from mature conventional T cells as well (Seddon & Mason, 1999; Apostolou & Von Boehmer, 2004; Cobbold et al, 2004). A recent paper has challenged the notion that Tregs are specific for self-antigens (Pacholczyk et al, 2007), finding that 70% of the most frequent conventional CD4 TCRs were also expressed by regulatory T cells. A study in humans also found a significant overlap in TCR repertoire between regulatory and responder CD4⁺ T cells (Fazilleau et al, 2007)(Vukmanovic-Stejic et al., 2006).

On average, ninety to ninety-five percent of regulatory T cells in adult humans express CD45RO (Taams et al, 2001) thus placing them in the memory compartment in humans (Akbar et al, 1988; Merckenschlager & Beverley, 1989). The remaining ten percent of Tregs have a naïve phenotype, expressing CD45RA. A high percentage of circulating human and mouse Tregs express CLA and they are found in normal skin from both humans and mice. This suggests that cutaneous Tregs may have an important immunoregulatory function at this site even in the absence of infection or inflammation. It was shown that after transfer of Tregs into Treg cell-deficient scurfy mice, Tregs upregulated expression of cutaneous homing receptors such as CLA and migrated to the skin. Loss of fucosyltransferase VII(FuT7) enzyme, which leads to generation of part of the CLA molecule, dramatically reduced Treg cell accumulation within the skin, and resulted in selective onset of severe cutaneous inflammation (Dudda et al., 2008). Skin resident Treg cells are therefore essential for maintaining normal cutaneous immune homeostasis.

1.3. Innate immune cells in human skin and their blood precursors

The innate arm of the immune system consists of monocytes, polymorphonuclear (PMN) and natural killer (NK) cells. Neutrophils are the first inflammatory cells to accumulate at the site of simple skin trauma (Peters et al., 2008). Blood monocytes represent 10% of circulating leucocytes in human blood and give rise to macrophages and some DCs in skin.

1.3.1. Blood Monocytes and their relationship to tissue macrophages and DCs

Monocytes, macrophages and DCs are all phagocytic cells thought to be interrelated and collectively referred to as the mononuclear phagocyte system (MPS) (Sasmono et al., 2003). The MPS was initially defined as a population of cells, derived from a bone marrow progenitor, that differentiate and enter the blood as monocytes and then enter tissues to become resident tissue macrophages and antigen-presenting cells (van Furth and Cohn, 1968). Indeed the best known function of monocytes is as a systemic reservoir of myeloid precursors for the renewal of some tissue macrophages and antigen-presenting dendritic cells (Randolph et al., 1999; Geissmann et al., 2003; Varol et al., 2007). However it is now recognised that not all DCs arise out of circulating monocytes (Auffray et al., 2009). The potential mechanisms for the renewal of individual subsets include a) self-renewal of resident post-mitotic cells, b) migration, homing, and limited proliferation of adult bone-marrow-derived progenitor cells in peripheral tissues (Massberg et al., 2007); and c) the extravasation and differentiation of circulating precursors such as blood monocytes. These mechanisms are not mutually exclusive and they are likely to depend on environmental cues such as inflammation. Langerhans cells of the epidermis are an example of the first possibility, in that most are self-regenerating within the skin (Merad et al., 2002). This was shown after syngeneic bone marrow transplantation where most Langerhans cells remain host-derived. Conventional dendritic cells (cDCs) are present in all lymphoid organs and renew in the steady state from a bone marrow precursor without a monocyte intermediate (Waskow et al., 2008; Fogg et al., 2006; Liu et al., 2007). A third group of cells represents short lived cells that

differentiate from blood monocytes in response to inflammation or infection such as monocyte-derived DC or TNF- α and iNOS-producing (Tip)-DCs (Randolph et al., 1999; Geissmann et al., 2003; Serbina et al., 2003). An overview of the mononuclear cell subsets located in the epidermis and dermis of normal human skin is shown in Figure 1-2.

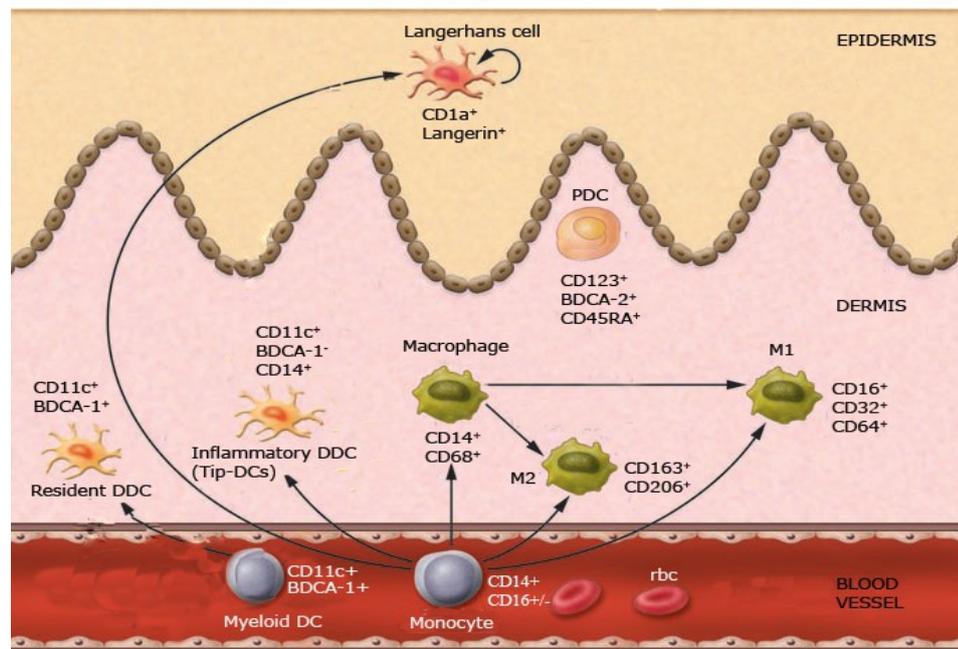


Figure 1-2: Cellular components of the innate immune system in human skin during steady state and inflammation.

Non-inflamed skin contains epidermal Langerhans cells, BDCA-1⁺ resident dermal DCs, plasmacytoid DCs and macrophages. In addition inflamed skin contains a population of inflammatory DCs (TNF- α and iNOS-producing DCs called Tip-DCs). Currently the macrophage population expressing CD68 and CD14 can be further subdivided into classically activated macrophages (M1), and alternatively activated macrophages (M2). Common markers used to identify these leucocyte populations are indicated. Circular arrows indicate the self-renewing potential of LCs under conditions of tissue homeostasis. Circulating blood monocytes are potential precursors of LCs, macrophages and inflammatory DCs. Circulating BDCA-1⁺ DCs are putative precursors for resident dermal DCs. (Figure adapted from Nestle, F.O. and Nickoloff, B.J., 2007)

1.3.2. Blood Monocyte Subsets

There are three well-defined subsets of human monocytes defined by the expression of CD14 and CD16 (CD14⁺CD16⁻, CD14⁺CD16⁺ and CD14^{low}CD16⁺) (Auffray et al., 2009; Passlick et al., 1989). The two main subsets both express CD14 and are either positive or negative for expression of CD16. They express distinct chemokine receptors as shown in Table 1-3 below. There are two main subsets of mouse monocytes with important species-specific differences to human monocytes. First, the relative frequencies of the two major subsets are different in mice and humans. Under resting conditions, CD14⁺ monocytes predominate in the bloodstream of humans, whereas Ly6C⁺ and CX3CR1⁺ monocytes in mice are present in roughly similar proportions (Serbina et al., 2008). Second, human monocyte subsets are similar in chemokine receptor expression to known mouse monocyte subsets but the function of each human monocyte subset and the equivalent mouse counterpart are reversed rendering any comparison in monocyte biology between the two species confusing (Serbina et al., 2008; Auffray et al., 2009). The CD14⁺CD16⁻ subset in humans is similar to the CD115⁺Ly6C⁺ (Gr1⁺) subset in mice but their functions differ in that in humans this subset is not inflammatory whereas in mice they give rise to tissue inflammatory DC, Tip-DC and M1-type macrophages (Geissmann et al., 2003). The CD14⁺CD16⁺ subset in humans is phenotypically similar to the CD115⁺Ly6C⁻ (Gr1⁻) subset in mice with similar expression of CX3CR1 (high) and CCR2 (negative). In mice they have been found to patrol blood vessels in the steady state and are capable of extravasation into tissue and responsible for a very early inflammatory response during infection. They also differentiate into alternative macrophages (M2 type) after extravasation. In humans this subset of monocytes is pro-inflammatory (Geissmann et al., 2003). However within this subset of human CD16⁺ monocytes there is a third well defined subset that shows low expression of CD14 (CD14^{low}CD16⁺) and no expression of CD64 or CD32, poor phagocytic function and does not release TNF- α or IL-1 (Auffray et al., 2009).

The study of monocyte subsets is hampered by the fact that monocytes adapt to their environment and change phenotypes easily. Also isolating them and culturing them

in vitro notably affects their phenotype and behaviour. An important development in their study has been the ability to track these cells *in vivo* by performing intravital studies in mice (Auffray et al., 2007). This technique using intravital microscopy techniques allowed imaging of dermal blood vessels *in vivo* in mice genetically engineered to express green fluorescent protein (GFP) on their monocytes. This showed that murine CD115⁺Ly6C⁻(Gr1⁻) monocytes exhibit a constitutive crawling on the luminal side of the dermal endothelium, in steady-state conditions (Auffray et al., 2007). They are therefore ideally located to survey endothelial cells and surrounding tissues. During mouse peritoneal infection with *Listeria monocytogenes*(*Lm*) it has been shown that these monocytes are the main blood cell type that extravasates into the peritoneum during 1 and 2 hrs after infection and are the only producers of TNF- α , a cytokine central to macrophage-mediated inflammation (Auffray et al., 2007). Therefore they are major players in the initial innate immune response to infection. It would be interesting to find out if similar patrolling macrophages are present in human dermal endothelium in equal numbers in young and old skin.

Table 1-3: Human and mouse monocyte subsets

HUMAN MONOCYTE SUBSET	CHEMOKINE RECEPTORS	CYTOKINES PRODUCED IN RESPONSE TO LPS <i>in vitro</i>	FUNCTION	SIMILAR MOUSE SUBSETS (FUNCTION)
CD14^{high}CD16⁻ (80-90% of blood monocytes)	CCR2 ^{hi} CX3CR1 ^{lo}	IL-10	Not good producers of TNF- α /IL-1	CD115 ⁺ Ly6C ⁻ (Gr1 ⁻) CX3CR1 ^{hi} CCR2 ⁻ (give rise to tissue inflammatory DC's and M1 macrophages)
CD14⁺CD16⁺ (~10% of blood monocytes)	CCR2 ^{lo} , CX3CR1 ^{hi} CD64 ⁺ CD32 ⁺	TNF- α IL-1	Pro-inflammatory	CD115 ⁺ Ly6C ⁺ (Gr1 ⁺) (Patrol blood vessels in the steady state, give rise to AAM in tissues)

1.3.3. Macrophages

Macrophages play a role in both innate and adaptive immunity through their ability to recognize “danger signals” via toll-like receptors (TLRs) and other pattern-recognition receptors (PRRs); through their effector mechanisms, including phagocytosis, nitric oxide production, and killing of bacteria; and through their ability to produce a wide array of cytokines and chemokines. In the past CD14 and CD68 have been used to identify macrophages in the skin but it is now recognised that these surface markers are also shared by dermal DCs (Zaba et al., 2007). Moreover, macrophages can process antigen after activation and present antigen derived peptides via MHC class II molecules to CD4⁺ T cells. Importantly, macrophages are involved in both the initiation and the resolution of an

inflammatory response, and two corresponding activation states for macrophages have been described *in vitro* (Goerdts and Orfanos, 1999; Gordon, 2003). The initial inflammatory response is carried out by macrophages that produce high amounts of proinflammatory cytokines and reactive oxygen species (Goerdts and Orfanos, 1999). These macrophages are also referred to as classically activated or M1 macrophages and can be generated *in vitro* by activation with IFN- γ /LPS. The resolution phase is associated with macrophages that produce mainly anti-inflammatory cytokines and have a higher phagocytic capacity, and an increased expression of the mannose receptor CD206 and/or the haemoglobin scavenger receptor CD163 (Tiemessen et al., 2007). These macrophages are often referred to as M2 or alternatively activated macrophages (AAM) and can be obtained *in vitro* after macrophage stimulation with IL-4/IL-13 (Stein et al., 1992; Edwards et al., 2006), a combination of immune complexes and LPS (Edwards et al., 2006), or IL-10 or glucocorticoids (Buechler et al., 2000). The three different induction methods lead to more or less distinct M2 macrophage subsets (also referred to as M2a, M2b, and M2c, respectively), each with a typical cytokine/chemokine and cell surface marker profile (Sica et al., 2006), although recent data indicate that a certain degree of versatility exists between the subsets (Lumeng et al., 2007; Arnold et al., 2007). The excessive presence or activity of either M1 or M2 subsets may cause damage to the host, because for M1 subsets this could promote immune responses to healthy tissue resulting in inflammation, or, conversely for M2 subsets, prevent an appropriate immune response leading to hampered tumour immunity (Sica et al., 2006; Van Ginderachter et al., 2006). Understanding the mechanisms behind the homeostatic control of macrophage function is, therefore, of fundamental importance and it has recently been shown that CD4⁺CD25⁺FoxP3⁺ Tregs have a role to play in inducing alternative activation (Tiemessen et al., 2007).

1.3.4. Dendritic cells

1.3.4.1. *Blood dendritic cells*

The myeloid blood DC population represents 5% of monocytic-like cells (0.5% of PBMCs in humans). These blood DCs express class II antigens and CD11c and are negative for CD14 and CD16. They stimulate T cell proliferation *in vitro* (Banchereau et al., 2000; Banchereau and Steinman, 1998).

1.3.4.2. *Conventional DCs*

The term conventional DCs (cDCs) is used to refer to DCs present in all lymphoid organs, such as the spleen and lymph nodes. They have a short half-life and renew in the steady state from a bone marrow precursor without a monocytic intermediate. Lymphoid tissue-resident DCs are the most studied DC populations in mice, but little information is available on their human counterparts. In mice, splenic DCs constitutively express MHC class II and the integrin CD11c (Shortman and Liu, 2002).

1.3.4.3. *Dermal DCs*

These cells have the capacity to take up cutaneous antigens, mature and migrate to draining lymph nodes, and present these antigens to T and B cells (Dubois et al., 1998; Nestle et al., 1998; Kissenpfennig et al., 2005). This process is important both during primary skin infections and also in the secondary memory cutaneous immune response. There is no single or specific marker for these cells although the integrin CD11c is probably the best tool we currently have to identify them (Zaba et al., 2007). Dermal DCs have been classified into resident dermal DCs and an additional group of DCs that appear or develop during inflammation (termed “inflammatory” dermal DCs) (Zaba et al., 2009; Shortman and Naik, 2007). They differ in their expression of BDCA-1 (Figure 1-1).

1.3.4.3.1. Resident dermal DCs

These are identified by their expression of BDCA-1(CD1c). BDCA-1 colocalises with nearly all CD11c⁺ cells. In the steady state, they are relatively immature with modest T cell stimulatory ability, but their immunostimulatory capacity can be greatly increased with DC maturing stimuli (Zaba et al., 2007). These BDCA-1⁺ DCs are also CD11c⁺ HLA-DR⁺CD45⁺CD14⁻ and are also CCR7⁺ and are responsive to the lymph node chemokine CCL19, suggesting that these cells can migrate to draining lymph nodes for antigen presentation (Angel et al., 2006). They are thought to arise from circulating BDCA-1⁺ DCs (Johnson-Huang et al., 2009).

1.3.4.3.2. Inflammatory dermal DCs

These inflammatory dermal DCs are CD11c⁺ BDCA-1⁻. This indicates that they may be derived from circulating DC precursors migrating into the skin due to inflammatory or chemotactic signals. Considerable evidence however suggests that these cells originate from circulating monocytes (Randolph et al., 1999; Geissmann et al., 2003; Serbina et al., 2003; Sunderkotter et al., 2004). They have been mainly studied in the context of psoriatic inflammation where a 30-fold increase in CD11c⁺ DCs in the dermis is seen (Zaba et al., 2007). In psoriasis these DCs have been shown to produce mediators, such as TNF- α and inducible nitric oxide synthase (iNOS) and have been termed Tip-DCs (Lowes et al., 2005). Tip-DCs were first described in a murine model of *Listeria monocytogenes* infection where following infection, Ly6C⁺(Gr1⁺) blood monocytes egress massively from bone marrow to the bloodstream in a CCR2-dependent fashion and differentiate into cells that produce TNF- α and inducible nitric oxide synthase (Serbina et al., 2003).

1.3.4.4. Langerhans cells

Langerhans cells (LCs) are dendritic antigen-presenting cells of the epidermis and constitute the first immunological barrier against pathogens and environmental insults. The exclusive identifying feature of these cells is the presence of Birbeck

granules, intracellular organelles only observable with electron microscopy (Birbeck et al., 1961). Langerin (CD207) is a type II C-type lectin that is present on the cell surface of Langerhans cells and involved in the formation of Birbeck granules (Valladeau et al., 2000). Other useful but non-specific markers for Langerhans cells are CD1a (Fithian et al., 1981), an MHC I-like molecule that presents microbial lipids to T cells (Mizumoto and Takashima, 2004), MHC Class II (De Panfilis et al., 1988) and CD39, a membrane ATPase (Wolff and Winkelmann, 1967).

As described above, LCs self-replenish within the epidermis under steady state conditions (Merad et al., 2002) but in inflammation leave the skin in great numbers and therefore need to be replenished by blood precursors, most likely monocytes (Ginhoux et al., 2006). They are traditionally thought to be the main antigen presenting cell in models of contact hypersensitivity; LCs are thought to capture antigen they encounter in the epidermis, antigen-laden LCs migrate subsequently via dermal lymph vessels to the draining lymph node (Stoitzner et al., 2002). Human skin is daily exposed to an array of environmental haptens, but it rarely results in contact hypersensitivity. Therefore, tolerance induction must be the default setting of the T cell response against encountered haptens. Even though LCs were described 140 years ago, their exact *in vivo* function still remains elusive. Recent scientific evidence obtained using LC-depleted genetically modified mice adds to potential confusion by demonstrating that following application of hapten to the LC-deficient mice the contact hypersensitivity response was diminished (Bennett et al., 2005), enhanced (Kaplan et al., 2005) or unchanged (Kissenpfennig et al., 2005). Therefore, it remains controversial whether LCs are dispensable for the induction of skin immunity. Also Langerin⁺ DCs in the dermis and skin-draining lymph nodes have been recently described in mice which do not merely represent LCs *en route* to lymph nodes and are distinct from LCs in terms of being CCR2⁺ rather than CCR6⁺ and are also radio-sensitive as opposed to radio-resistant like LCs (Bursch et al., 2007). Dermal Langerin⁺ cells can traffic from the skin to draining lymph nodes both in the steady state and after the application of hapten to the skin (Kissenpfennig et al., 2005). These cells are also capable of participating in skin immune responses. It is possible that LCs in general are required for maximal contact hypersensitivity

responses but nonepidermal Langerin⁺ DCs can mediate the contact hypersensitivity response in the absence of epidermal LCs (Bursch et al., 2007).

In our skin model using intradermally injected skin test antigen, it is likely that antigen presentation is mediated by dermal DCs and not LCs in the epidermis as the mode of injection of antigen bypasses the epidermis. We therefore mainly focus on dermal dendritic cell biology throughout the study.

1.3.4.5. *Plasmacytoid dendritic cells (pDCs)*

This subset represents 0.5% of PBMCs in human and are the most potent IFN- α -producing cells in response to viral pathogens (Cella et al., 2000; Siegal et al., 1999). Human pDCs express very low to no level of CD11c, they express CD4 and CD45RA antigens, the c-type lectin receptor BDCA2, and the molecule BDCA4, a neuronal receptor often used to isolate pDCs, and high levels of the interleukin-3 (IL-3) receptor (CD123) (Colonna et al., 2004). These cells are normally found in the circulation and are rare in normal skin, but accumulate under conditions of chronic skin inflammation such as psoriasis and are implicated in its pathogenesis (Nestle et al., 2005).

1.4. Pattern recognition receptors

Innate immune cells rely on pattern recognition receptors (PRRs) to detect the presence of pathogens (Gilliet et al., 2008). PRRs are host receptors that can sense pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS) and initiate signalling cascades that lead to an innate immune response. PRRs can be membrane bound, for example Toll-like receptors (TLRs), or soluble cytoplasmic receptors such as Nod-like receptors.

1.4.1. Toll-like receptors

TLRs are a family of molecules found spanning the cell membrane and consisting of an ectodomain, a transmembrane domain and a cytoplasmic domain known as the TIR (Toll/IL-1 receptor) domain. Since the first mammalian TLR (now known as TLR4) was identified (Medzhitov et al., 1997), 10 TLRs have been identified in humans and 12 in mice (Gilliet et al., 2008). The TLR ectodomains bind to PAMPs, such as the bacterial cell-wall component lipopolysaccharide (a ligand of TLR4) (Medzhitov et al., 1997; Poltorak et al., 1998), bacterial flagellin (a ligand of TLR 5) (Hayashi et al., 2001), and lipoprotein and peptidoglycan (which are recognised by TLR2 complexed with TLR1 or TLR6) (Schwandner et al., 1999). These TLRs, which are located on the surface of host cells lead to immediate detection of and response to microorganisms in their environment (Akira and Takeda, 2004). Other TLRs including TLR3, TLR7, TLR8 and TLR9 are located in intracellular endosomal-lysosomal compartments, where they are involved in the detection of microbial nucleic acids (Gilliet et al., 2008). Myeloid DCs express all TLRs except TLR7 and TLR 9 which are selectively expressed by pDCs, suggesting that innate immune cells complement each other in their expression of a range of TLRs (Jarrossay et al., 2001). TLR activation by their ligands leads to the activation of multiple signalling pathways, and initiates the transcription of genes encoding cytokines, chemokines and co-stimulatory molecules (Gilliet et al., 2008) leading to the development of distinct T helper responses to microorganisms (Palm and Medzhitov, 2007).

1.5. Chemokines and cytokines and their role in memory responses in the skin

Cytokines released early on in the response are crucial for endothelial activation and early inflammatory cell transmigration. Cytokines and chemokines are thought to be involved in all phases of inflammation in the skin. Multiple cell types in the skin, including keratinocytes, T and B lymphocytes, monocytes, endothelial cells, fibroblasts, mast cells, dendritic cells, granulocytes and platelets are known to

produce cytokines (Bos Jan D, 2004). The initiation of the response to infection in the skin, known as the acute phase response, is thought to be dependent on the production of tumour necrosis factor alpha (TNF- α), IL-6 and IFN- γ (Janeway CA, 2009). TNF- α is secreted by a variety of cells including monocytes and macrophages, T cells, fibroblasts and endothelial cells (Tracey and Cerami, 1994). Following stimulation by exogenous factors such as bacterial lipopolysaccharide, viruses and other organisms large quantities of TNF- α are released within minutes and synthesis is rapidly increased under the influence of IFN- γ (Beutler et al., 1986).

TNF- α acts to increase vascular permeability and blood flow resulting in the observed erythema and induration at the site as well as increasing expression of various adhesion molecules E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) on the endothelium (Groves et al., 1995) with resulting recruitment of lymphocytes to the site of the immune response and also activation and chemotaxis of neutrophils and monocytes (Ming et al., 1987). IFN- γ produced predominantly by activated T lymphocytes increases MHC-II expression on monocytes and macrophages (Kelley et al., 1984) in addition to stimulating further release of TNF- α by monocyte activation (Nedwin et al., 1985). IL-6 is produced by activated monocytes and is important for T cell activation and differentiation (Diehl and Rincon, 2002; Horn et al., 2000).

Following acute initiation of the immune response in the skin further cellular activation and recruitment is mediated by the production of a range of cytokines and chemokines from several resident skin cell types. MCP-1 and MIP 1- α are classified as CC (cysteine-cysteine) chemokines and are thought to have an essential role in humans in the activation and chemoattraction of monocytes to sites of inflammation (Lu et al., 1998; Wolpe et al., 1988). The CXC (Cysteine-X-cysteine) chemokines, such as IL-8 and IP-10, are thought to attract and activate neutrophils (Schroder, 1995). A mixture of both CC and CXC chemokines have been shown to have an effect on the induction of directional migration of human T cells and T cell subsets including memory effector T cells (Rossi and Zlotnik, 2000).

1.6. Ageing and the immune system

Humans now live longer than ever before and human life-expectancy is still increasing. It has been predicted that by 2050 approximately 40% of the population will be over 60 years of age in Europe and the United States (Lutz et al., 1997). Ageing of the organism is associated with a number of physiological and pathological changes including skin atrophy, atherosclerosis and decreased fertility. Moreover the incidence and severity of infectious diseases, such as pneumonia (LaCroix et al., 1989), meningitis (Gorse et al., 1984), sepsis (Chattopadhyay and Al Zahawi, 1983), infection of the urinary tract (Ackermann and Monroe, 1996) or infection with respiratory syncytial virus (Barker and Mullooly, 1980) or influenza (Sprenger et al., 1993), are increased in elderly individuals. In fact, after suffering from urinary-tract infection or tuberculosis, the mortality rate of elderly patients is ten-fold higher than that of young patients (Yoshikawa, 1997). There is also reduced effectiveness of vaccination with infection being prevented in 70-90% of subjects aged <65 who had been vaccinated with the influenza vaccine compared to only 30-40% prevention in those aged >65 years (Murasko et al., 2002). All of these factors contribute to the increased morbidity and mortality in the elderly population and places ever increasing significant demands on the provision of healthcare for this age group. The information obtained out of studies of the immune system in the ageing population should therefore be of practical relevance to increasing the efficacy of vaccination in the elderly and restoring immunity in immunocompromised individuals.

1.6.1. Studying ageing and the immune system

There are two important considerations when studying the effects of ageing 1) mouse studies are limited in that mouse longevity is different to human longevity 2) in an attempt to isolate ageing changes from the external changes of disease and medication most studies on ageing include only the very healthy elderly. This is accomplished by the exclusion of subjects who have evidence of disease or use

medications by applying rigorous criteria as defined by the SENIEUR protocol (Ligthart et al 1984). These two issues will be discussed below.

1.6.1.1. Murine models

Murine models are generally considered to be the mainstay of *in vivo* immunological experimentation as they allow for elegant manipulation of the immune system and in many ways mirror the human immune system. The use of murine models to study ageing is still controversial however (Aspinall, 1999). Mice and men have dramatically different lifespans. Moreover, whereas telomere shortening is a marked characteristic of ageing in humans, mice have significantly (10X) longer telomeres (Kipling and Cooke, 1990) which makes telomere shortening less significant in the study of murine ageing. Also mice do not lose CD28 expression on T lymphocytes as they differentiate unlike CD8⁺ and CD4⁺ human T cells (Engwerda et al., 1994). Mice also have a reduced antioxidant defence and therefore may be more susceptible to the effects of oxidative stress with ageing compared with humans (Pawelec et al., 1999)

In the context of examining immune responses in the skin, DTH responses are assessed by the measurement of either footpad or ear swelling in mice compared with the forearm in humans. Previous experiments in humans have shown variation in the DTH response according to anatomical site (van Strien and Korstanje, 1994; Wammanda et al., 2006) and therefore comparison of a forearm in a human and a footpad or ear in a mouse is difficult. In rats the resident T cells in the skin are also different with gamma delta TCR bearing T cells being predominant compared with alpha beta TCR in humans (Elbe et al., 1996). The duration of the response is altered in mice with a peak response at 12 hours compared with 2-3 days in humans (Mestas and Hughes, 2004). This may be reflective of differences in antigen presentation and time required for antigen to be transported to the draining lymph nodes. Whereas the cutaneous DTH response consists primarily of lymphocytes in humans, it is neutrophil rich in mice (Crowle, 1975). Elicitation of the response also requires

much higher concentrations of antigen in mice compared with humans (Mestas and Hughes, 2004).

Therefore many of the hallmarks of human cell ageing are absent on mouse cells and the timeframe of an immune response is different in mouse skin compared to human such that any study using a mouse model to define human ageing must be interpreted with caution for the reasons mentioned above.

1.6.1.2. *The SENIEUR protocol*

One of the difficulties with analyzing data from human studies is the presence of confounding factors due to poor standardization of the groups studied. In an attempt to isolate aging changes from external changes of disease and medications, most studies on aging have included only the very healthy elderly. This has been accomplished by the exclusion of subjects who have evidence of disease or use medications, by applying rigorous criteria as defined by the SENIEUR protocol (Ligthart et al., 1984). The criteria of the SENIEUR protocol exclude subjects on the basis of unhealthy lifestyle choices; any clinical information that suggests the presence of infection, inflammation, malignancy, or other immune disorders; any laboratory data that suggests abnormal organ function; and administration of medications for the treatment of a defined disease. These stringent criteria exclude 90% of subjects aged >65 years and 25% of younger subjects (Wick and Grubeck-Loebenstein, 1997; Rowe and Kahn, 2000). Applying such strict inclusion criteria is therefore now thought to limit understanding of the mechanisms of vulnerability to infections in the elderly and might be the reason why to date there has been no compelling scientific evidence to show that changes observed between T cell phenotype and function in the old have direct relevance to the common infections seen in the aged population (Castle, 2000). The study of individuals who are excluded by the SENIEUR protocol but well characterized in terms of clinical comorbidity may be more relevant to the development of strategies to enhance quality of life with ageing. In this thesis, a modified version of the SENIEUR protocol was employed with a view to excluding significant comorbidity but selecting a

representative group of old individuals. Our exclusion criteria are described in detail in Chapter 2: Materials and Methods.

1.6.2. Ageing and its effect on T cells

T cells develop in the thymus. Thymic involution starts as early as the first year of age in humans and by the time of middle age, most parenchymal tissue is replaced by fat, although functional thymic tissue remains in humans at least until the sixth decade of life. Therefore both in mice and humans, there is extremely limited generation of naive T cells with increasing age (Linton and Dorshkind, 2004; Haynes et al., 2000; Jamieson et al., 1999). This means that immunity is maintained by turnover of existing populations of cells. The quantity of T lymphocytes stays relatively stable over time and with increasing age as there are homeostatic mechanisms in place to replace or remove the expanded populations of effector T cells (Akbar et al., 2000). However, it has been shown that despite maintenance of normal T cell numbers with age, there is a considerable decrease in CD4 and CD8 mediated responses (Grubeck-Loebenstein and Wick, 2002; Effros et al., 2003). For example, naive CD4 T cells in the aged secrete less IL-2, leading to decreased expression of CD25, and show reduced proliferation and incomplete differentiation to T helper type 1 or type 2 effector cells (Linton et al., 1996). In addition CD4 memory T cells derived from aged naive precursors divide and produce cytokine poorly, whereas CD4 memory T cells derived from young mice function well into old age.

The proportionate representation of particular antigen specific T cells within the T cell pool can be altered with ageing with large expansions of terminally differentiated effector memory cells (Fletcher et al., 2005). This is particularly true for T cells that are specific for agents that cause persistent infection such as Cytomegalovirus(CMV), in which repeated episodes of cellular expansion can lead to huge clones of highly differentiated, senescence prone and poorly functional CMV-specific CD8⁺ T cells (Ouyang et al., 2004). This is to the detriment of immunity to other non-chronic infections and in the case of cytomegalovirus (CMV)

infection might be detrimental to host survival as CMV seropositivity has been shown to be predictive of decreased survival of ageing individuals (Pawelec et al., 2009; Wikby et al., 2002).

Another key difference between T cells in young and old is that a significant proportion of effector memory T cells in the old are CD45RA⁺ (Pawelec et al., 2009; Almanzar et al., 2005), a marker usually associated with naive T cells (as described above-section 1.2.3 “Memory T cell phenotype”). This phenomenon is mainly seen in the CD8⁺ T cells and to a lesser extent in CD4⁺ T cells (Amyes et al., 2003). In young subjects these CD8⁺CD45RA⁺ revertant T cells have similar telomere lengths to the central memory pool and do not require proliferation to mediate effector function and are resistant to apoptosis (Dunne et al., 2005). This might be beneficial in that it minimises the impact of telomere erosion and replicative senescence on memory T cell populations to persistent infections. On the other hand the memory CD8⁺CD45RA⁺ T cells, that are significantly increased in old subjects, have very short telomeres and might be poorly functional suggesting that during the course of ageing, these populations are eventually driven to end-stage differentiation (Akbar and Fletcher, 2005; Plunkett et al., 2005).

1.6.3. Ageing and its effect on Innate Immunity

Monocytes and macrophages from aged mice show a reduced functional potential. Both splenic and activated peritoneal macrophages from aged mice express much lower amounts of Toll-like receptors and alterations in the secretion of various chemokines and cytokines that include IL-6 and tumour necrosis factor (Lloberas and Celada, 2002; Plowden et al., 2004). Studies on monocytes from whole blood have shown reduced production of TNF- α after stimulation with LPS in old humans (Gon et al., 1996) and mice (Effros et al., 1991). Also, monocytes and macrophages from old mice have been shown to inhibit T cell proliferative responses (Pawelec et al., 1998). Very little is known about dendritic cell changes with age, although the number of Langerhans cells in the epidermis has been reported by some to decrease with age (Uyemura et al., 2002; Sprecher et al., 1990).

1.6.4. Ageing and its effect on skin structure and cellular constituents

Cutaneous ageing is a complex process and comprises both intrinsic ageing, thought to be genetically determined, and extrinsic ageing caused by environmental factors such as exposure to ultraviolet irradiation. Although there are microscopic differences between intrinsically and extrinsically aged skin, similar changes at a macroscopic level are seen with increased skin fragility, loss of elasticity, increased transparency, wrinkling and increased laxity (Leyden, 1990). These changes are thought to occur as a result of reduced proliferative capacity, cellular senescence and reduced biosynthetic capacity of skin-derived cells such as fibroblasts in addition to the increased production of degradative enzymes including the matrix metalloproteinases (MMPs) (Jenkins, 2002; Berneburg et al., 2000; Rabe et al., 2006).

Collagen is the most abundant extracellular component of the skin, accounting for 80% of dry weight, and provides the strong tensile properties of the dermis. An elastic fibre network provides elasticity accounts for 2-4% of extracellular matrix of sun-protected skin. Glycosaminoglycans/ proteoglycan macromolecules play a role in hydrating the skin and biological signalling. All three of these components are affected by ageing. In intrinsic skin ageing a reduction in the number and biosynthetic capacity of fibroblasts is observed with an associated gradual loss of elastic tissue in the papillary dermis and a reduction in collagen content (Jenkins, 2002). In extrinsic skin ageing there is an accumulation of elastotic material, with thickened, tangled granular amorphous structures thought to be degraded elastic fibres, and dysregulation of elastin and fibrillin production. This results in an associated gross distortion of the dermal matrix (Jenkins, 2002; El Domyati et al., 2002). As with intrinsic ageing, a reduction in the collagen network is also seen.

1.7. Aims and Objectives

Immunity declines with age (Yoshikawa, 2000). This may explain the increase in frequency of cutaneous tumours and infections in older individuals (Laube, 2004;

Diffey and Langtry, 2005). Most studies directed at identifying age-related defects in old humans have focused on circulating leukocyte populations but it is not clear if the results obtained can be extrapolated to the behaviour of leucocytes within tissues during immune responses. Clearly a more informative experimental system is required to define the nature of attenuated cutaneous immunity during ageing in humans.

Given the demographic increase in age in the population it is essential that we have not only the tools to assess immunity but also have an understanding of the changes that occur in immunity with ageing.

AIM

To characterise the effects of ageing on the immune system in the skin in healthy old individuals.

OBJECTIVES

The objectives of this project were to:

1. Study the correlation between clinical DTH responses to the intradermal injection of antigen in the skin and peripheral blood responses to antigen in the old and young.
2. Characterize the lymphocytic response to the intradermal injection of antigen in the old.
3. Compare T lymphocyte migration from peripheral blood into skin in young and old subjects.
4. Characterise the innate immune response to the intradermal injection of antigen in young and old subjects

Chapter 2. Materials and Methods

2.1. Volunteer recruitment

This work was approved by the Ethics Committee of the Royal Free Hospital. Healthy young individuals under the age of 40 (n=71; 34 females, 36 males, median age=30 years,) and old individuals over 70 years (n=81, 49 females, 32 males median age=79.50 years) were recruited for the study. All volunteers were Caucasian and living in the Greater London area. Exclusion criteria based on a modified version of the SENIEUR protocol were employed to recruit old people in order to reduce confounding factors due to associated significant comorbidity (Ligthart et al., 1984). For our study, we adhered to all the original clinical exclusion criteria detailed in the SENIEUR protocol (Table 2-1) but to obtain a more representative sample of the elderly population we did not screen for and omit elderly volunteers based on biochemical or haematological abnormalities and we also did not exclude individuals on prescribed medications for defined disease, unless these medications were known to be immunosuppressive. All volunteers provided written informed consent and study procedures were performed in accordance with the principles of the declaration of Helsinki.

Table 2-1: Exclusion Criteria

Past history of TB
Significant co-morbidity (renal impairment or failure, heart failure, diabetes)
Past history of inflammatory skin disorder
Past history of neoplasia in the last 10 years
Previous treatment with chemotherapy or radiotherapy
Immunosuppressive medication
Recent infection or immunisation (within last month)
Pregnancy and breast feeding
Previous history of hypersensitivity to skin testing

2.2. Skin testing

Delayed type hypersensitivity responses (DTH) were induced by intradermal injection of antigen on non sun exposed skin of the medial proximal volar forearm. Increased resistance to injection and appearance of a pale skin bleb at the site of injection were signs of correct intradermal injection of antigen. The injection site was sampled by skin biopsy or skin suction blister at an allotted time point between 0 and 7days after the skin test injection.

2.2.1. Candida skin testing

Candin skin test solution 0.02ml (Allermed Laboratories Inc) was injected in 40 young and 34 old subjects.

2.2.2. Mantoux test

Tuberculin purified protein derivative (PPD) 0.1ml (1 unit) of 10U/ml (Evans Vaccines Ltd) was injected in 20 young and 32 old individuals in the first part of the study. For the Treg blister experiments (see chapter 6). Mantoux test from Statens Serum Institute (SSI) was used 0.1 ml of 2TU/0.1ml (n= 15 young) due to lack of availability of the Evans Vaccine manufactured Mantoux test in the UK in 2007.

2.2.3. Assessment of skin test responses

Induration, palpability, and the change in erythema from baseline were measured and scored on day 3. Baseline skin erythema at the site of antigen injection was measured using a DermaSpectrometer (Cortex Technology, Hadsund, Denmark). This is a portable, handheld device that allows the measurement of the skin erythema index (EI) by measuring light absorption coefficients (Figure 2-1B). The mean of 3 measurements was recorded. At Day 3 and at the time of sampling (if different from Day 3) the change in EI from baseline, palpability and size of induration were measured. The change in EI was calculated by subtracting the baseline measurement

from the EI taken when measuring the response. The size of induration was determined by measuring the maximum diameter of the indurated skin. The change in EI, induration and palpability were then scored according to Table 2-2 and the sum of the scores was combined to give an overall clinical score. Non-responding individuals were defined as having a clinical score of 0 at Day 3.

Table 2-2: Clinical Scoring System

Clinical Score	0	1	2	3	4	5	Score
Erythema-index (EI)	0	1-5	6-10	11-15	>16		
Size of Induration (mm)	0	1-5	6-10	11-15	16-20	>21	
Palpability	Nil	Just palpable	Easily	Marked	Very marked		
							Total

2.3. Skin sampling

Skin suction blisters were raised or 5mm punch biopsies were taken from the site of skin testing. Samples were collected at various time points between 0 and 7 days after injection of antigen. Each volunteer was allotted to a specific sample time-point and either paired skin suction blisters and punch biopsies or blisters or biopsies alone were taken (Figure 2-1 and **Error! Not a valid bookmark self-reference.**). Repeated skin testing to the same skin test antigen in the same individual was avoided in order to prevent boosting of the immune response from repeated antigenic exposure (which has previously been observed by our group).

2.3.1. Suction blisters

2.3.1.1. *Suction blister induction*

Skin suction has previously been shown to result in the formation of a split between the epidermis and dermis at the level of the lamina lucida (Kiistala, 1968; Vermeer et al., 1979). Suction blisters were induced by the application of negative pressure of

25-40kPa (200-300mmHg below atmospheric pressure) via a suction chamber (Medical Engineering, Royal Free Hospital, UK) centred over the site of testing for 2-4 hours using a clinical suction pump (VP25, Eschmann, Lancing, UK) (**Error! Not a valid bookmark self-reference.**). The pump has a gauge that allows for constant negative pressure to be applied to the skin. Suction cups were made of 3 main components: A template, a cup made of nylon and a see through Perspex lid. Rubber O-rings were fitted around the lid and template to allow an airtight seal to be formed between all 3 components when fitted together. The pump has sterile disposable tubing and liners that were changed in between volunteers. Skin suction chambers with apertures of 15mm, 12.5mm and 10mm were used according to the size of the response and skin elasticity. In all cases, the size of suction chamber aperture selected ensured that the whole of the area of induration was sucked up into the chamber with minimal incorporation of normal surrounding skin.

Suction was applied until a unilocular blister measuring 10-15mm was formed over the site of the skin test. The blister was then protected overnight with a rigid adhesive dressing assembled using a Comfeel plus ulcer dressing (Coloplast, Peterborough, UK), a universal top (Sterilin, Fisher Scientific UK Ltd, Loughborough, UK), Micropore tape (3M healthcare, Loughborough, UK) and Tubigrip bandaging (Seton Healthcare Group plc, Oldham, UK).

Blister fluid was aspirated from the blister at 18-24 hours after induction using a sterile 23G needle and a 2ml syringe (Tyco Healthcare UK Ltd, Gosport, UK). The fluid was aspirated at the 18-24 hour time point in order to ensure maximal accumulation of cells within the blister fluid from the site of antigenic challenge in the skin. The recorded time of sampling was the time from skin injection to blister fluid aspiration. The volume of fluid recovered from the blister was recorded and suspended in 1.5ml conical tubes (Alpha Laboratories Ltd, Eastleigh, UK). The aspirated blister site was dressed with Betadine dry powder spray (Seton Healthcare Group plc, Oldham, UK) and a Mepore dressing (MoInlycke Health Care Ltd., Dunstable, UK). Volunteers were advised to leave the dressing in place and to keep it dry for 24 hours before removing it and leaving the wound open to the air. The

suction cups were dismantled after use and disinfected in Barrycidal 36 (Heraeus Instruments Ltd, Brentwood, Essex, UK) for a minimum of 24 hours.

2.3.1.2. *Suction blister cell isolation*

The blister fluid was microcentrifuged at 650xg (3000 rpm) for 4 minutes (Microcentaur, MSE, Sanyo) to pellet the cellular contents. The supernatant was removed and aliquotted in to 1ml cryogenic tubes (Nunc, Thermofisher Scientific, Roskilde, Denmark) and stored at -80°C until analysed. The blister cell pellet was resuspended in 500µl of RPMI 1640(GIBCO, BRL Life Technologies, Paisley, UK) containing 10% human AB serum, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine (all from Sigma Aldrich, Gillingham, Dorset, UK).

2.3.1.3. *Counting of blister cells*

Blister white and red cell numbers were quantified using a haemocytometer. Cellular viability was assessed by trypan blue exclusion. A 10µl aliquot of blister cell suspension was mixed 1:1 with Trypan blue (Sigma- Aldrich, Gillingham, Dorset, UK) and viable non-stained cells were counted.



Figure 2-1: The clinical DTH response in the skin and skin sampling

(A) The skin test response to the intradermal injection of antigen is characterized by the development of erythema and induration at the site of injection, reaching a peak at 2-3 days. (B) The erythema index at the site of the response was measured at baseline, at day 3 and at the time of sampling using a DermaSpectrometer. The mean of 3 measurements was recorded. (C) Following skin testing on the inner aspect of the forearm, skin samples were collected at various time points from 0-15 days after injection. Skin suction blisters were raised primarily to obtain either cutaneous lymphocytes or monocytes. (D) Skin biopsies were also taken. Whenever possible, paired biopsies and blisters were taken from opposite forearms. Venous blood was also taken for PBMC isolation.

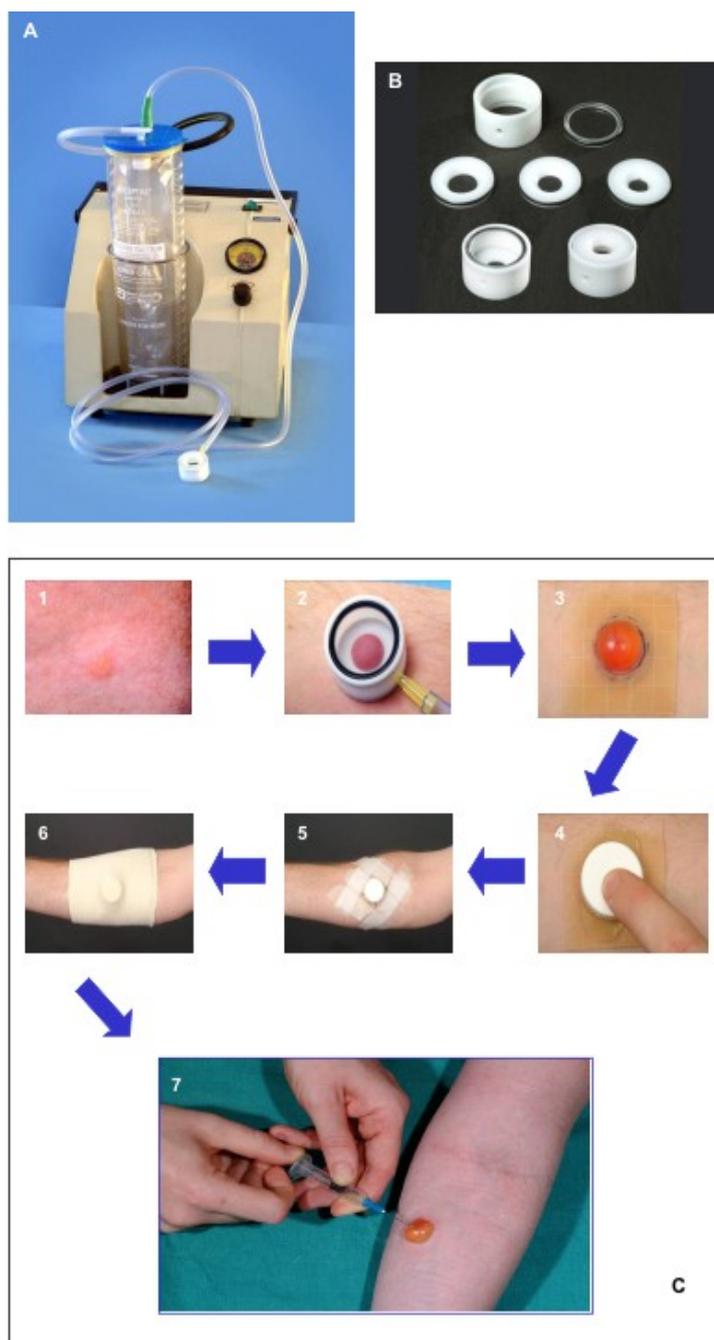


Figure 2-2: Skin suction blister induction.

Skin suction blisters were induced over the site of skin testing (1). A suction cup was centred over the site of injection (2) and a negative pressure of 25-40kPa (200-300 mmHg) below atmospheric pressure was applied until a unilocular blister (3) was formed. The blister was dressed with a 5X5cm Comfeel dressing (3), a trimmed universal container top (4), micropore tape (5) and a tubigrip bandage (6). Blister fluid was aspirated the following day using a sterile 23G needle and a 2ml syringe (7).

2.3.2 Skin biopsy

2.3.2.1. *Biopsy procedure*

A 5mm punch biopsy was taken from the centre of the site of injection of antigen or normal skin (Figure 2-1D). The surrounding skin was infiltrated with 1% lidocaine hydrochloride local anaesthetic (Hameln Pharmaceuticals Ltd, Gloucester, UK) prior to biopsy. The wound was closed with 4/0 Surgipro polypropylene suture (Tyco Healthcare UK Ltd., Gosport, UK). The skin was transported from the clinic to the lab in sterile saline within 20 minutes of obtaining it to prevent dessication of the skin sample.

2.3.2.2. *Biopsy storage and sectioning*

Biopsies were mounted in OCT(optimal cutting temperature compound; Bright Instrument Company Ltd, Huntingdon, UK) on cork disks, orientated so that the epidermis was perpendicular to the cork disk, and snap frozen in isopentane (Sigma-Aldrich, Gillingham, Dorset, UK) cooled in a bath of liquid nitrogen. The samples were then stored in a freezer at -80°C. 6µm frozen sections were cut at -20°C using a Bright 5040 microtome (Bright Instrument Company Ltd., Huntingdon, UK) on to poly-L-lysine coated glass slides (Sigma-Aldrich, Gillingham, Dorset, UK). Poly-L-lysine coated slides were used in order to promote strong adhesion of the skin section to the slide. Two sections were mounted on to each slide. The sections were then left overnight to air-dry and then fixed in fresh acetone for 10 minutes, followed by 99% Ethanol for 10 minutes. The sections were air-dried for 10 minutes and then cling-filmed wrapped and stored until use in a freezer at -80°C.

2.4. Blood sample

2.4.1. PBMC isolation

Heparinised blood was collected from young and old volunteers prior to skin testing or at the time of blister aspiration. Heparinised blood was mixed 1:1 with Hanks Balanced Salt Solution (HBSS) (GIBCO, BRL Life Technologies, Paisley, UK) and layered on to Ficoll-Paque (Amersham Biosciences UK Ltd, Chalfont St. Giles, UK) in 50ml Falcon tubes. This was centrifuged for 20 minutes at 800xg with no brake. The buffy coat at the interphase layer was harvested and then washed twice for 10 minutes in excess HBSS by centrifugation at 650xg for the first wash and 300xg for the second wash. The cells were finally resuspended in complete medium as for the blister cells (RPMI 1640 [GIBCO, BRL Life Technologies, Paisley, UK] containing 10% human AB serum, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine [all from Sigma Aldrich, Gillingham, Dorset, UK]). Typically, 1-2 x10⁶ cells were isolated per 1ml of venous blood. For experiments where CLA expression on antigen specific cells was analysed, unsupplemented X-Vivo medium (Biowhittaker, Cambrex Bioscience, Walkersville) was used to prevent the down-regulation of CLA upon cell stimulation.

2.4.2. CD4 isolation for migration experiments

CD4 T cells were isolated using Magnetic Cell Sorting MACS (Miltenyi Biotec, Bisley, Surrey, UK) separation columns and by positive selection using CD4 microbeads (Miltenyi Biotec). MACS allows the purification of cell subsets from complex cell mixtures. Cells can be specifically labelled using MACS MicroBeads. These are extremely small biodegradable super-paramagnetic beads that do not activate or influence cell function or viability. After magnetic labelling, the cells are passed through a separation column, which is placed in a strong permanent magnetic field. The column matrix serves to create a high-gradient magnetic field. The magnetically labelled cells are retained in the column, while non-labelled cells pass through. After removal of the column from the magnetic field, the magnetically

retained cells can be eluted. Purified cells can be used for in vitro cell culture or flow cytometry. MACS MicroBeads are submicroscopic and therefore the light scatter characteristics of labelled cells are unaffected. There are two general approaches to magnetic cell sorting: depletion and positive selection.

2.4.2.1. Positive selection of cell subsets

This involves the isolation of target cells as the positive magnetically labelled fraction and was used to purify CD4⁺ and CD25⁺T cells. PB CD4⁺ T cells were isolated as described above. The cells were washed in MACS buffer centrifuging at 650 ×g for 5 minutes. For CD25⁺ purification, the cell pellet was resuspended in 90µl of MACS buffer per 10⁶ total cells, to which 10µl of CD25 Microbeads per 10⁶ total cells was added. For CD4⁺ purification, the cell pellet was resuspended in 80µl of MACS buffer per 10⁶ total cells, to which 20µl of CD4 Microbeads per 10⁶ total cells was added. The cells were vortexed and incubated for 10 minutes at 4°C before washing with MACS buffer by adding 10× the labelling volume and centrifuging at 650 ×g for 5 minutes. MS and LS columns were used for the positive selection of up to 10⁷ and 10⁸ labelled cells respectively. MS columns were used for CD25⁺ cell selection and LS columns for CD4⁺ cell selection. The cell pellet was resuspended in 500µl or 1000µl of MACS buffer per 10⁷ or 10⁸ CD4⁺ T cells respectively. MS columns were prepared by applying 500µl of MACS buffer. The cell suspension was applied to the column, after which the column was washed 3 times with 500µl of MACS buffer. The effluent unlabelled fraction representing enriched CD25⁻CD4⁺ T cells was collected. The column was then removed from the magnet and 1ml of MACS buffer was flushed through the column using a plunger. This effluent was also collected and consisted of the positive magnetically labelled fraction representing enriched CD25⁺ T cells. The LS column was prepared by rinsing with MACS buffer. The cell suspension was applied to the column. For CD4⁺ T cell isolation, only the positive fraction was collected. The LS column was removed from the magnet and 5ml of buffer was pipetted into the column and the labelled fraction

was immediately flushed out of the column by applying the plunger. This effluent consisted of the positive magnetically labelled CD4⁺ T cells.

2.5. Immunohistology

2.5.1. Antibodies used in the study

Table 2-3: Antibodies used for Indirect Immunohistochemistry

Antigen	Source	Clone ^a	Isotype	Dilution	Specificity	Detection, developing agent
CD3	Dako	UCHT-1	IgG1k	1:10	All T cells	Horseradish peroxidise conjugated polyclonal rabbit anti-mouse antibody, DAB
CD4	BD	Pure leu-3a	IgG1k	1:10	T helper/inducer cells	Horseradish peroxidise conjugated polyclonal rabbit anti-mouse antibody, DAB
CD8	Dako	DK25	IgG1k	1:50	T cytotoxic/suppressor cells	Horseradish peroxidise conjugated polyclonal rabbit anti-mouse antibody, DAB
Ki67	Dako	Ki-67	IgG1k	1:10	proliferating cells (except those in G0 phase)	Horseradish peroxidise conjugated polyclonal rabbit anti-mouse antibody, DAB
CD163	Acris	5C6-FAT	IgG1	1:100	Macrophages	Biotinylated horse anti-mouse antibody, avidin-biotinylated enzyme complex (VECTASTAIN ABC, Vector Laboratories); AEC
CD11c	BD	B-ly6	IgG1	1:100	Dendritic cells	Biotinylated horse anti-mouse antibody, avidin-biotinylated enzyme complex (VECTASTAIN) AEC
Isotype control	IgG1k	107.3	IgG1k	1:10/1.50/1.100	Isotype control	

^a= all are murine monoclonals unless stated

Abbreviations: DAB= 3,3'-diaminobenzidine tetrahydrochloride,

AEC=3-amino-9-ethylcarbazole

Table 2-4: Antibodies used for indirect immunofluorescence

Antigen	Source	Clone ^a	Isotype	Dilution	Specificity	Amplification/detection ^b
INDIRECT IMMUNOFLUORESCENCE						
E-selectin (CD62E)	Abcam	ENA2	IgG1	1:50	Endothelial cells	Goat anti-mouse Ig G(H+L)-AlexaFluor546, 1:200
VCAM-1 (CD106)	Abd serotec	1.G11B1	IgG1	1:50	Endothelial cells, bone marrow stroma, follicular dendritic cells, osteoblasts, mesothelium	Goat anti-mouse Ig G(H+L)-AlexaFluor546, 1:200
ICAM-1 (CD54)	Abd serotec	84H10	IgG1	1:50	Most types of cells	Goat anti-mouse Ig G(H+L)-AlexaFluor546; 1:200
CD163	Acris	5C6-FAT	IgG1	1:100	Macrophages	Goat anti-mouse IgG1 Alexa Fluor 568, 1:250
CD11c	BD	B-ly6	IgG1	1:100	Dendritic cells	Goat anti-mouse IgG1 Alexa Fluor 568, 1:250
Foxp3-biotin	e-bioscience	PCH101 rat	IgG2a	1:100 o/n	Regulatory T cells Activated T cells	Streptavidin-Cy3 1:200 (Cedarlane laboratories Limited CLCSA1010 www.cedarlanelabs.com)
CD4 (Pure Leu-3a)	BD		IgG1k	1:10, o/n	Helper T cells, Regulatory T cells, monocytes, macrophages, dendrit	Donkey anti-mouse Ig G AlexaFluor488, 1:200
Ki67	Dako	Mouse antihuman IgG1k	IgG1k	1:100 /n	Expressed in nucleus of cycling cells (except G0 phase of cell cycle)	Donkey anti-mouse Ig G AlexaFluor488, 1:200
TNF- α FITC	BD	6401.1111	IgG	1:10	TNF- α	Goat Ig G A488 anti-fluorescein/Oregon, 1:200
DC-SIGN (CD209) FITC	BD	DCN46	IgG2b	1:50	macrophages, immature dendritic cells	Goat Ig G A488 anti-fluorescein/Oregon, 1:200
Isotype control	BD	MOPC-31C	IgG1	1:50	Isotype control	
DIRECT IMMUNOFLUORESCENCE						
CD31FITC	BD	WM59	IgG1	1:50	Endothelial cells	Nil additional
Isotype control	BD	MOPC-21	IgG1 FITC	1:50	Isotype control	

^a= all are murine monoclonals unless stated

^b=all amplification/detection antibodies are from Invitrogen/Molecular probes unless stated

o/n=overnight

2.5.2. Indirect Immunofluorescence

In this technique the primary unconjugated antibody is allowed to bind to the antigen in the tissue section. A secondary fluorescently labelled antibody, raised in another animal host and specific for the animal and immunoglobulin class of the primary antibody is then applied to the section and allowed to bind the primary antibody. Table 2-4 shows the primary antibody and secondary amplification/detection combinations used in our study. In the case of CD31 staining we used a directly conjugated CD31FITC antibody which did not need amplification. For TNF- α FITC and DC-SIGN FITC a secondary anti-FITC antibody was used to amplify the signal.

Frozen 6 μ m skin sections were left in a moist staining box at room temperature, wrapped in cling film, for 15 minutes. The cling film avoids rapid condensation and consequent antigen destruction. The slides were then unwrapped carefully to avoid damaging the tissue sections and the sections were ringed with polysiloxane (Dakocytomation, Glostrup, Denmark) which acts as a water repellent. A drop of 1xPBS (Sigma Aldrich) (dissolve 2 PBS tablets in 400ml sterile distilled water) was added to each ringed section to prevent drying. The slides were washed for 5 minutes in 1 xPBS. The PBS on the sections was removed by tapping the slides gently on the side and a drop of Dako non-serum protein block (Dakocytomation, Glostrup, Denmark) was added to the ringed sections for 20 minutes. This step minimises non-specific antibody binding. Slides were again tapped vertically to get rid of excess blocking fluid. Primary antibodies diluted in 1 xPBS were added at 40 μ l per section and incubated for 1 hour at room temperature in the dark. In the case of FOXP3 staining, this primary antibody was left to incubate overnight as better staining was obtained with longer incubation times. Slides were washed twice for 10 minutes in the dark in 1xPBS, and then tapped vertically to get rid of excess fluid and any remaining excess fluid was soaked up with filter paper placed to the side of the specimen with care being taken not to disturb the specimen. The staining was then amplified with the appropriate secondary antibody. We followed a slightly different protocol for TNF- α and CD163/CD11c double labelling of skin tissue as this staining was carried out at Professor James Krueger's laboratory at the Rockefeller University

in New York using our collaborator's reagents and techniques. Day 3 candida stimulated skin were blocked in 10% normal goat serum (Vector Laboratories) for 30 minutes. Primary antibody CD163 or CD11c was diluted in 10% goat serum and was incubated overnight at 4°C and amplified with: goat anti-mouse IgG1 conjugated with Alexa Fluor 568, followed by overnight incubation with directly conjugated TNF- α FITC and amplified with goat anti-FITC AlexaFluor 488.

2.5.2.1. Double labelling

If double staining and using two primary antibodies it is important that the antibodies are raised in different species and the secondaries are raised in the same species. This is to avoid any one secondary antibody binding to either primary. Using double labelling with two primaries on any one section enables proportions of single and double stained cells to be counted using a fluorescence microscope.

2.5.2.2. Control slides

Antibodies conjugated with a fluorochrome often gave background epidermal fluorescence. Dermal collagen fibres gave green autofluorescence. Appropriate isotype controls (**Table 2-4**) were used as well as imaging of unstained skin tissue and both of these controls allowed assessment of the degree of autofluorescence.

When choosing fluorochrome combinations for colocalisation studies obtained with double labelling as above, their spectra must be unambiguously distinctive. Bleed-through is the passage of fluorescence emission in an inappropriate detection channel caused by an overlap of emission spectra (Bolte and Cordelieres, 2006). Figure 2-3 Figure 2-1 illustrates the phenomenon of bleed-through when using a commonly used fluorochrome combination FITC and AlexaFluor546. For this reason, we always ensured we had single-labelled control slides for each fluorochrome used eg FITC and AlexaFluor546. In this way we could check for bleed through between fluorochromes by switching from visualising the control slide with the FITC filter (green) and then switching to the AlexaFluor filter (red) and ensuring that no FITC is

detected or captured during image acquisition using same setting as for experimental slides when visualising the slide with the AlexaFluor filter.

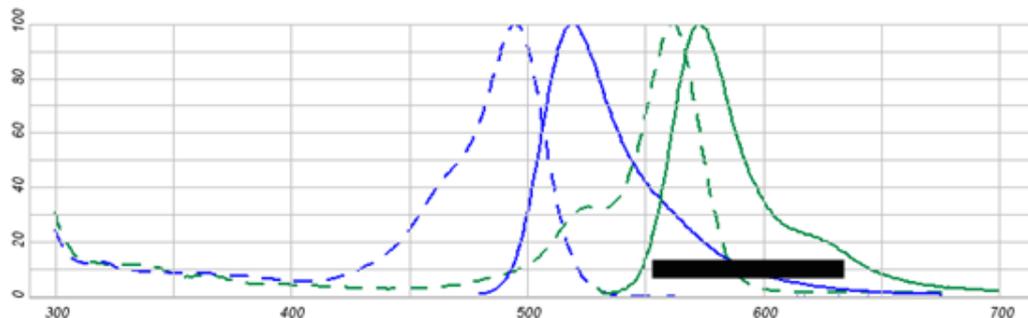


Figure 2-3: Explanation of bleed-through illustrated with the fluorochrome couple fluorescein iso-thiocyanate/AlexaFluor546.

Excitation spectra of FITC (broken blue line) and AlexaFluor546(broken green line). Emission spectra of FITC (solid blue line) and AlexaFluor 546(solid green line). The black bar marks the typical detection window of Alexa546. Note the overlap of FITC and Alexafluor 546 in this detection window (this represents what would appear as bleed through)

2.5.2.3. *Biotin-Streptavidin Cy-3 technique*

An indirect biotin/ streptavidin method was used to detect FOXP3 expression on skin tissue sections. In this technique, primary biotinylated rat anti-human antibody is allowed to bind with FOXP3 in the tissue sections. A secondary reagent consisting of streptavidin conjugated to a fluorochrome (in this case Cy3) is then added. Streptavidin has a strong affinity for biotin. Up to 150 biotin molecules can be complexed to a single second layer antibody, allowing for amplification of the staining signal. The staining steps employed for this technique were performed in precisely the same way as for the indirect immunofluorescence technique described above.

2.5.3. Indirect Immunohistochemistry

2.5.3.1. *Indirect immunoperoxidase*

The indirect immunoperoxidase technique was used to detect numbers and distribution of T cells and to detect cellular proliferation in the skin biopsies. In this technique a primary unconjugated antibody is bound to the antigen of interest in the tissue section. A second horseradish-peroxidase conjugated antibody, raised in another animal host that is specific for the animal and immunoglobulin class of the primary antibody, is then bound to the primary antibody. The complex that is formed is visualized following incubation with an appropriate chromagen or substrate. In this study, the chromagen 3, 3'-diaminobenzidine tetrahydrochloride (DAB) was used. This is converted by horseradish- peroxidase into an insoluble brown substrate, identifying the cell/ antigen of choice.

Skin sections from normal and candida injected skin (n=25 young, 26 old) were stained with optimal dilutions of purified primary antibody. The primary antibodies used with this technique were mouse anti-human CD3 antibody (Dakocytomation), purified mouse anti-human CD4 antibody (BD Pharmingen), purified mouse anti-human CD8 antibody (Dakocytomation) and purified mouse anti-human Ki67antibody (Dakocytomation) Rabbit anti-mouse horseradish-peroxidase conjugated antibody (Dakocytomation) was then added. The staining signal for these antibodies was developed using chromagen 3'-diaminobenzidine tetrahydrochloride.

Frozen tissue sections were defrosted and placed in a moist staining box. The sections were ringed with polysiloxane (Dakocytomation, Glostrup, Denmark) as a water repellent. 50µl of 1x phosphate buffered saline (PBS) (GIBCO, Invitrogen, Paisley, UK) containing 10% human serum (Sigma Aldrich, Gillingham, Dorset, UK), 10% Aprotinin (Sigma- Aldrich, Gillingham, Dorset UK) and a pre-titrated concentration of primary antibody was carefully pipetted on to each slide. The antibodies used are listed in Table 2-3. Non-specific antibody binding was minimized by the addition of 10% human serum. 10% Aprotinin (a protease inhibitor

derived from bovine lung) was added to block any endogenous immunoperoxidase activity within the tissue. The slides were then left to incubate for 45 minutes at room temperature. The fluid on the sections was removed by gently tapping the slide and the sections were then washed with fresh 1xPBS for 5 minutes, followed by formalin buffered saline (4.5g sodium dihydrate phosphate, 6.5g di-sodium hydrogen orthophosphate, 100ml of 40% formaldehyde and 900ml of 1x PBS) for 10 minutes and finally 1xPBS for 5 minutes. 4 μ l of horseradish peroxidase conjugated rabbit anti-mouse secondary antibody in 50 μ l of 1xPBS with 10% human serum was then added to each section. The sections were incubated at room temperature for a further 45 minutes and then washed as previously.

1 drop of Filtered Nickel DAB solution (95 mg of 3,3'-diaminobenzidine, 1.6g NaCl, 0.136g Imidazole, 2g nickel sulfate dissolved in 180ml of Tris/HCL buffer with pH corrected to 7.4 with 1M TRIS) containing 1 μ l/ml of 10% H₂O₂ (added immediately prior to use) was then added to each section using a Pasteur pipette. This was left for up to 7 minutes prior to rinsing with PBS for 3 minutes. Slides were then placed in Tris Cobalt to intensify the stain (1.2g TRIS base, 1g Cobalt Chloride dissolved in 180ml distilled H₂O with pH corrected to 7.2 with HCl) for 5 minutes and then rinsed in tap water. Sections were counterstained with 0.1% Nuclear fast red for 5 minutes, rinsed in tap water and then dehydrated by washing for 10 seconds in 70% ethanol, 90% ethanol, twice in neat ethanol and then cleared in 50:50 CitrocLEAR/ ethanol mix and finally neat CitrocLEAR (HD Supplies, Aylesbury, Bucks, UK). Sections were left in the neat CitrocLEAR for a further 10 minutes and then mounted in styrolite.

2.5.3.2. *Biotin-Avidin technique*

This method is more sensitive than the technique described above and was therefore used to identify the distribution of TNF- α , CD163 and CD11c in skin sections. With this technique the primary unconjugated antibody is allowed to bind to the antigen in the tissue section. This technique then employs the strong ability of biotin to complex with several molecules in two steps: first a secondary biotin conjugated

antibody, raised in another animal host and specific for the animal and immunoglobulin class of the primary antibody, is applied to the section and allowed to bind to the primary antibody, secondly avidin linked to a biotinylated enzyme (“avidin:biotinylated enzyme complex”, which consists of many biotinylated enzyme molecules crosslinked by avidin into a three-dimensional array) is added to the previously formed primary antibody-secondary biotinylated antibody reactions. When the substrate 3-amino-9-ethylcarbazole (AEC) is subsequently added, the enzyme linked to avidin and in the presence of hydrogen peroxide catalyses the colour producing reaction such that AEC changes to a dark brown pigment. AEC is a chemical derived from DAB and both give a dark brown pigment. AEC is mounted using aqueous mounting medium because the AEC is degraded in organic mounting media such as those we utilised for DAB staining. This also means that if air bubbles form under the coverslip one can dissolve the mountant in water and reapply the coverslip for further visualisation and imaging of section.

Frozen 6µm skin sections were prepared for staining as described in Section 2.5.2. The slides were immersed in PBS (0.1M, pH 7.5) and left to stand in PBS wash for 5 minutes. The sections were then incubated for 20 minutes with 50 µl per skin section of 10% normal horse serum (prepared from the same species in which the secondary antibody was made). Excess serum was then blotted from sections. The primary antibody was pre-diluted in 1% normal horse serum and added to the tissue sections at the required dilution and left to incubate overnight at 4°C. The sections were then rinsed twice by immersion in PBS and then left to stand in PBS for 5 minutes. This technique employs a commercially available Avidin-biotinylated enzyme complex (VECTASTAIN ABC, Vector laboratories) which is prepared at this stage by allowing 10 µl of agent “A” (Avidin DH) and 10 µl of agent “B” (biotinylated enzyme) to react in 1000 µl of 1% normal horse serum diluted in PBS. The substrate AEC is prepared prior to commencing of staining by dissolving 120mg of AEC in 15ml of N,N-dimethyl formamide (SIGMA). 0.1M acetate buffer is prepared by adding 79ml of 0.1M sodium acetate (13.61 g/L distilled H₂O and 21ml of 0.1M Acetic acid). The mixture is filtered through a 0.2 micron filter with a syringe and 2.5 µl of 30% H₂O₂ is added to activate the reagent. The horse anti-mouse

biotinylated antibody is diluted at the required dilution (1:200) and incubated with the sections for 30 minutes at room temperature. The sections are then rinsed twice by immersion in PBS and then left to wash for 5 minutes. At this stage to ensure quenching of endogenous peroxidase the sections are incubated for 15-20 minutes in 0.3% H₂O₂ (Henry Schein Hydrogen peroxide solution (1:10 dilution of 3% stock solution in distilled H₂O and mixed well in graduated cylinder). The sections are then washed as before. Sections are then incubated for 40 minutes at room temperature with VECTASTAIN ABC (40µl per section). The slides are washed as before. The AEC reagent is now applied and the sections monitored until the desired staining intensity develops. The slides are then washed in distilled water and left to dry. Once dry the sections are mounted in Aqueous mounting medium.

2.5.3.3. Control slides

Three control preparations were used each time staining was performed. As a negative control, a section of both tonsil and skin tissue was incubated with normal mouse serum instead of primary antibody. In addition, where possible, a negative isotype control antibody for the same animal species as the primary antibody was also used at the same concentration on single sections of skin and tonsil (Table 2-4). Tonsil tissue was also stained as a positive control. Tonsil provides a rich source of cells including those stained for in the skin sections and allows for the distribution and staining of cells to be tested against tissue architecture.

2.5.4. Imaging and Quantification/Assessment of Immunofluorescence micrographs

2.5.4.1. Image acquisition and processing

Images were acquired using appropriate filters of a Leica DMLB microscope with Leica N PLAN 20x /0.40 objective and a Cool SNAP-Pro cf Monochrome Media Cybernetics camera, controlled by Image-Pro PLUS 6.2 software. In the case of

CD163 and CD11c and TNF- α double labelling, the images were acquired in our collaborator's laboratory at the Rockefeller University in New York, using appropriate filters of a Zeiss Axioplan 2I microscope with Plan Apochromat 20 \times 0.7 numerical aperture lens and a Hamamatsu orca ER-cooled charge-coupled device camera, controlled by METAVUE software (Universal Imaging).

One important factor to take into consideration when quantifying immunofluorescence is that procedures and parameters must be standardised. Therefore to ensure that immunofluorescence micrographs acquired from skin sections from young and old subjects were interpretable for analysis of staining intensity, the saturation, sensitivity and gain settings during acquisition were standardised on the image acquisition software. Staining for each antibody and each skin section from the same candida injection time-point was done on the same day, in order to avoid differences in staining intensity due to day-to-day fluctuations.

Image processing when necessary eg to produce images for printing, was performed by using commercially available software (Adobe Photoshop CS3, San Jose, CA). With the use of the Adobe Photoshop "levels" and "threshold" functions, the background was optimised (non-specific extracellular signalling was reduced to a uniform black background). These settings, used to optimise image quality on a control image from a young skin section, showing good expression of the marker being tested, were then used as standards for the processing of all subsequent cell images. All optimisation of image quality was performed on the whole image and not to selected features that needed to be highlighted.

2.5.4.2. Image qualitative analysis and quantification of data used for colocalisation purposes

Immunofluorescence microscopy images were used for analysis in the case of E-selectin, VCAM-1, ICAM-1 expression on CD31 positive endothelial cells in skin tissue and for calculating proportions of cells expressing a particular marker out of the total population of interest e.g. CD163⁺ TNF⁺ macrophages. Wherever the

staining was all or none, we manually counted cells (using Adobe Photoshop CS3 manual count function) that were positive for the cell type identifying marker, such as CD163 then manually counted cells that were doubly positive for the second marker of interest (eg TNF- α). For expression that was characterised by a variation in intensity of staining such as ICAM-1 we asked two independent observers to blindly assess the staining and label each slide as high intensity/moderate/low.

Specialised software for quantitative analyses of immunofluorescent images is available and is generally based on global statistic analysis of pixel intensity distributions. However we felt that there are many potential pitfalls to analysing our immunofluorescent data in this way. The majority of colocalisation situations demand customised approaches as different tissues contain a plethora of structures with multiple morphologies. Firstly most software packages have been created with specific tissue and protein expression in mind (Belichenko et al., 1996; Bolte and Cordelieres, 2006). Skin tissue poses its own particular problems in that there is background autofluorescence caused partly by non-specific staining and partly by protein autofluorescence such as the epidermis and dense concentrations of cells, as well as collagen fibrils in the dermis. Both these types of fluorescence disturb the fluorescence of interest to a degree which varies in different areas. This makes it difficult to obtain pixel intensity measurements that we could be sure were coming from the area of interest eg the dermal endothelium and not background collagen fibril in close juxtaposition to the endothelium in the dermis. Another advantage of “naked eye” analysis of micrographs is that in some skin sections it was clear that there were some areas where the staining hadn’t worked but there was good interpretable staining in the rest of the section. An image analysis program would not be able to factor this in and would give false low pixel readings for an area of the skin section which should have been discounted in the first place.

2.5.5. Quantification of Immunohistochemistry

Immunohistochemistry was analysed using a light microscope (Nikon Eclipse E600) and the 5 largest perivascular cellular infiltrates in the upper and mid-dermis were

photographed using Nikon DXM1200F camera and Eclipse Net software version 1.16.3 for Nikon. The number of positive cells in these infiltrates was counted using Adobe photoshop CS3 software. The mean number of cells for the 5 perivascular infiltrates photographed per section was then calculated. The number of CD163⁺ and CD11c⁺ cells/mm was counted manually using computer-assisted image analysis (NIH Image 6.1; <http://rsb.info.nih.gov/nih-image>).

2.6. Skin Explant Culture

Punch biopsies (5 by 5 mm) were obtained from the forearm as before and bisected with care. Each half was placed in medium alone or medium enriched with TNF- α (110,000 U/ml) and IFN- γ (100U/ml) for 24 hours. The biopsies were then frozen in OCT(optimal cutting temperature compound; Bright Instrument Company Ltd). 6 μ m sections were cut and left to dry overnight and then fixed in ethanol and acetone and stored at -80°C as before.

2.7. Flow cytometry

2.7.1. Surface staining by direct Immunofluorescence

Four-parameter analysis of blister and blood T cell phenotype was performed on a FACSCalibur™ (Becton Dickinson). PBMCs were stained with antibodies to CD3 (Dakocytomation), CD4, CLA, CCR4, CD11a (all from BDPharmingen). Appropriate isotype controls were used. Details of antibodies and dilutions used are found in Table 2-3.

2.7.2. Intracellular staining

2.7.2.1. *Detection of antigen specific cells*

For the detection of antigen specific cells, PBMCs (1×10^6) or blister cells were stimulated with PPD at a final concentration of $10 \mu\text{g/ml}$ or Candida antigen at a final concentration of $40 \mu\text{g/ml}$ for 15 hours at 37°C in a humidified 5% CO_2 atmosphere. Brefeldin A (Sigma- Aldrich) was added at a final concentration of $5 \mu\text{g/ml}$ after 2 hours of incubation. Unstimulated controls were also included. The cells were fixed and permeabilized (Fix & Perm $\text{\textcircled{R}}$ Cell Permeabilisation Kit; Caltag Laboratories) before staining for CD4 and IFN- γ as described (Reed et al., 2004).

2.7.2.2. *Detection of TNF- α producing cells*

For the analysis of macrophage function PBMC and blister cells were washed and resuspended in RPMI 1460 supplemented with 1% penicillin/streptomycin, 1% glutamine and 10% heat-inactivated foetal calf serum (Cambrex, Nottingham, UK). Cells were stimulated with LPS (100ng/ml ; Sigma) or medium as a control for 4hrs in the presence of Golgi Stop (Becton Dickinson, San Jose, California). Cells were stained for cell surface markers for 30min at 4°C , fixed in 2% paraformaldehyde and permeabilized with 0.5% saponin, then labelled with either anti-IgG1 APC or TNF- α APC (eBioscience). Cells were analyzed on FACS Canto II using FACS Diva software (Both Becton Dickinson) and further analyzed using FlowJo software (TreeStar, Inc).

Table 2-5: Antibodies used for flow cytometry

Antigen	Source	Clone ^a	Isotype	Dilution	Specificity
CD14APC	Serotec	UCHM-1	IgG2a	1/35	Monocytes, macrophages, dendritic cells
CD163FITC	Santa Cruz	GHI/61	IgG1	1/20	Monocytes, macrophages
CD45RA-FITC	BD	L48	mIgG1	1/25	Naïve T lymphocytes, all B lymphocytes, Nk lymphocytes
IFN- γ -APC	BD	B27	mIgG1	1/200	Activated Th0 and Th1 Cd4+ T lymphocytes, activated Cd8+ T lymphocytes, NK cells
IFN- γ -FITC	BD	25723.11	mIgG2b, κ	~1/5	Activated Th0 and Th1 Cd4+ T lymphocytes, activated Cd8+ T lymphocytes, NK cells
IL-2-FITC	BD	5344.111	mIgG1, κ		Activated CD4+ Th0 and Th1, some Cd8+ T cells
CD4-PE	Dako	MT310	mIgG1, κ	~1/20	Helper T cells, Tregs, monocytes, macrophages, Langerhans cells, dendritic cells
CD45RA-PE	Dako	4KB5	mIgG1, κ	~1/20	Naïve T lymphocytes, all B lymphocytes, Nk lymphocytes
Ki67-FITC	BD	B56	mIgG1	1/10	Expressed in nucleus of cycling cells
Foxp3-APC	eBioscience	PCH101	mIgG1	1/10	Regulatory T cells Activated T cells
CLA FITC	BD Pharm	HECA-452	Rat IgGM	1/10	Lymphocytes in the skin, small subset of circulating lymphocytes, all monocytes, granulocytes
DC-SIGN APC	BD	DCN46	IgG2b	1/50	Monocytes, Macrophages, dendritic cells
CD14 PeCy7	BD	M5E2	IgG2a, κ	1/100	Monocytes, macrophages, dendritic cells
CD11c PE	BD	B-ly6	IgG1, κ	1/100	NK cells, B and T cells, Monocytes, macrophages, Dendritic cells, granulocytes

2.8. T Lymphocyte transendothelial migration

2.8.1. Endothelial culture

Normal non-transformed human dermal microvascular endothelial cells (HDMEC-c) (PromoCell, Heidelberg, Germany) derived from young human dermis were cultured at 37°C in a humidified 5% CO₂, in endothelial cell basal medium supplemented with 5% FCS (supplement pack C39220, PromoCell, Heidelberg, Germany). The preparation of endothelial cell cultures was carried out in collaboration with Dr Anna-Pia Papageorgiou, Institute of Child Health, UCL. Briefly the endothelial cells were stored frozen until needed. To initiate cell culture 5 ml of endothelial cell basal medium were added to a 25cm² culture flask and this was warmed to 37°C. A frozen tube of endothelial cells was agitated in a 37°C waterbath until 90% thawed and then agitated outside the waterbath until completely thawed. Endothelial cells were pipetted into the culture flask at 10,000-20,000 cells/cm². The medium was replaced the next day and every 2 to 3 days subsequently. The cell cultures were considered ready for splitting 24 hours after reaching 70-90% confluence. For splitting 5 ml of endothelial cell basal medium were added to a new 25cm² culture flask and incubated at 37°C for at least 30 minutes. The endothelial culture flask was placed in a sterile hood and the medium removed taking care not to disturb the endothelial monolayer with the pipette. The medium was replaced with 5ml HEPES Buffered Saline Solution (HEPES-BSS) and washed for 30 seconds by swirling the flask. The HEPES-BSS was then removed and replaced with 100µl/cm² (i.e. 2.5ml in 25cm² flask) trypsin/EDTA solution. The flask was then examined under the microscope. When ~50% of cells were loose, the side of the container was tapped to release the rest of the cells. This procedure was performed at room temperature. When the endothelial cells were detached, an equal volume of Trypsin Neutralising Solution was added and the cell suspension transferred to a centrifuge tube. This was then centrifuged at 220g for 4 minutes at room temperature and the resultant clear upper layer was carefully removed. The cells were resuspended gently in 1.5ml medium and diluted to a total volume of 2ml and then counted. The cell suspension was then redistributed to new flasks (250,000-500,000 cells/flask) and maintained at 37°C in

5% CO₂ and the medium replaced every 2-3 days until they reached 50% confluence when they were used for migration assays. The cell culture was checked for mitotic figures/clusters, indicating that the cells were in a proliferative state.

2.8.2. Activation of endothelial cells for migration studies

HDMEC monolayers were grown to confluency in 96 well plates and stimulated with TNF- α (100ng/ml)(110,000 U/ml) and IFN- γ (100U/ml) for 24 hours prior to migration assay. By FACS analysis, TNF- α and IFN- γ treated cells were >99% and 96% positive for ICAM-1 and VCAM-1 respectively, after 24 hours of treatment.

2.8.3. Migration experiments

CD4⁺ T cells, isolated from young and old PBMCs by negative selection and kept overnight in X-vivo medium (to prevent downregulation of CLA) were added (20,000 cells per well) with a minimum of 6 wells per condition per experiment. To evaluate the level of migration, cocultures were placed on the stage of a phase-contrast inverted microscope housed in a temperature controlled (37°C), 5% CO₂ gassed chamber (Zeiss, Herts, U.K.). For each well a 200 x 200-mm field was randomly chosen and recorded for 5 min spanning the 4-h time point using a camera linked to a time-lapse video recorder. Recordings were replayed at 160 x normal speed, and lymphocytes that had migrated through the monolayer were identified and counted. Lymphocytes on the surface of the monolayer were identified by their highly refractive morphology (phase-bright) and rounded or partially spread appearance. In contrast, cells that had migrated through the monolayer were phase-dark and highly attenuated. Data were expressed as the percentage of total lymphocytes within a field that had migrated through the monolayer

2.9. In Vitro Cell Culture

2.9.1. Measurement of cellular proliferation by [³H] thymidine incorporation

For measurement of cellular proliferation by [³H] thymidine incorporation, PBMCs were added to 96 well round bottomed tissue culture plates (Falcon, Becton Dickinson Labware) at a concentration of 10⁵ cells/well. The cells were incubated at 37°C in a humidified 5% CO₂ atmosphere for 5-6 days (5 days for PPD antigen, 6 days for candida and VZV antigens) before adding [³H] thymidine (Amersham Biosciences UK Ltd, Chalfont St. Giles, UK). The mean of triplicate wells was calculated and used for the purpose of data analysis.

2.9.2. Monocyte cell culture with TLR ligands

PBMCs were obtained from healthy young and old subjects (n=3 young, n=3 old) as described. The PBMCs were suspended at 1 x10⁶/ml in RPMI +10% human serum in 24 well flat bottomed plates and incubated at 37°C in a humidified 5% CO₂ atmosphere for 18 or 72 hours TLR ligands: LPS (100ng/ml, Sigma) or Pam₃CSK₄ (1µl/ml, Invitrogen) or Candida antigen (5µg/ml, Greer). Unstimulated controls were also used. Prior to surface staining for extracellular DC-SIGN, CD14, CD11c and CLA, the 24 well plate was placed on ice for 1 hour so that macrophages which are known to adhere to plastic in warm environments, start to detach back into solution. To ensure further detachment of macrophages the sides of each well were scraped with the tip of a Pasteur pipette and the cell suspension aspirated and prepared for extracellular marker staining for flowcytometry as described before.

2.10. Cytokine assays

Concentrations of cytokines (IL-6, IL-10, TNF, IFN-γ) in blister fluid and serum were analysed using the BDTM Cytometric Bead Array (CBA) Cell Signalling Flex Set System and were analysed using a BD FACSArrayTM bioanalyser with FCAP

Array Software (all BD Biosciences, San Jose, California, USA). This technique employs the use of particles with discrete fluorescent intensities and sizes to detect soluble analytes and enables multiplexed quantitative analysis of multiple proteins from a single sample using flow cytometry.

The assay was performed in a 96 well round bottomed plate (Falcon, Becton Dickinson Labware, New Jersey, USA). 50µl of mixed capture beads each coated with a cytokine-specific antibody was mixed with 50µl of mixed cytokine-specific PE-conjugated antibodies in addition to 50µl of blister fluid, serum or dilution series of analyte recombinant protein. The plate was gently vortexed and then incubated at 4 hours in the dark, at room temperature, in order for sandwich complexes to form. The plate was then centrifuged and the supernatant aspirated from each well. 150µl of wash buffer was then added to each well and the plate was then inserted in to the BD FACSArray™ bioanalyser for analysis. This machine automatically assigns an alpha-numeric position on a grid for each bead population using the NIR and Red channels. A standard curve for each analyte is created based on bead size and PE-fluorescence intensity for the dilution series of the recombinant protein samples. This enables the quantitative calculation of the concentration of the analytes from the test samples bound to the beads.

All samples were analysed in one experiment in order to minimise variation in measured concentrations due to different experimental conditions on different days. In addition, blister and serum samples were stored at -70°C immediately after collection from volunteers and were only defrosted for the purpose of analysis in order to minimize changes in cytokine concentration due to repeated freeze thaw cycles.

2.11. Statistics

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA). Non-parametric tests were predominantly utilised as the data could not be assumed to be normally distributed.

The Kruskal-Wallis test was used to compare three or more unpaired groups and a 2-tailed Mann-Whitney test was used when comparing only two unpaired groups. The Wilcoxon matched pairs test was used when comparing two groups of matched data.

Chapter 3. Decreased clinical and cellular response to injected recall antigen in old skin

3.1. Introduction

Immunity is known to decline with age (Yoshikawa, 2000). This may explain why there is an age-associated increase in the frequency of skin tumours and infections (Diffey and Langtry, 2005) (Laube, 2004). Most studies directed at identifying defects in the immune system during ageing have focused on circulating leucocyte populations but it is not clear if the results obtained can be extrapolated to the behaviour of leucocytes within tissues. In this chapter we elicited cutaneous delayed type hypersensitivity responses to bacterial and fungal skin test antigens in young and old subjects. This enabled us to compare the kinetics of this memory immune response in the skin and to determine the correlation between local cutaneous immunity and peripheral blood leucocyte function in young and old subjects.

The intra-dermal injection of recall antigens such as tuberculin purified protein derivative (PPD) (Orteu et al., 1998; Reed et al., 2004; Vukmanovic-Stejic et al., 2008) or candida albicans induces a delayed type hypersensitivity reaction (DTH) in the skin. DTH responses against Candida or vaccine antigens (Tetanus) can be used clinically as a measure of overall cell mediated immunity. The Candin skin test, using a sterile solution produced from the culture filtrate and cells of two strains of *Candida albicans*, has been licenced for the evaluation of cellular immune responses in the skin in patients with HIV (Ahmed and Blose, 1983). In this chapter we therefore use Candin skin test injected intradermally to compare cell mediated immunity in young and old subjects. Our group has previously characterised the cutaneous response to the injection of tuberculin purified protein derivative (PPD), known as the Mantoux test (MT), in terms of the kinetics of CD4⁺ T cell accumulation in the response (Reed et al., 2004) (Orteu et al., 1998). In humans, a positive clinical response to the injection of PPD antigen in to the skin is typically characterised by erythema and swelling that peaks at 48-72 hours and resolves within 14 days (Reed et al., 2004). This clinical response in the skin has been used as a

measure of systemic immunity *in vivo* (Turk JL., 1980). Previous studies in old subjects, predominantly performed on nursing home residents, have indicated an increase in the percentage of negative DTH reactions to the Mantoux test (MT) and other antigens (Castle et al., 1990; Dorken et al., 1987; Rodysill et al., 1989). Previous work carried out in our group has shown that subjects over 70 years have poor clinical responses to the injection of PPD antigen intradermally when compared to subjects under the age of 40. Older subjects also show reduced percentages of antigen specific cells for PPD when cells obtained from suction blisters raised over the skin test are stained for IFN- γ production *in vitro* (Dr Katie Lacy, unpublished data). Moreover in young individuals, good PBMC proliferative responses *in vitro* to PPD antigen were associated with a good clinical response to the MT. However, in the old group, there was dissociation between the observed absent clinical response in the skin and PBMC responses. In spite of good proliferative PBMC responses (equivalent to those seen in the young) twenty-two old individuals failed to develop a clinical response to the MT. This indicates that, in the old, the absent clinical skin response to the MT may not be reflective of global immunity to the antigen and may be reflective of local age- specific changes in cutaneous immunity.

The BCG (Bacillus of Calmette and Guerin) vaccine immunisation programme in the UK did not start until the 1940's and therefore most individuals over the age of 70 will not have routinely been vaccinated. Therefore another explanation for the observed differences between the young and old observed previously in our group (Dr Katie Lacy) could be that all individuals in the young group had been immunised with the BCG vaccine whereas only one individual in the old group had been vaccinated. Previous studies have demonstrated that the route of primary exposure to antigen may determine the ability of different tissues to subsequently develop memory responses to that antigen. PPD comprises of a complex mixture of peptides and carbohydrates derived from *M. tuberculosis* and immunity can be acquired in several ways; namely previous and/or latent infection with TB, BCG vaccination and exposure to environmental mycobacteria. The young individuals were most likely to have developed immunity to the PPD antigen as a result of BCG vaccination (administered percutaneously) whereas immunity in the old group was likely to be

due to previous exposure to tuberculosis or environmental mycobacteria. Of interest, however, one old individual in this study had been vaccinated and did not have a clinical response to the Mantoux test in spite of good peripheral blood response to the antigen. This suggests that percutaneous administration of the BCG vaccine may not determine subsequent ability to respond to the antigen in the skin in the old. In view of this possible confounding factor with regards to accurately comparing skin immunity in young and old subjects we subsequently decided to use candida skin test antigen to induce DTH responses for comparison in young and old subjects. Candida is a commensal on skin and mucous membranes and therefore both young and old subjects are equally exposed to this organism suggesting that both age groups could potentially mount a secondary cutaneous immune response to it on re-challenge. Old individuals are particularly prone to candida infections of the skin (Ronald Marks, 1999). The most common clinical subtypes that affect the elderly include vulvovaginal candidiasis, intertrigo and candida paronychia (Robert A. Norman, 2001).

Also our group has previously shown that non-immune individuals do not mount a DTH response to antigen despite the fact that equal levels of antigen as well as trauma to the skin occurs (Orteu et al., 1998) (Reed et al., 2004). This highlights the importance of memory T cell recruitment for the amplification step of this response to take place (Reed et al., 2004). Memory T cell recruitment into the skin of young and old subjects will be looked at throughout the course of this chapter.

3.2. Candida Skin testing

3.2.1. Clinical response to the Candida skin test in young and old individuals

A total of 40 young and 34 old individuals were tested with the Candin skin test with intradermal injection of Candin skin test solution into the inner aspect of the upper forearm. A dose of 0.02ml of the Candin skin test solution was selected for use in the study as this was the smallest dose/volume that could be administered accurately intradermally without the need for diluting the commercial preparation with an

associated risk of contamination of the skin test preparation. Once again, a clinical score was assigned for each individual tested based on erythema, diameter and palpability of the clinical response (Table 2-2). In both young and old groups, a peak in clinical response to the Candida skin test was seen at 48- 72 hours.

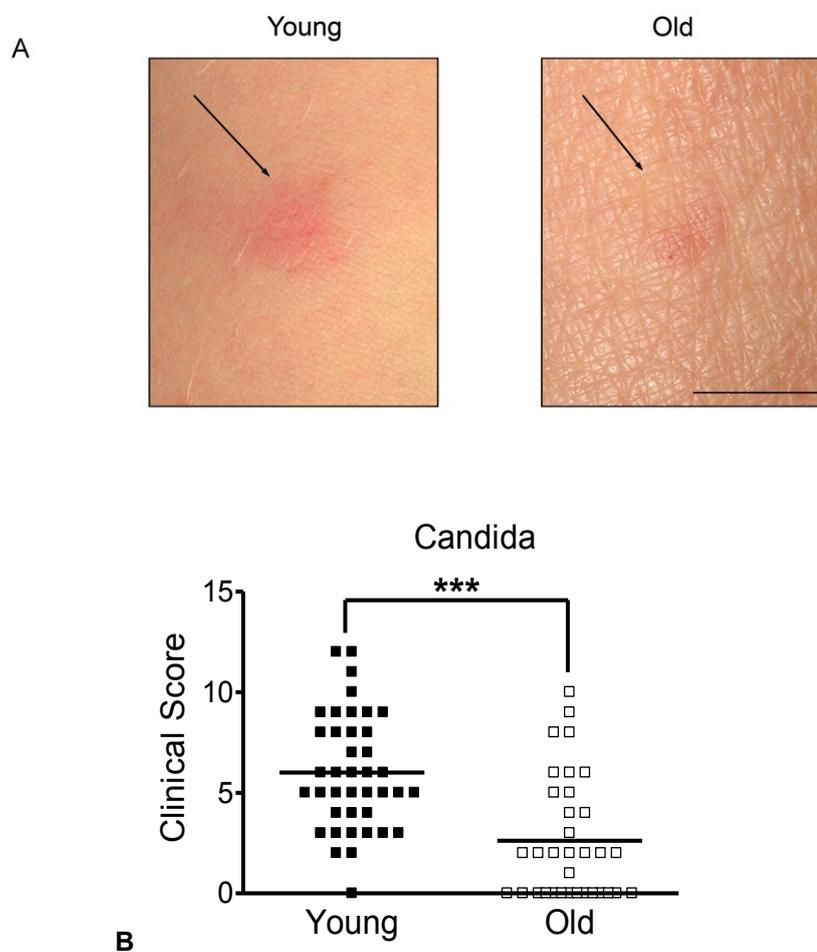


Figure 3-1: Cutaneous clinical responses to the Candida skin test are reduced in old individuals.

Young and old volunteers were injected intradermally with 0.02ml of Candida skin test antigen ((n=40 young, 34 old)) (A) A photograph of the injection site at day 3 following candida skin test antigen injection. (B) Induration diameter, erythema index and palpability of the lesion were assessed and graded at day 3 and a clinical score assigned based on these parameters (***) $P < 0.0001$, Mann Whitney test). (Dr KE Lacy collected half of the data points shown in this graph)

A marked reduction in clinical response to antigen injection was observed in the old compared with the young at Day 3 ($P < 0.0001$; Mann Whitney test) (Figure 3-1 A/B) and at all time points following the injection of 0.02ml of Candin skin test solution

3.2.2. Clinical response to the Candida skin test correlated to peripheral blood response in young and old individuals

The ability of old and young volunteers' PBMCs to proliferate in response to Candida antigen *in vitro* for 5-6 days was assessed for a range of concentrations of Candida antigen up to 20 $\mu\text{g/ml}$. No statistical difference was found between young and old PBMC proliferation at all concentrations of candida antigen tested including the 20 $\mu\text{g/ml}$ concentration (Mann Whitney $P = 0.69$). The clinical response to the skin test was then correlated with PBMC proliferative responses. This demonstrated that, as with the MT, there were significant numbers of old volunteers who failed to respond clinically to Candida antigen injection in spite of good peripheral blood responses (Figure 3-2:). This indicates that they have defective local immune responses to secondary antigen challenge in the skin that does not reflect a global loss of systemic immunity. The majority of young individuals tested had both good peripheral blood responses and skin responses to the Candida antigen.

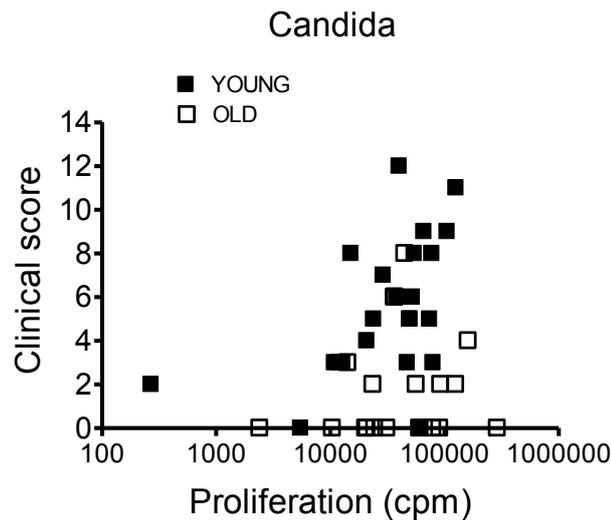


Figure 3-2: PBMC proliferation in response to Candida antigen *in vitro* plotted against clinical scores to Candida skin test antigen.

PBMC from old individuals proliferate well in response to Candida antigen *in vitro* despite poor clinical scores. Freshly isolated PBMC were stimulated with candida (n=23 young, 20 old) *in vitro* for 5-6 days. Proliferation was assessed by [³H] thymidine incorporation and plotted against the corresponding clinical score. (Dr KE Lacy collected half of the data points shown in this graph)

3.2.3. Differences in T cell number accumulation during the candida skin test in young and old subjects

We next determined whether the defective clinical response in old subjects was associated with decreased CD4⁺ T cell infiltration after antigen challenge since optimal secondary immune responses in the skin are dependent on antigen-specific CD4⁺ T cells.

3.2.3.1. CD3⁺ and CD4⁺ T cell numbers

We carried out skin punch biopsies on normal skin and at 24hrs, 3 and 7 days after candida skin test antigen injection in at least 4 young and 4 old volunteers at each timepoint tested. H&E staining in young and old skin sections at day 3 revealed

perivascular collections of lymphocytes which were much larger in the young skin sections compared to the old (Figure 3-3:A). We then performed immunohistochemical staining for CD3, CD4 on young and old skin sections obtained from 24hr, day 3 and day 7 biopsies. In the young, after the injection of intradermal Candida, CD3⁺ and CD4⁺ cells were seen to accumulate perivascularly within the dermis (Figure 3-3:). The peak of the cellular response for candida skin test injection in the young was at Day 3 which corresponds to the peak clinical response. Furthermore, there were reduced numbers of CD3⁺ (Figure 3-3:B) and CD4⁺ T lymphocytes (Figure 3-3:C) in perivascular infiltrates in old skin sections at all time-points studied (Figure 3-4:) indicating that the results obtained were not due to a shift in the kinetics of the response in old individuals.

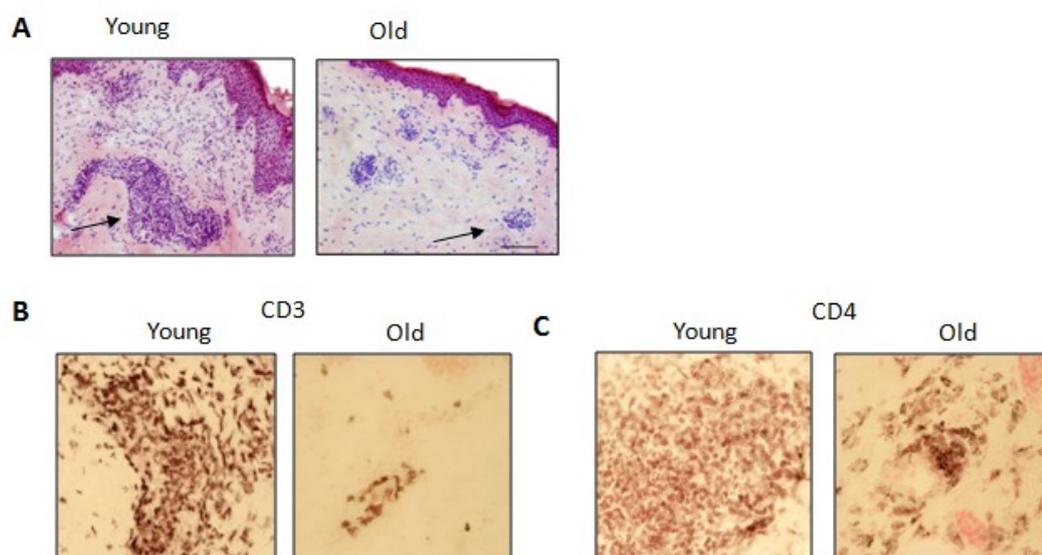


Figure 3-3: Cellular infiltrate at the site of cutaneous DTH response is reduced in the old.

Young and old volunteers were injected with 0.02ml Candida skin antigen test and 5mm punch biopsies were performed on days 1, 3 or 7 post injection (n=20 old, 22 young with 4-7 volunteers per timepoint). (A) H&E staining of skin sections from young and old skin on day 3 following Candida injection showing perivascular infiltrates (black arrow). Scale bar=100 μ m. Day 3 biopsies (n=5 young, 4 old) were stained for CD3 (B) and CD4 (C) using an indirect immunoperoxidase immunohistochemical technique (original magnification: x200).

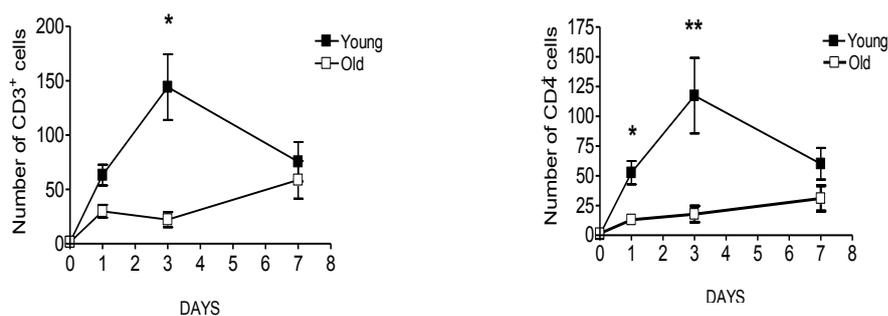


Figure 3-4: CD3⁺, CD4⁺ T cell numbers at the site of the Candida antigen skin test

The number of CD3⁺, CD4⁺ and CD8⁺T cells within perivascular infiltrates was determined by counting the number of positive cells within the 5 largest perivascular infiltrates per skin section. Mean±SEM of 4-7 individuals per time point is shown (*P=0.01, **P=0.007 Mann Whitney test).

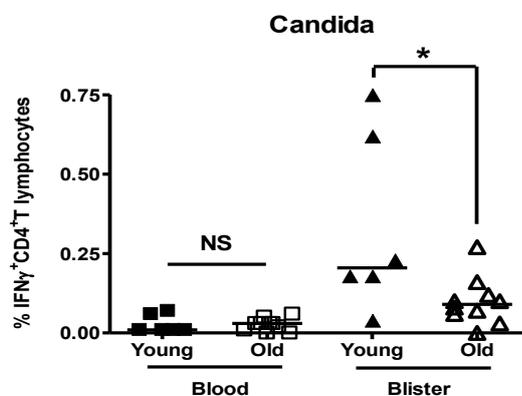


Figure 3-5: Candida-specific T cell infiltration in the skin following the Candida skin test in young and old.

The percentage of Candida specific CD4⁺ T cells in the peripheral blood and skin was determined in the old and young by examining for intracellular cytokine staining using flow cytometry. Skin blister and PBMC were stimulated with Candida antigen overnight in the presence of brefeldin A. Unstimulated controls were also performed. Live CD3+CD4+ cells were gated and at least 10,000 CD4+T cell events were acquired. The percentage IFN- γ producing antigen specific CD4⁺ T lymphocytes in old blisters is significantly reduced compared to young (n=6 young, 10 old; Mann Whitney test *P=0.04). Horizontal bars represent the median. At all time points measured the percentage of antigen specific (CD4⁺ IFN- γ) cells was significantly higher in the SB cell population compared with the blood.

3.2.3.2. *Local in situ T cell proliferation at site of candida response in young and old skin*

We have previously shown that T cell proliferation is likely to occur *in situ* in the skin during the Mantoux response in humans and not only in the draining lymph nodes (Reed et al., 2004). We therefore hypothesised that there is reduced T cell proliferation in old skin during a candida immune response. We carried out indirect immunoperoxidase staining for Ki67, a nuclear protein expressed during cell replication in young and old skin sections from normal skin biopsies and different time-points after candida injection (n=at least 4 old and 4 young subjects per timepoint). We found that Ki67 expression peaks at day 7 though later time-points were not studied for candida skin test histology. (Figure 3-6) There is a significant reduction in Ki67⁺ cell numbers at day 3 in skin sections from old individuals (Figure 3-6 A and B). This could occur however because the total number of CD3⁺ T cells in the old is reduced and therefore there are obviously fewer proliferating cells. However when the number of Ki67⁺ cells is expressed as a percentage of total CD3⁺ T cells quantitated from consecutively stained skin sections no significant difference is detected for Ki67 expression in young and old candida injected skin (Figure 3-66C). The percentage of CD3⁺Ki67⁺ cells on day 0 could not be accurately assessed due to very low CD3 numbers found on histology. However it was noted that very few cells were positive for Ki67 expression at Day 0.

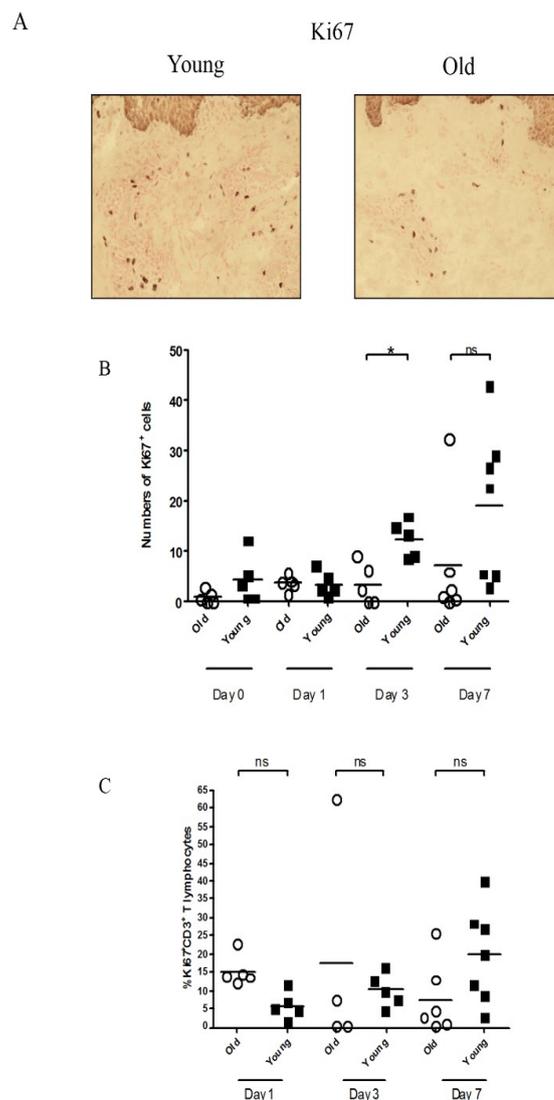


Figure 3-6: Kinetics of T cell proliferation during a cutaneous response to *Candida* skin test antigen in young and old.

Young and old volunteers were injected with 0.02ml *Candida* skin antigen test and 5mm punch biopsies were performed on days 1, 3 or 7 post injection (n=20 old, 22 young with 4-7 volunteers per timepoint) Biopsies were stained using antibodies for Ki67 and the indirect immunoperoxidase technique. Ki67⁺ cells were seen predominantly within the perivascular infiltrates. Ki67⁺ cells within frames centred over the 5 largest perivascular infiltrates per section were counted and the mean number of Ki67⁺ cells per frame per individual was calculated. **A.** There was a reduction in the mean number of Ki67⁺ cells in the old compared with the young (Mann Whitney test *P=0.03). **B.** However when the Ki67⁺ cells are expressed as a percentage of total CD3⁺ cells counted in the same individual using consecutive skin sections there is no significant difference between young and old CD3⁺ cell proliferation rates.

3.2.4. Differences in numbers of candida-specific memory T cell accumulation in young and old

We showed previously that large numbers of antigen-specific CD4⁺ T cells, identified by their ability to secrete IFN- γ after re-challenge, accumulated and proliferated at the site of antigen injection (Reed et al., 2004). We found that old and young subjects have similar proportions of PPD and candida specific CD4⁺T lymphocytes in the blood however; the former had significantly lower proportions of Ag-specific CD4⁺ T cells in the skin (Figure 3-5:). This indicates that the inability to mount a clinical response to recall antigen challenge older individuals is associated with decreased cutaneous antigen-specific CD4⁺ T cell infiltration.

3.2.5. Clinical response to the candida skin test correlated to numbers of T cells *in situ*

In order to confirm our findings that low cell numbers on histology corresponded to poor clinical responses we performed correlation analysis between these two parameters. Figure 3-7 shows that there is a significant correlation for T cell number and clinical score in both young and old individuals (CD3: Spearman's correlation coefficient = 0.793, P=0.0007 and CD4: Spearman's correlation coefficient = 0.748, P=0.002). Two old individuals had high clinical scores compared to the rest of the subjects in their age group and were found to have correspondingly higher T cell numbers on histology. This further supports our findings that the clinical score is linked to the number of T cells accumulating within the skin response for Candida skin test injections.

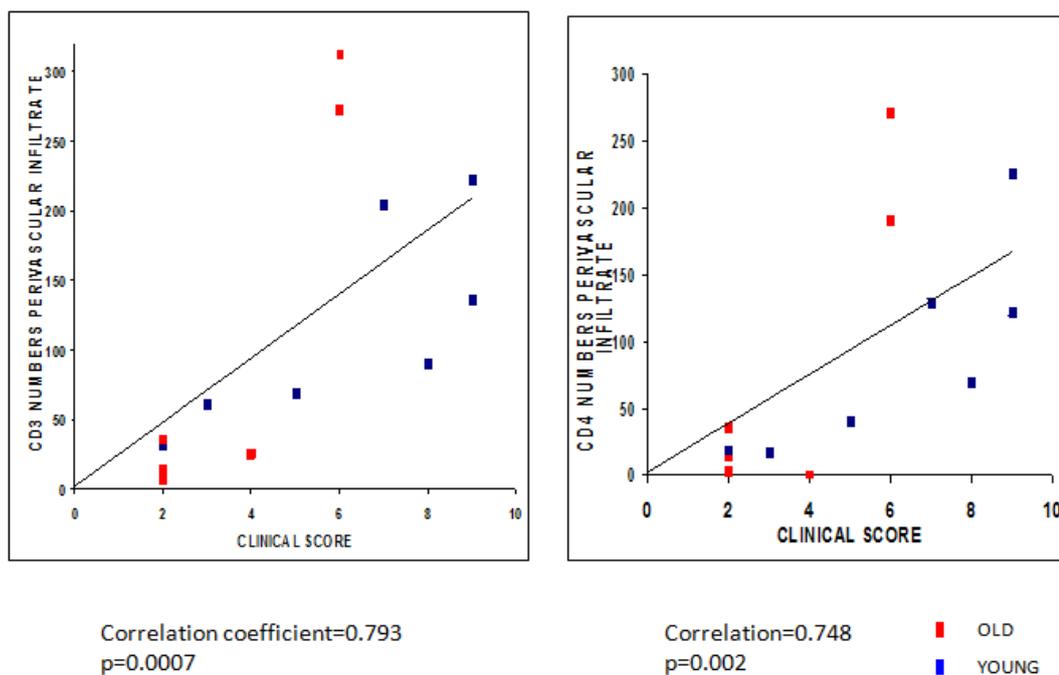


Figure 3-7: T cell number found on histology of Candida skin test injected skin correlates to the clinical score in both young and old.

The number of CD3⁺ and CD4⁺ T cells counted perivascularly was correlated to clinical score (n=7 young, 6 old). There was a significant correlation for T cell number and clinical score in both young and old individuals (CD3: Spearman's correlation coefficient = 0.793, P=0.0007 and CD4: Spearman's correlation coefficient = 0.748, P=0.002). The two old individuals with high clinical scores had correspondingly higher T cell numbers on histology.

3.3. Discussion

In order to study young versus old cutaneous immune reactions our group has developed a skin model that allows assessment of the DTH response as it evolves in the skin in terms of the clinical and histological characteristics of the immune response. Previous studies investigating the changes to the immune system with age have focussed on peripheral blood samples. Our skin model has allowed us to determine the *in vivo* kinetics of T cell accumulation in young healthy skin during a response to the Mantoux test and candida skin test. We were then able to compare the findings in young skin to those seen in old subjects to determine and investigate crucial differences. Moreover by investigating PBMCs from young and old

individuals in parallel to skin we can directly compare localised skin immunity to that of the peripheral blood compartment. The suction blister technique in addition allows assessment of function and antigen specificity of the T cells in the response. Our results indicate that older subjects have decreased ability to mount a clinical response to recall antigen challenge in the skin, which is also associated with a decreased antigen-specific CD4⁺ T cell accumulation. However this study demonstrates for the first time that in the old volunteer cohort this does not equate to decreased immunity, as the numbers of antigen-specific T cells in peripheral blood and their ability to respond to antigen is not different in subjects in both age groups. This points to a skin specific rather than a general decline in cell-mediated immunity and suggests that the DTH response is not a good test for the presence of immunity to a particular antigen in the old.

The DTH response to recall antigen in the skin has been used as a marker of systemic immunity (Takahashi et al., 2003; Almeida et al., 2001). Numerous studies have documented age-associated changes in different types of immune cells including T cells, B cells and antigen presenting cells that may result in defective immunity with ageing (Grubeck-Loebenstein and Wick, 2002). In particular, changes in the memory T cell pool with ageing which include large clones of terminally differentiated memory T cell which are less able to divide and persist *in vivo*, may result in the loss of immune memory (Fletcher et al., 2005). However, the equal ability of young and old PBMCs to proliferate in response to both PPD and candida antigens suggests that healthy old individuals used in this study were not significantly immunocompromised. It is possible that PBMC proliferation is not totally representative of an individuals' ability to respond to infection. However, previous studies investigating global parameters of immunity in healthy old individuals also indicate that the decline in immunity is modest in this population (Castle et al., 1990). This data is further supported by the prior observation that anergic old individuals have been able to mount an adequate secondary immune response following infection with acute TB (Creditor et al., 1988). A negative skin test response to cutaneous challenge with antigen in the old is therefore not necessarily reflective of globally reduced immunity.

In view of the dissociation between clinical and cellular responses seen in the young the aim of this study was to establish whether the reduced clinical response in the skin in the old was truly reflective of reduced lymphocytic responses at the site of the response. We used Candida skin test antigen instead of the MT to ascertain that the differences observed in the clinical and histological response in the skin in young and old subjects were attributable to changes in the cutaneous immune system with age and not to different rates of vaccination with BCG. A reduction in lymphocyte numbers was seen at 1, 3 and 7 days after skin testing with Candin. Importantly, a positive correlation between clinical response and numbers of cells within perivascular infiltrates for both young and old individuals was demonstrated with reduced numbers of lymphocytes in all individuals who failed to develop a clinical response to the injection of antigen. This data is important as it further indicates that the clinical DTH responses in the skin are dependent on T lymphocyte responses at the site. We have however only studied lymphocytic responses at a set number of time points. It remains possible that lymphocyte numbers peak after day 7 in the old due to a slower response time, although this is unlikely as there is no detectable clinical response in the old past day 7.

In old subjects, reduced proliferative capacity of T lymphocytes present in the skin may be responsible for reduced cellular response. In particular, a lack of cellular proliferation at the site may be responsible for the reduced expansion of the CD4⁺ T lymphocyte population in the skin. Published data from human studies has indicated that a significant proportion of the expansion of lymphocyte numbers within the skin is mediated by localised T cell proliferation (Reed et al., 2004). This model is further supported by the results from this study that show that there is a marked increase in the percentage of Ki67⁺ T cells at day 3 and day 7 in the skin. Previous work in our group has shown no detectable increase in Ki67⁺ cells within the peripheral blood population during a DTH response in the skin (Dr Katie Lacy, unpublished data). Moreover the similar percentages of CD3⁺Ki67⁺ cells in both old and young confirm that the difference in T cell numbers seen is not due to decreased T cell proliferation in old skin.

In this study we have demonstrated a marked reduction in the percentage of antigen-specific cells present in the skin during the Candin skin test in the old compared with the young. The Candin skin test is thought to induce Th1 type lymphocytic responses in the skin with the production of predominantly IFN- γ . It is also possible that the observed reduction in antigen specific cells in the old may not be due to a reduction in antigen-specific cells *per se* but rather an inherent inability of these cells to produce IFN- γ . Although no difference in IFN- γ production was found for peripheral blood CD4⁺ T lymphocytes in the young and old groups, the skin microenvironment in the old could render T lymphocytes refractory to restimulation with antigen and also affect their ability to produce IFN- γ . It is also possible that the old skin suction blister cells may have contained reduced numbers or defective antigen presenting cells, required for restimulation of T cells by the antigen in our *in vitro* model. Further investigation, using autologous irradiated APCs from PBMCs added to the skin suction blister cells, would help to exclude absent or defective antigen presenting cells within the skin suction blister cell population as a reason for the apparent reduction in antigen specific cells.

Low percentages of antigen specific cells were identified during the course of the response to the Candin skin test in the young. It was not possible to use the same reagent as used for the skin test for *in vitro* work. This was thought to be attributable to the presence of preservative agents within the skin test that were toxic to the SB cells *in vitro*. It is therefore possible that the agent used to test for *in vitro* responses did not contain the full range of epitopes that were present in the skin test resulting in an underestimation of the actual number of antigen specific cells present. Previous studies have indicated that intracellular cytokine staining only detects between 30 to 90% of tetramer positive cells (Hislop et al., 2001) and it is therefore possible that our data represents an underestimate of the percentage of antigen specific cells present in the skin. Of interest, other studies have found that less than 1% of cells infiltrating the skin are antigen specific (Kalish and Johnson, 1990).

Another possibility to explain the presence of reduced T cell numbers in old skin is that T cells in old subjects are migrating into skin in equal numbers to young initially

but a larger percentage of them is undergoing apoptosis early on in the response compared to the young. Although assessment of apoptosis rates in young and old T cells *in situ* was not performed in this study, the fact that significantly reduced numbers of CD4⁺ T cells are noticed as early as 24 hours post injection in the old subjects compared to the young, makes it unlikely that increased apoptosis rates account for the significantly reduced numbers of CD4⁺ T cells found in old skin. It is more likely that T cells are not getting into old skin and this hypothesis will be explored in Chapter 4.

Chapter 4. Impaired memory T cell-endothelial interaction affects transmigration into ageing skin

4.1. Introduction

In order for a localised immune response to develop, circulating T cells need to migrate to the tissue where the microbes/antigen is found. In Chapter 3 we showed that there are reduced CD4⁺ T cell numbers in the skin, which is noticed as early as 24 hours post antigen injection and lasts throughout the course of the response in old subjects. As circulating antigen-specific T cells are not decreased in number or capacity to respond to antigen in the old, we hypothesised that T lymphocytes might be impaired in their capacity to migrate into the skin following antigen injection. This chapter studies the mechanism of T cell-endothelial interactions during a secondary memory cutaneous immune response *in vivo*.

Skin homing memory T lymphocytes express CLA which binds E-selectin expressed by dermal microvascular endothelium (Berg et al., 1991) and this interaction leads to rolling of the T cell on the endothelium. Subsequently Cd11a and VLA-4 ($\alpha_4\beta_1$) on T lymphocytes bind ICAM-1 and VCAM-1 on the endothelium respectively. Chemokines on endothelial surfaces signal to integrins CD11a and VLA-4 on the rolling lymphocyte to activate them. CCR4 is a chemokine receptor for skin homing of T cells as it allows binding to its agonist thymus and activation-regulated chemokine, TARC, secreted within inflamed skin tissue (Imai et al., 1997). We hypothesised that the migration process was impaired in old subjects ie either circulating T cells in old subjects were deficient in their expression of adhesion molecules and/or endothelium in old skin was defective in its expression of adhesion molecules.

The endothelial cells (ECs) that line the postcapillary venules are responsible for local recruitment of T lymphocytes into the skin. Flowing blood quickly dislodges lymphocytes that touch the vessel wall. Endothelial cells that line the high endothelial venules in lymph nodes or Peyer's patches constitutively express

adhesion molecules which support the homing of naïve lymphocytes, whereas endothelial cells in the dermis permit little or no lymphocyte binding unless they are exposed to inflammatory mediators. The cutaneous endothelium therefore only solicits the entry of T lymphocytes when faced with danger signals and this is a homeostatic mechanism to protect skin and other organs from unnecessary inflammation. In this chapter we also explore the importance of endothelial activation in cutaneous T cell transmigration using *in vitro* migration assays across both activated and non-activated endothelium.

4.2. Expression of skin homing receptors on CD4⁺ T cells is not reduced during ageing

In view of the reduced number of T cells found at the site of a cutaneous immune response in skin from old subjects one hypothesis is that the expression of adhesion molecules on circulating leucocytes in old individuals was reduced. We therefore assessed expression of CLA, CCR4 and CD11a on the leucocyte cell surface in PBMCs from young and old subjects.

4.2.1. CLA expression on T lymphocytes in peripheral blood.

CLA expression on circulating T cells in the young and old was investigated in order to determine whether decreased CLA expression could be responsible for the observed reduced lymphocytic response in the skin. CLA⁺ lymphocytes bind to E-selectin on inflamed skin.

PBMCs from young and old individuals were stained for CD3, CD4 and CLA and analysed using flow cytometry (Figure 4-1). No significant difference was identified between the groups for percentage CLA expression on total CD4⁺ T lymphocytes (Mann Whitney $p=0.054$) although a small increase in the expression of CLA on the total CD3⁺ lymphocyte population was seen in the old (Mann Whitney: $P=0.01$, Figure 4-1).

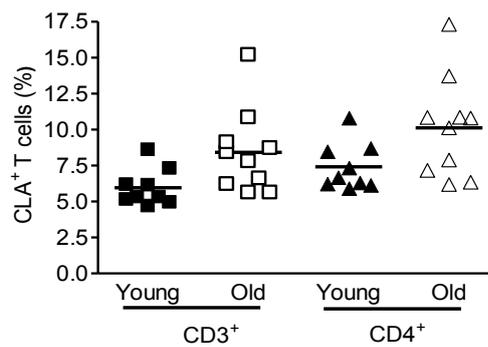


Figure 4-1: Expression of CLA on circulating CD3⁺ and CD4⁺ T lymphocytes in the young and old.

Expression of old peripheral blood leukocytes' skin homing receptor CLA not reduced. Immunostaining of peripheral blood mononuclear cells from old and young with antibodies to CLA was analysed by flow cytometry. The percentage CLA expression amongst CD3 and CD4 subsets was similar for young and old (n=9 young, 10 old, each symbol represents an individual and lines indicate the mean).

4.2.2. CCR4 expression

We next investigated CCR4 expression on skin homing (CLA⁺) and non-skin homing (CLA⁻) CD4⁺ T lymphocytes isolated from the peripheral blood in the young and old by flow cytometry (Figure 4-2). In both groups, CCR4 expression was found to be significantly higher on the CLA⁺ T cells (Mean old: 70.37% vs 22.45%; mean young: 60.65% vs 13.7% for CLA⁺ and CLA⁻ cells respectively). No difference was found for percentage of CD4⁺ T cells expressing CCR4 in young and old groups (Mann Whitney CLA⁺ cells: P=0.15, CLA⁻ cells P=0.2) (Figure 4-2A). The level of expression of this chemokine receptor (MFI) is not reduced but actually increased on the old T cells (Figure 4-2B, Mann Whitney P=0.03), further indicating that the lack of infiltration into the skin is does not result from decreased chemokine receptor expression.

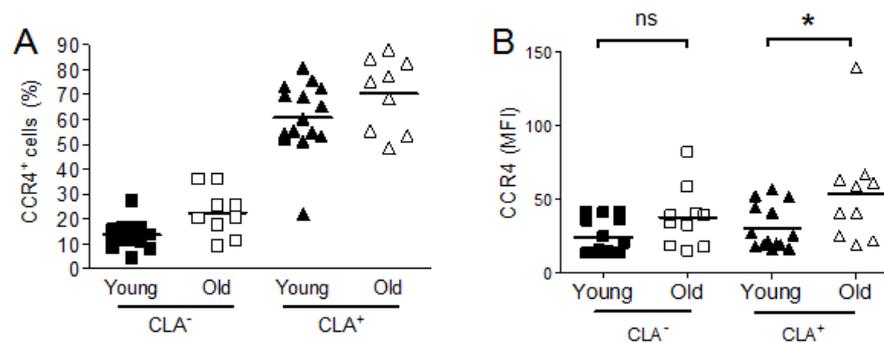


Figure 4-2: Expression of CCR4 on CLA⁺ and CLA⁻ CD4⁺ T lymphocytes in the young and old.

Immunostaining of peripheral blood mononuclear cells from old and young with antibodies to CCR4 was analysed by flow cytometry. CCR4 expression was compared on both CLA⁺ and CLA⁻ CD4⁺ T lymphocytes isolated from the peripheral blood in the young and old. In both groups, CCR4 expression was found to be significantly higher on the CLA⁺ T cells. No difference was found for CCR4 expression between the young and old groups (n=15young, 9 old, each symbol represents an individual and lines indicate the mean). The level of expression of this chemokine receptor is not reduced but actually increased on the old T cells (MFI, Mann-Whitney P=0.03).

4.2.3. CD11a (LFA-1) expression on peripheral blood T lymphocytes.

After selectin mediated rolling on the endothelium, lymphocytes attach firmly to the endothelium through the adhesion of beta 2 integrins, including LFA-1, expressed on the lymphocyte cell surface and adhesion molecules such as ICAM expressed on the endothelium. The expression of CD11a, a subunit of LFA-1, on both CLA⁺ and CLA⁻ circulating peripheral blood CD4⁺ T lymphocytes was compared in young and old using flow cytometric analysis. (Figure 4-3). No difference was found for CD11a expression between young and old CLA positive (Mann Whitney p=0.21) and negative cells (Mann Whitney p=0.48) (Figure 4-3).

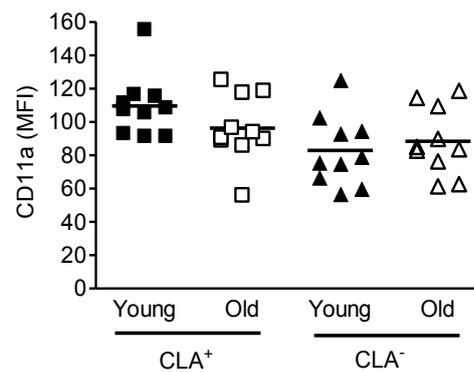


Figure 4-3: Expression of CD11a on CLA⁺ and CLA⁻ CD4⁺ T lymphocytes in the young and old.

Immunostaining of peripheral blood mononuclear cells from old and young with antibodies to CD11a was analysed by flow cytometry. No difference was found for CD11a expression (MFI) between young and old CLA positive and negative cells (n=10 young, 9 old), each symbol represents an individual and lines indicate the mean)

In conclusion the decreased migration of T cells into the skin after antigen challenge in old subjects is therefore not due to the decreased expression of key cell surface molecules on circulating CD4⁺ T cells.

4.3. Capacity of CD4⁺ T cells to migrate is not reduced during ageing

To further investigate the physical capacity of T cells from peripheral blood of old subjects for migration across endothelium, we used phase-contrast microscopy to assess the migration of these cells across primary human dermal microvascular endothelial cells (HDMEC) *in vitro*.

4.3.1. Endothelial monolayer culture and activation and control studies using pre-activated T cells for migration

The HDMEC monolayer was grown to confluence on 96 well plates as described in Chapter 2 Materials and Methods. It was then pre-activated for 24 hours with TNF- α and IFN- γ to stimulate E-selectin, ICAM-1 and VCAM-1 expression (Swerlick et al.,

1992). Adhesion molecule expression and CD4 cell migration was minimal when using unactivated endothelium compared to activated endothelium (Figure 4-4a-c). Control assays using stimulated (IL-2 overnight exposure-5ng/ml and plate bound CD3-OKT3 0.5 μ l/ml and CD28-1 μ g/ml) versus unstimulated T cells were carried out simultaneously. CD4⁺ T cell stimulation prior to migration assay made no difference to % total migration rates (Figure 4-4d).

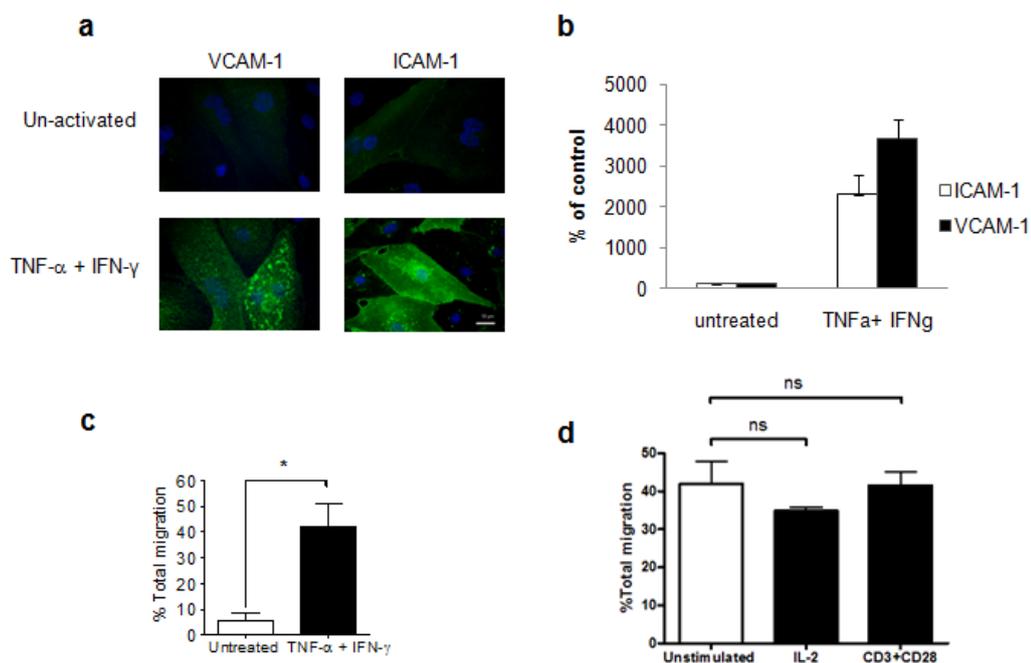


Figure 4-4: CD4⁺ T cell migration across dermal microvascular endothelium assays.

Activation of HDMEC is necessary for migration assays. HDMEC was grown to confluence in 96-well plates and endothelial monolayers were activated with TNF- α (110,000 U/ml) and IFN- γ (100 U/ml) for 24 hours prior to addition of T cells. **(a)** Photomicrographs of immunostaining of HDMEC monolayer for VCAM-1 and ICAM-1 (green) on unactivated (top panel) and activated (bottom panel) endothelium (Scale bar=10 μ m). **(b)** Graph showing ICAM-1 and VCAM-1 upregulation upon HDMEC activation. **(c)** Bar graph showing increased total CD4⁺ T cell migration across stimulated HDMEC compared to unstimulated endothelium. **(d)** Bar graph showing similar levels of stimulated (IL-2 or plate-bound CD3 and CD28 exposure overnight) versus non-stimulated T cell migration across activated endothelium.

4.3.2. CD4⁺ T cell migration

Unactivated CD4⁺ T cells from young and old subjects were incubated with activated HDMEC for 4 hours and video recordings of the culture wells were taken using phase-contrast visualisation and time-lapse microscopy. We found that CD4⁺ T cells from old subjects migrated more efficiently than those from young subjects across activated endothelium (Figure 4-5).

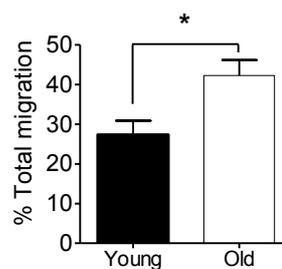


Figure 4-5: Comparison of young and old subject peripheral CD4⁺ T cell migration across dermal endothelial monolayer *in vitro*

To evaluate the level of migration, CD4⁺ T cells isolated from young and old volunteers were plated onto confluent dermal endothelial monolayers for 4 hours at 37°C and 5%CO₂. (n=at least 6 wells per experimental condition, n=3 young, 3 old). The percentage total migration for old vs young CD4⁺T cells across stimulated endothelium was significantly different (Mann Whitney *P=0.02).

Furthermore, this migration was blocked by the addition of anti-ICAM-1 and anti-VCAM-1 antibodies, (Figure 4-6) supporting the direct requirement for these molecules in the migration process (McHale et al., 1999). This confirmed that the defect in the accumulation of antigen-specific CD4⁺ T cells in the skin of old donors after antigen challenge was not due to alterations in the migration capacity of the T cells themselves.

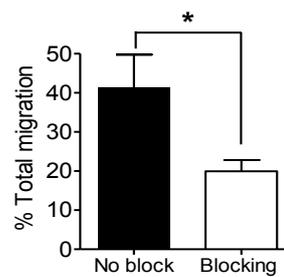


Figure 4-6: Effect of adding blocking antibodies on CD4⁺ T lymphocyte migration.

Graph showing effect of adding blocking antibodies on CD4⁺ lymphocyte migration to ICAM-1, VCAM-1 and E-selectin on total CD4⁺ lymphocyte migration across endothelial monolayers *in vitro* (n=3; at least 6 wells per experimental condition, Paired t test *P=0.04).

4.4. Reduced expression of adhesion molecules by dermal endothelium during a DTH response in old subjects.

Since we found no defect in the migratory capacity of and adhesion molecule expression of circulating T cells from old subjects compared to young we next hypothesised that the lack of T cell infiltration into the skin in these individuals was due to defective endothelial cell activation.

The activation of dermal blood vessel endothelial cells induces expression of E-selectin, ICAM-1 and VCAM-1, the ligands to CLA, LFA-1 and VLA-4 respectively on T cells (Berg et al., 1991; Berlin et al., 1995; Alon et al., 1995). The interaction of these molecules with their counter receptors is essential for the rolling, adhesion, and extra-vasation of the T cells from blood into the skin (von Andrian and Mackay, 2000). Skin biopsies from day 3 post-candida skin test injections from young and old skin were stained for these adhesion molecules in conjunction with CD31 to highlight the dermal endothelial vessels (Newman, 1997).

4.4.1. E-selectin expression

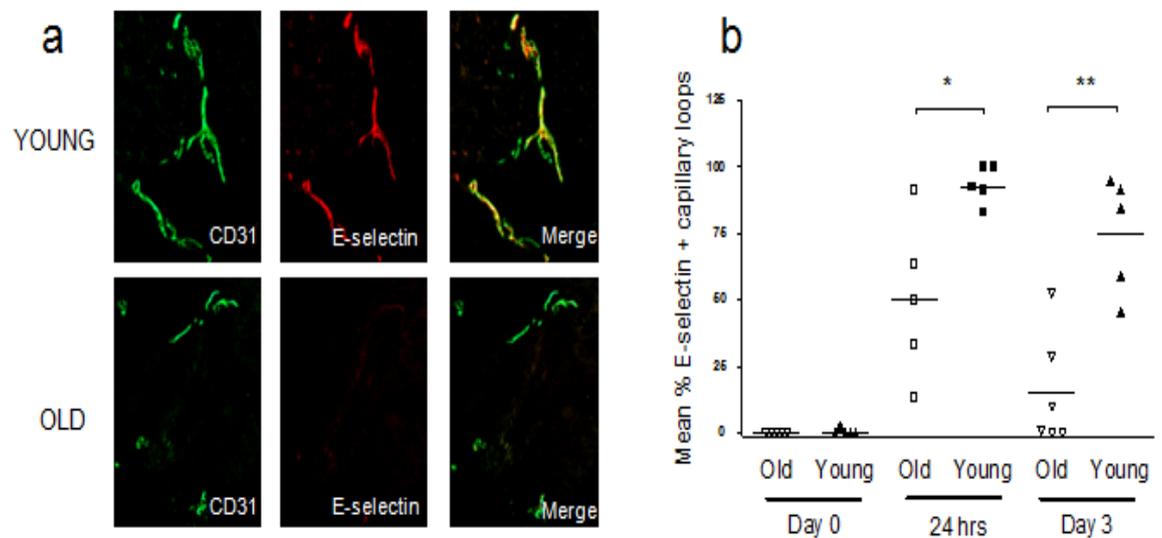


Figure 4-7: Dermal endothelium in old individuals shows reduced expression of adhesion molecule E-selectin.

(a) Dermal endothelium in old individuals shows reduced expression of adhesion molecule E-selectin. Histological sections ($6\mu\text{m}$) from skin injected with candida ($n=5$ young, 6 old) at day 3 post injection were stained with antibody to CD31 to highlight dermal capillary loops and endothelial adhesion molecule E-selectin. Original magnification: $\times 200$. Double immunofluorescence staining of representative biopsies (young and old) shows CD31 (green) and E-selectin (red). Capillary loops expressing adhesion molecules appear yellow (merge). (b) Graph shows percentage of capillary loops expressing E-selectin at time-point 0, 24hrs and Day 3 post candida skin injection; each symbol represents an individual and lines indicate the mean (* $P=0.01$ ** $P=0.008$, young vs old Mann Whitney test).

E-selectin is not constitutively expressed on endothelium at Day 0 (Figure 4-7b) although expression was noted to increase at 24hrs post candida injection with 93% of dermal capillary loops expressing E-selectin in skin from young subjects. There was a significant reduction in the proportion of capillary loops that expressed E-selectin in old skin at 24hrs (Figure 4-7b) and at day 3 (Mann Whitney $P=0.008$) (Figure 4-7a,b). Therefore the first crucial steps of tethering and rolling of the

lymphocyte on dermal endothelium would be impaired in old skin not expressing E-selectin post-candida injection and would explain the reduced number of T cells found in skin from old subjects injected with candida.

4.4.2. VCAM-1 expression

VCAM-1(CD106) is an endothelial adhesion molecule belonging to the immunoglobulin superfamily. This is absent on resting endothelial cells and its expression is induced by cytokines. It interacts with VLA-4, expressed by leucocytes to enable the steps of both tethering and rolling (although this is less efficient than the tethering and rolling mediated by the selectin group of adhesion molecules) and also arrest and firm binding of T cell to endothelium. Dermal endothelial cells from the site of injection in old subjects also showed significantly reduced VCAM-1 expression at day 3 post Candida antigen injection compared to young controls (Mann Whitney $P < 0.015$, Figure 4-8).

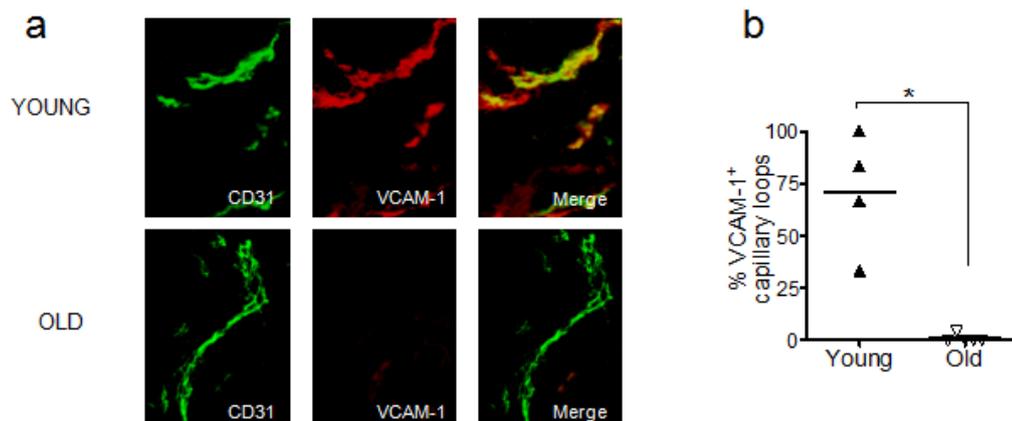


Figure 4-8: Dermal endothelium in old individuals shows reduced expression of adhesion molecule VCAM-1.

(a) Histological sections (6 μ m) from skin injected with candida (n=5 young, 6 old) at day 3 post injection were stained with antibody to CD31 to highlight dermal capillary loops and endothelial adhesion molecule VCAM-1. Original magnification: x200. Representative staining of CD31 (green) and VCAM-1 (red) in young and old skin on day 3 following candida injection. (b) Graph shows

percentage of capillary loops expressing VCAM-1; each symbol represents an individual and lines indicate the mean (Mann Whitney *P=0.015, young vs old).

4.4.3. ICAM-1 expression

The expression of ICAM-1 was found on all dermal capillary loops throughout skin sections from old subjects but the intensity of staining was reduced in skin sections from 6 out of 6 old compared to young donors (Figure 4-9) as visualised independently by 2 investigators. The defective accumulation of dermal T cells during the response to recall antigen in the skin in old donors is therefore linked to the reduced expression of adhesion molecules that are essential for T cell migration on the endothelium of dermal vessels.

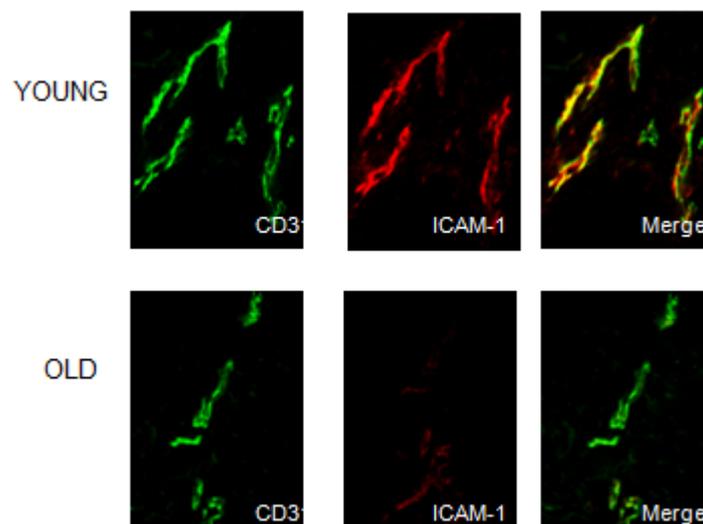


Figure 4-9: Dermal endothelium in old individuals shows reduced expression of adhesion molecule ICAM-1.

Histological sections (6 μ m) from skin injected with candida (n=5 young, 6 old) at day 3 post injection were stained with antibody to CD31 to highlight dermal capillary loops and endothelial adhesion molecule ICAM-1. Original magnification: x200. Double immunofluorescence staining of representative biopsies shows CD31 (green) and ICAM (red).

4.5. Endothelial activation using TNF- α and IFN- γ in young and old skin explant culture

To determine if the reduction in endothelial adhesion molecule expression in the skin of old donors was caused by an intrinsic defect in the endothelium of these subject or due to a lack of activation by pro-inflammatory cytokines we obtained biopsies from normal young and old skin (n=3 young, 3 old) and incubated the tissue with medium containing TNF- α and IFN- γ or medium alone for 16 hours (Figure 4-10).

We found that there was an increase in the intensity of E-selectin, ICAM-1 and VCAM-1 stains on the endothelium in the presence of TNF- α and IFN- γ in all the old and young subjects (as assessed independently by 2 investigators). A representative VCAM-1 staining profile is shown in Figure 4-10. Similar results were obtained for E-selectin and ICAM-1 (data not shown). This suggests old endothelium is able to express adhesion molecules in the presence of appropriate cytokines. We next investigated whether the number or surface area of capillary loops in the skin was reduced in the old.

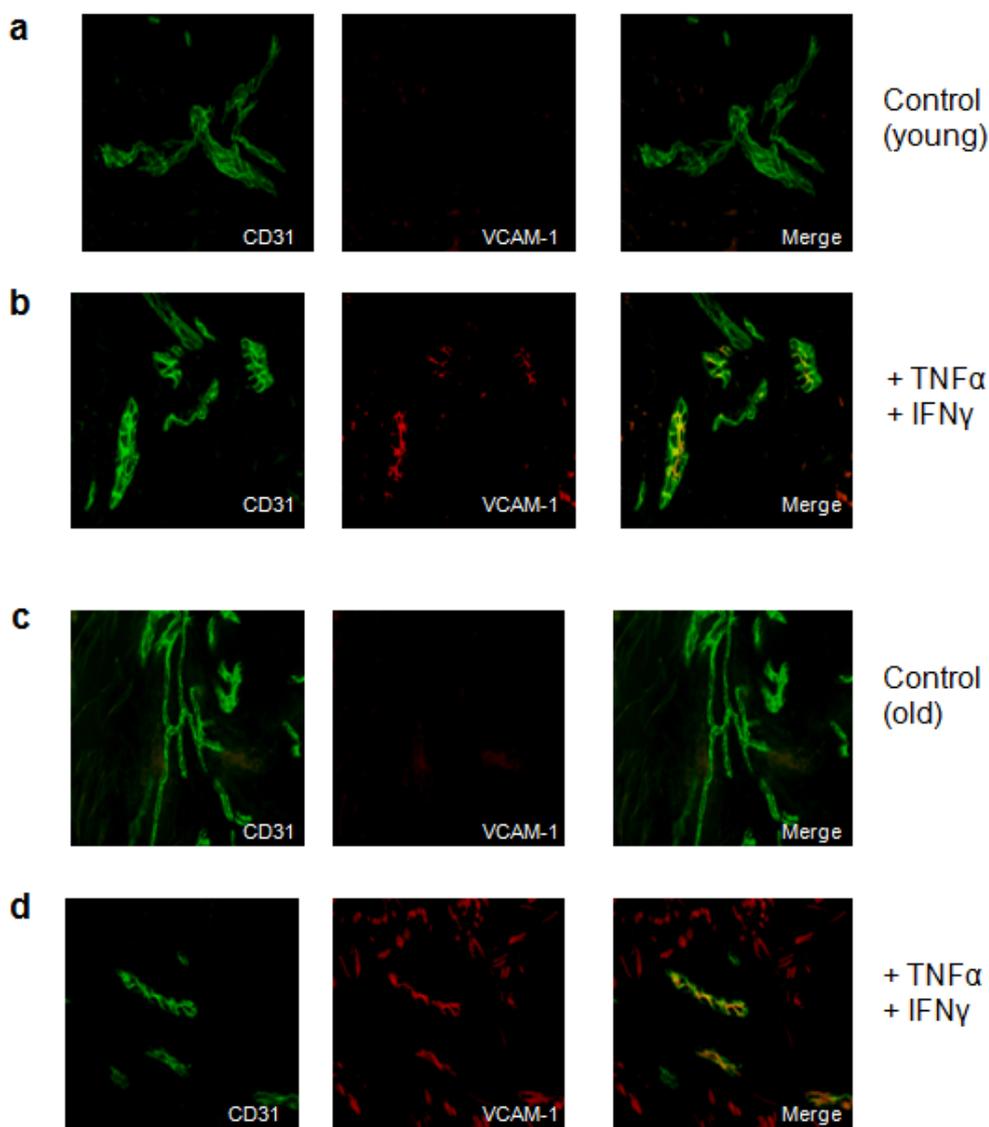


Figure 4-10: Old endothelium can upregulate adhesion molecules *in vitro*.

Young and old normal skin explants were cultured in RPMI+10% human serum or in RPMI+10% human serum supplemented with TNF- α (110,000 U/ml) and IFN- γ (100 U/ml) for 16 hours at 37°C and 5% CO₂. Histological sections from skin explants (n=3 young, 3 old) were stained with antibodies to CD31 and E-selectin, ICAM-1 and VCAM-1. Representative VCAM-1 staining is shown for young (a,b) and old (c, d).

4.6. Surface area of endothelial cells in young and old dermis

Structural changes that occur in the skin with ageing, including atrophy of the epidermis or dermal connective tissues might mean that the vascular network in elderly skin is also affected in terms of number of dermal capillary loops and total surface area of dermal endothelium across which T cell transmigration can occur. This could explain reduced transmigration of T cells into old skin during an immune response. Moreover the vascular endothelium is a source of numerous cytokines such as IL-1 α and β , IL-6, IL-8, G and GM-CSF, gamma IP-10, IFN- γ , MCP-1,2, and 3, M-CSF, MIF, MIP-2, OSM, PDGF, and TNF- α and reduced numbers or surface area of endothelial cells could also account for reduced inflammation seen in old skin. To explore this possibility, we counted CD31⁺ endothelial loops in micrographs of young and old skin (n=4 young, 6 old). We found that there was no significant difference in the number of dermal endothelial loops (Figure 4-11). We then measured the total surface area of dermal endothelial CD31⁺ loops in young and old skin using a surface area measurement tool on Image J (NIH Image 6.1; <http://rsb.info.nih.gov/nih-image>). We found no significant difference in the surface area of young and old dermal endothelial networks. Therefore the endothelium shows no defect in function or surface area in old subjects compared to young.

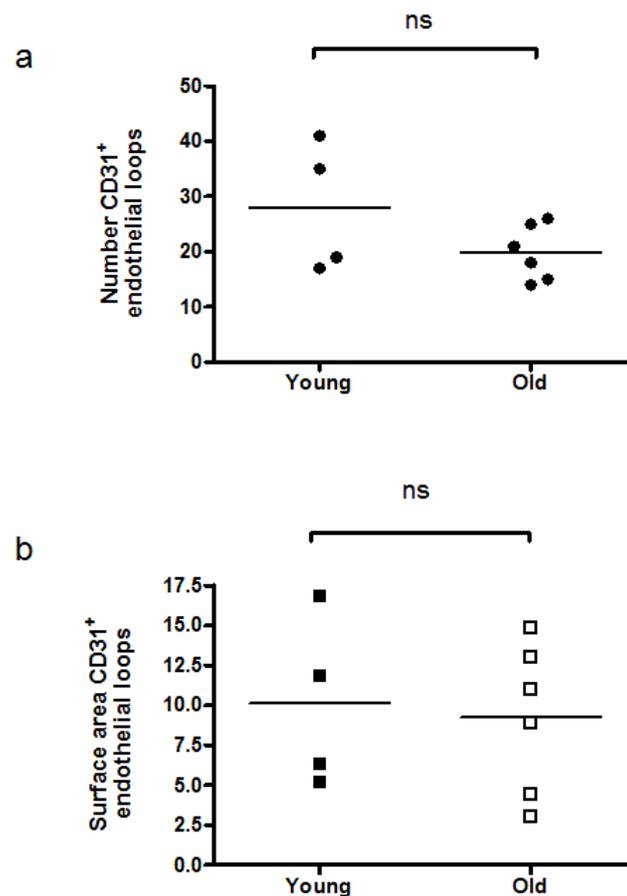


Figure 4-11: Dermal CD31⁺ endothelial loop surface area and number is similar in young and old skin.

(a) Graph shows numbers of CD31⁺ endothelial loops/ per micrograph (x100 original magnification) of young and old skin at day 3 post-candida injection. Each symbol represents an individual and lines indicate the mean (n=4 young , n=6 old) (b) Graph shows total measured surface area of CD31⁺ endothelial loops in young and old skin section at day 3 post candida injection measured in mm² using Image J software (micrographs x100 original magnification). Each symbol represents an individual and lines indicate the mean.

4.7. Discussion

In this chapter we have shown that old circulating memory T cells have all the migratory machinery required to effectively transmigrate across dermal endothelium. However there is much reduced expression of endothelial adhesion molecules, E-selectin, VCAM-1 and ICAM-1 during a secondary cutaneous immune responses in

old subjects. We have shown that these molecules are essential for T cell transmigration as blocking them in *in vitro* migration assays leads to significantly reduced migration of both young and old T cells across dermal endothelial monolayers *in vitro*. The lack of endothelial activation in skin from old subjects therefore leads to decreased transmigration of T cells into skin in the older individuals. This might account for the decreased immune response of elderly skin to infection and skin cancer (Diffey and Langtry, 2005; Laube, 2004).

Clark et al have recently (Clark et al., 2008) shown that in squamous cell carcinomas of the skin which are very common in the elderly and in immunosuppressed individuals, there is reduced local expression of E-selectin in blood vessels within and surrounding the tumour. Moreover the tumours contained few CLA⁺ T cells. This finding correlates with our observation of reduced expression of endothelial adhesion molecules in elderly individuals during the course of a secondary cutaneous immune response in the skin and also shows that problems with T cell migration into skin lead to cutaneous immunosuppression and consequent pathology.

The density of superficial capillary loops decreases with age in association with atrophy of the superficial dermis, however overall blood flux has been shown to increase with an expansion of the parallel vasculature in the deeper dermis (Kelly et al., 1995; Li et al., 2006). In one study, old individuals (60-74 years) were found to have a 66% reduction in capillary density on the volar (photoexposed) aspect of the forearm compared to a young cohort (Li et al., 2006). Similar findings have been demonstrated in mice (Vollmar et al., 2000). In our hands however we found no difference in the number or surface area of dermal endothelial loops between young and old skin sections (Figure 4-11). We also found no difference in the number of perivascular infiltrates in young and old skin tissue (data not shown, Dr Katie Lacy thesis). Our study uses only skin tissue from the same non-photoexposed skin site in both young and old cohorts. The changes others have noticed between young and old skin vasculature density might be attributed to different anatomical sites studied for young and old and also failing to factor in different degrees of photoexposure for the individuals and sites studied (Chung et al., 2002; Toyoda et al., 2001).

Some authors suggest endothelial dysfunction with ageing. Ageing has been shown to result in a decreased expression of eNOS, the constitutive isoform of nitric oxide (NO) synthetase, responsible for endothelium derived NO synthesis (Matsushita et al., 2001). NO is thought to be a potent inhibitor of cellular adhesion, in particular monocytes and leukocytes, by the inhibition of the expression of adhesion molecules such as VCAM-1 (Peng et al., 1998). A reduction in endothelial nitric oxide production in the old has been proposed as a possible mechanism for development of atherosclerosis (Matsushita et al., 2001). However, we have shown that endothelium in skin explants from old subjects is capable of upregulating expression of adhesion molecules to a similar extent as the young if exposed to TNF- α and IFN- γ *in vitro*. The concentration of TNF- α and IFN- γ used in the skin explants culture experiment were the same ones used to stimulate HDMEC *in vitro*. This is considerably higher than physiological concentrations of these cytokines during secondary cutaneous memory immune responses (see Chapter 5) and one might argue that if skin explants from old individuals are exposed to lower concentrations of TNF- α and IFN- γ they might not upregulate endothelial adhesion molecules to a similar extent as in the young. It is unlikely that lower doses would have caused perceptible upregulation of adhesion molecule expression as the *in vitro* concentrations of cytokines used, reflect careful titrations adopted in the case of HDMEC stimulation *in vitro*. Therefore because skin biopsies are thicker than HDMEC monolayers it is unlikely that lower rather than higher cytokine concentrations would be required. Also the situation *in vivo* is different to *in vitro* and one cannot extrapolate too much between the two, for example *in vivo* one finds a complex network of interacting cytokines as opposed to the two cytokines tested here so that one cannot achieve the *in vivo* settings *in vitro*. Also each 5 mm skin punch biopsy obtained from volunteers was bisected and the two halves placed in control/experimental solution containing cytokines and due to the small size of the biopsy there is no tissue left over to carry out titrations of the cytokine concentration in the media.

The clinical importance of leucocyte-endothelial adhesion cascade is demonstrated by the leucocyte adhesion deficiency syndrome, a genetic defect either in $\beta 2$ integrins (type 1) or in selectin ligands (type 2) where neutrophils cannot stop or roll,

respectively. As a consequence recurrent infections, particularly of the skin, is seen (Etzioni et al., 1992). Blocking T cell migration into skin has also been used therapeutically to control skin diseases such as psoriasis, which is associated with increased T cell migration into the dermis and epidermis. Efalizumab, which blocks CD11a from interacting with ICAM-1 is widely used in dermatological practice to treat psoriasis (Papp et al., 2001). It was shown to decrease numbers of epidermal and dermal T cells (Gottlieb et al., 2000). Similarly lack of endothelial adhesion molecule expression in old individuals may predispose ageing skin to decreased cutaneous immunosurveillance by T cells.

Therefore we have shown that T cell migration into cutaneous secondary memory immune responses in old subjects is impaired due to lack of expression of endothelial adhesion molecules. Moreover we have shown that the old endothelium *per se* is not defective but that the defect arises due to a lack of adequate activating signals within the skin microenvironment in old individuals. In the next two chapters we explore the mechanism of this defective skin conditioning in the old.

Chapter 5. The innate immunity and its effects on secondary memory cutaneous immune responses in ageing skin

5.1. Introduction

Secondary cutaneous immune responses are antigen specific, such that if an individual was never exposed to candida antigen before re-challenge with the candida skin test, a T cell infiltrate does not develop at the site of skin injection with antigen. However if an individual had been exposed to candida antigen in the past one would expect that the presence of antigen specific cells in their circulation would ensure the development of a secondary cutaneous immune response upon antigen injection in the skin. Therefore in the traditional model of secondary immunity the presence of functional antigen specific cells *per se* would seem to be enough for the generation of a secondary response to that particular antigen. We have shown however that although the majority of old individuals retain normal functional activity in their candida antigen specific memory T cell compartment in the blood, they are unable to develop a secondary response to candida at the site of antigen injection in the skin. We have also shown in Chapter 4 that there is inadequate endothelial activation in old skin during a secondary cutaneous immune response. In this case, it is possible that despite the adaptive arm of the immune system in the old being capable of effectively responding to antigen, in the absence of pro-inflammatory cytokine release within skin tissue, which we have shown is necessary for effective endothelial activation, there is no activation of the endothelium and memory T cells are unable to transmigrate into the site of antigen injection to generate an immune response.

TNF- α is essential for endothelial activation. It has been shown to upregulate expression of E-selectin, ICAM-1 and VCAM-1 40-80 fold on dermal microvascular endothelium *in vitro* (Johnson et al., 2006) and this has also been demonstrated in *in vivo* models of delayed type hypersensitivity in mice (McHale et al., 1999). Both IFN- γ and IL-6 have also been shown to upregulate adhesion molecule expression.

(Swerlick et al., 1992; Wung et al., 2005). We therefore hypothesised that there might be reduced levels of pro-inflammatory cytokines necessary for endothelial activation in skin from old subjects compared to young.

The macrophage is crucial for early pro-inflammatory cytokine production, including TNF- α , during a secondary memory cutaneous immune response. Macrophages initiate an effective immune response against microbes by recognising pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRPs) (Taylor et al 2005 901-944) such as TLRs. They are phagocytes and can kill most micro-organisms. By producing TNF- α and presenting antigens to T cells (Pozzi LA, 2071-2081), both macrophages and DCs induce the adaptive immune response leading to the expansion and differentiation of lymphocytes specific for invaders. We hypothesised therefore that there might be a defect in the number or function of macrophages and dendritic cells found in skin from old subjects.

5.2. Early inflammatory cytokine levels during cutaneous secondary memory immune responses

We measured the concentration of TNF- α , IFN- γ , IL-6 and IL-10 in blister fluid aspirated from blisters induced at Day 3 over the site of Candin injection (n=6 young, n=11 old). The cytokine concentrations were calculated using the BDTM Cytometric Bead Array (CBA) Cell Signalling Flex Set System (Figure 5-1). A significant reduction in the concentration of TNF- α , IFN- γ and IL-6 was found in the old following Candin injection compared with the young. Concentrations of IL-10 did not differ between young and old suggesting that the lack of inflammation in the old is not due to overproduction of the inhibitory cytokine IL-10. The observed differences in pro-inflammatory cytokine levels could also be due to the fact that old skin blisters more easily and therefore generates less trauma and cytokine release. However in parallel control experiments (performed in our group by Dr Katie Lacy), using saline injection in the young and old (n=3 both groups), lower levels of TNF- α were found in blister fluid from both old and young subjects after saline injection compared to injection of Candin, indicating that TNF- α production was induced by

the injection of antigen rather than by trauma alone. Therefore there is a true difference in pro-inflammatory cytokine levels in blister fluid post-candida injection in young and old skin.

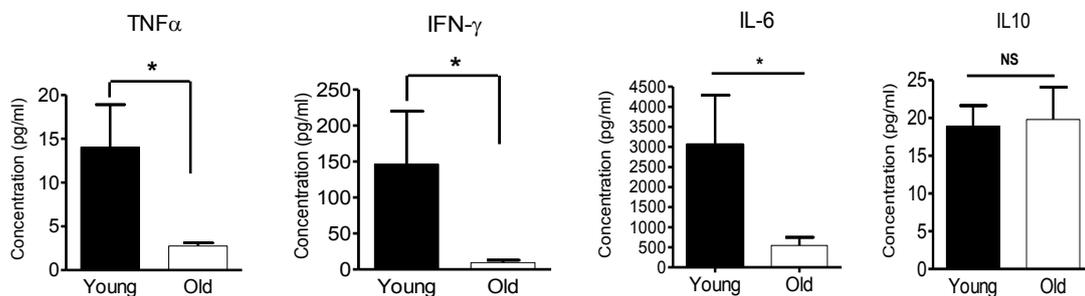


Figure 5-1: Early pro-inflammatory cytokines are reduced in old skin blisters.

Skin suction blisters were raised over the site of Candida antigen injection at day 3 (n=10 old, 6 young). Levels of TNF- α , IFN- γ , IL-10 and IL-6 were measured in the blister fluid using cytometric bead array. The assay was kindly performed by Dr John Curnow, University of Birmingham. The graph shows the mean \pm SEM of 6 experiments for Young and 10 experiments for old. A significant reduction in the level of TNF- α (Mann Whitney *P=0.01), IL-6 (Mann Whitney *P=0.03) and IFN- γ (Mann Whitney *P=0.01) was noted in the old group compared with the young. The level of IL-10 was not altered.

5.3. Distribution of TNF- α expression in skin tissue

In order to confirm the above findings and also to establish whether TNF- α was mainly being produced in the epidermis (suggesting a keratinocyte origin) or dermis (suggesting T lymphocyte or monocyte derived cell origin) we carried out immunohistochemical staining of young and old skin sections for TNF- α . Normal skin sections (n=3 young, 3 old), 24 hour (n=3 young, 3 old) and day 3 (n=3 young, 3 old) candida injected skin were stained using an indirect immunoperoxidase technique for TNF- α . There was pronounced TNF- α staining in the 24 hour and day 3 candida young skin sections but none in the old (Figure 5-2). Moreover the

keratinocytes did not seem to be expressing TNF- α in that all the TNF- α staining was limited to the dermis.

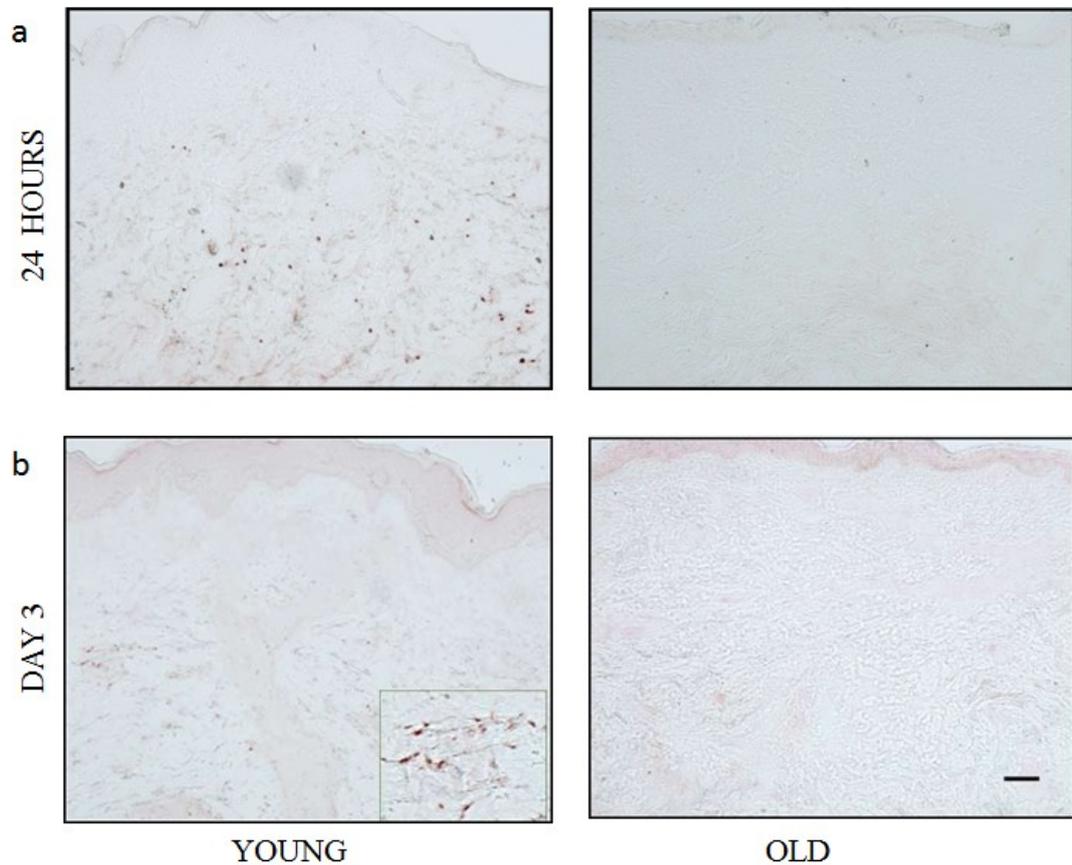


Figure 5-2: Lack of TNF- α expression in old skin compared to young.

TNF- α producing cells present in biopsies taken on day 3 following candida injection were identified using an indirect immunoperoxidase technique. The staining signal was developed using chromogen 3-amino-9-ethylcarbazole (Sigma-Aldrich). The TNF- α producing cells were limited to the dermis. Insert shows high power magnification of TNF- α positive cells in a perivascular cluster. Representative photomicrographs from 1 out of 5 experiments for young and old at 24 hours and day 3 post-candida injection are shown. Scale bar: 100 μ m.

5.4. Numbers of macrophages and dendritic cells in young and old skin by immunohistochemistry

Previous studies have shown that skin macrophages and DCs are mainly responsible for TNF- α and IL-6 secretion after cutaneous challenge with recall antigens while IFN- γ is secreted mainly by T lymphocytes (Chu et al., 1992). We have already shown (Chapter 3) that numbers of T lymphocytes are reduced in old skin and this might explain the lack of IFN- γ . We next wanted to assess if there was a reduction in numbers of macrophages or DCs to explain the observed decreased levels TNF- α , IFN- γ and IL-6. Many of the DC and macrophage markers overlap (Zaba et al., 2007). A recent study by Zaba *et al* has shown however that CD163 can be used to selectively stain for macrophages whilst CD11c is a good selective marker for DCs (Zaba et al., 2007).

We carried out single immunohistochemical staining for CD163 in normal, 24hour and day 3 post-Candida injection skin sections (n=5 young, 5 old at each time point studied) (Figure 5-3). There were similar numbers of CD163⁺ cells in skin from young and old subjects at all time-points tested. There was a trend for a higher number of CD163⁺ cells in skin sections from old subjects compared to the young. Single immunohistochemical staining for CD11c in normal, 24 hour and day 3 post-candida antigen skin test injection skin sections showed similar numbers in young and old skin at all time-points (Figure 5-4).

It is interesting to observe the differences in anatomical distribution of CD163⁺ macrophages and CD11c⁺ DCs. Macrophages were noted to be predominantly interstitial and present throughout the dermis at all time-points. CD11c⁺ DCs are found in the upper dermis in normal skin, at the junction between reticular and papillary dermis. At 24 hours and Day 3 post-candida skin test injection they are found in perivascular clusters throughout the dermis (Figure 5-4A) in a similar distribution to that seen for T cells (Chapter 3). This is ideal for cross-talk with infiltrating T cells to occur. The CD163⁺ macrophages form an outer border of cells surrounding the immediate perivascular clusters of cells (Figure 5-5A).

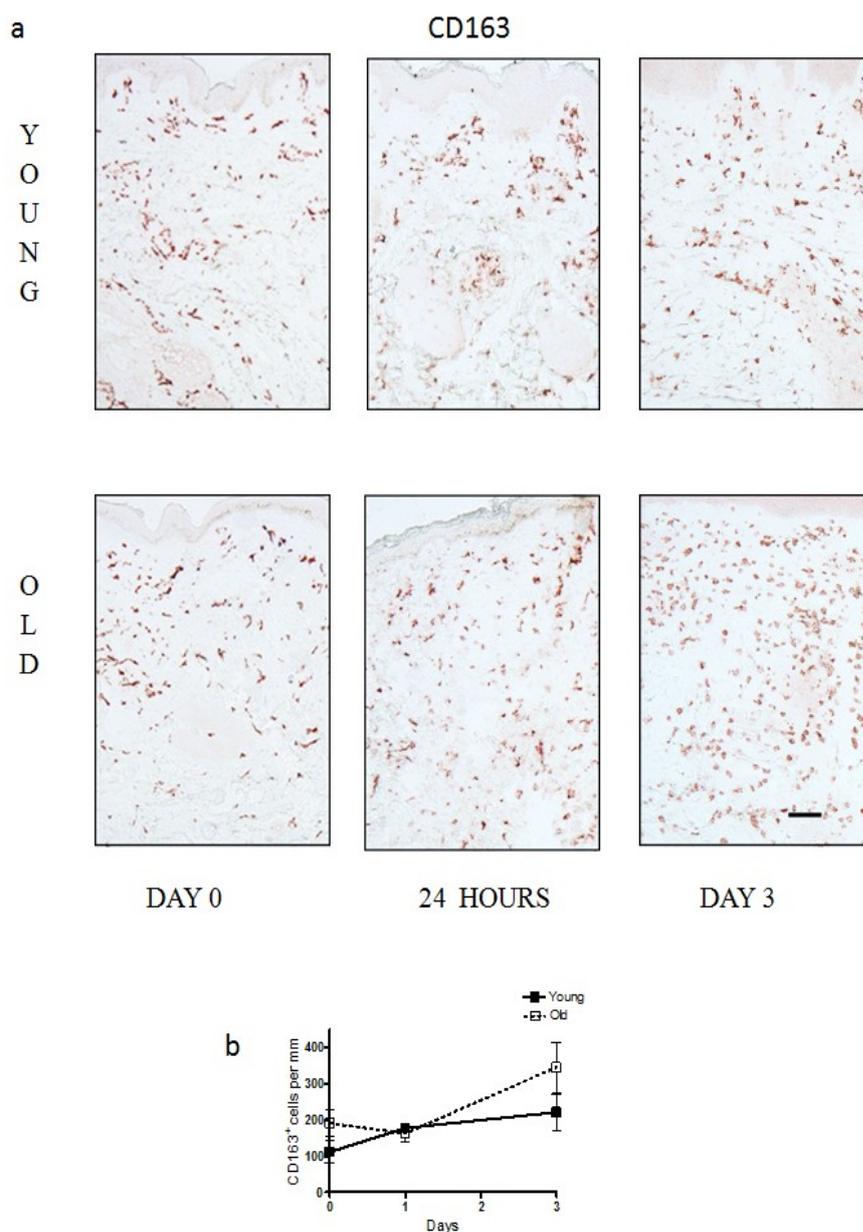


Figure 5-3: Similar number of CD163⁺ macrophages in skin from young and old subjects.

(a) The number of dermal CD163⁺ cells was determined by indirect immunoperoxidase staining of 6 μ m tissue sections. The staining signal was developed using chromagen 3-amino-9-ethylcarbazole (Sigma-Aldrich). Representative day 0, 24 hours and day 3 sections from young and old volunteers are shown. (b) Graph shows mean number of CD163⁺ macrophages per mm at all time points (n=5-7 per timepoint young and old, mean \pm SEM are plotted). Scale bar: 100 μ m.

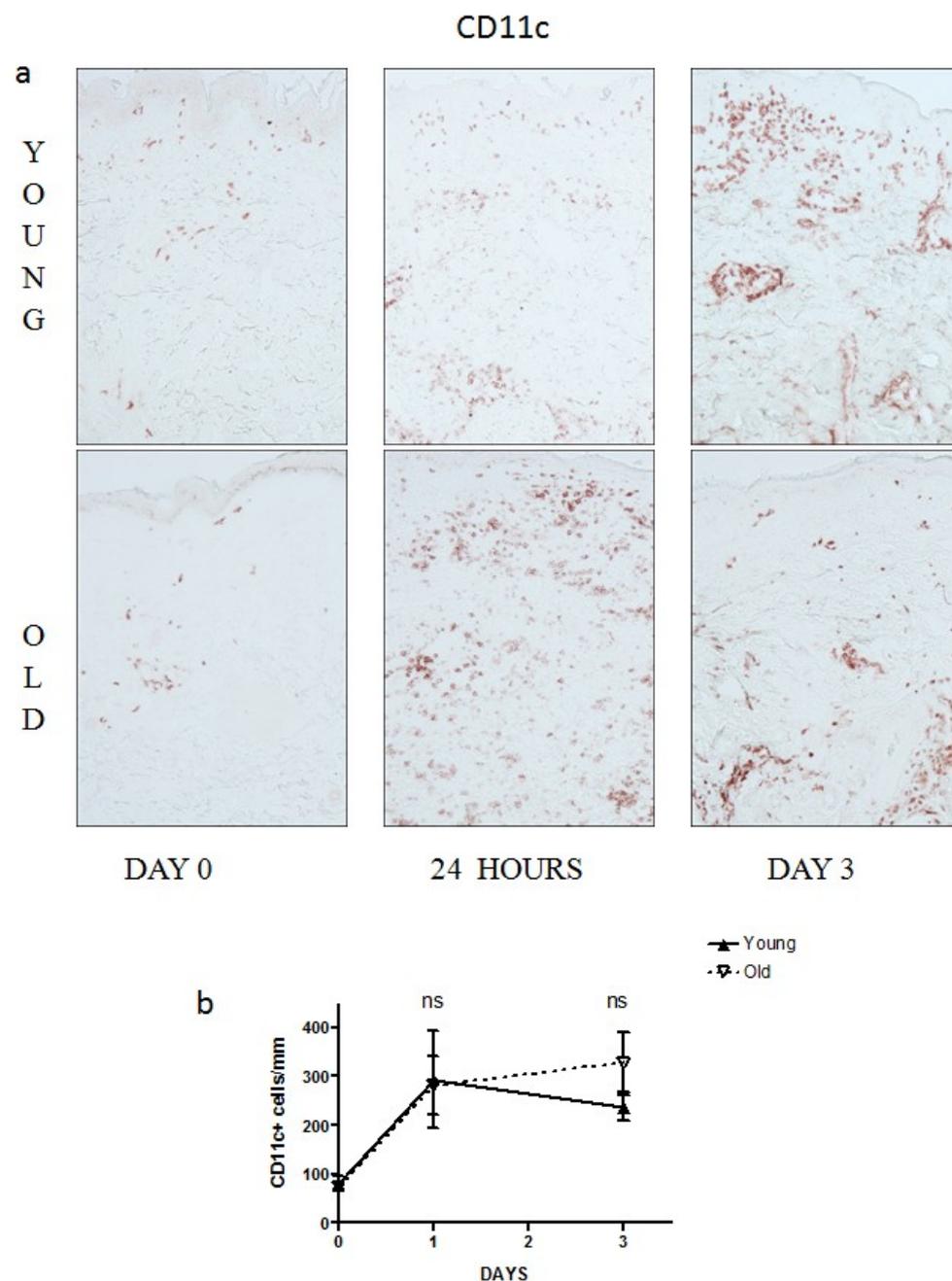


Figure 5-4: Similar number of CD11c⁺ dendritic cells in skin from young and old subjects.

(A) The number of dermal CD11c⁺ cells was determined by indirect immunoperoxidase staining of 6 μ m tissue sections. The staining signal was developed using chromogen 3-amino-9-ethylcarbazole (Sigma-Aldrich). Representative day 0, 24 hours and day 3 sections from young and old volunteers are shown. (B) Graph shows mean number of CD11c⁺ dendritic cells at all time points (n=5-7 per timepoint young and old, mean \pm SEM are plotted).

5.5. TNF- α expression by monocyte derived cells by immunohistochemical analysis of skin tissue

Having established that there were similar numbers of macrophages and DCs in young and old skin we hypothesised that there might be a defect in the production of TNF- α by either or both of these cell types during the secondary memory cutaneous response to candida injection. We therefore carried out double immunofluorescence staining for TNF- α and CD163 or CD11c (Figure 5-5 and Figure 5-6) on skin sections at 24hours and day 3 post-candida injection. Grey level images of single stains were obtained to improve optical resolution with the naked eye. Isotype control staining was carried out on young skin tissue injected with candida skin test antigen and at the same timepoint as experimental tissue and is shown for comparison with TNF- α staining. The double positive cells were clearly discernible and were counted using the manual count function on Adobe Photoshop CS3 (San Jose, California) in each perivascular infiltrate and were plotted as a percentage of total CD163⁺ cells in the infiltrate (see graph Figure 5-5b). This showed significantly reduced proportions of CD163⁺TNF- α ⁺ double positive cells in the old. The isotype control shows some background perivascular infiltrate autofluorescence in the green channel, which is also the TNF- α FITC channel (Figure 5-5a, greyscale images bottom panel). This does not affect the interpretation of the CD163⁺TNF- α ⁺ double positive staining cells as these cells are found on the periphery of the main perivascular infiltrate (Figure 5-5a, greyscale images left panel and merge panel). However in the case of CD11c⁺ cells which are part of the perivascular infiltrate (Figure 5-6, greyscale images left panel and merge panel), it is less clear if the perivascular cells are producing TNF- α or if there is autofluorescence as the intensity of greyscale staining of these cells appears the same both for isotype (Figure 5-6, greyscale images, bottom panel) and experimental TNF- α staining. It is therefore not possible to say if the CD11c⁺ cells in both young and old subjects are producing TNF- α . The images were carefully obtained using the same exposure times when acquiring isotype, young and old images. The results were confirmed by two independent investigators. Therefore CD163⁺ macrophages *in situ* in old skin fail to

express TNF- α both at early time-points (24hrs) and at the peak of the response (Day 3) to candida antigen injected intradermally.

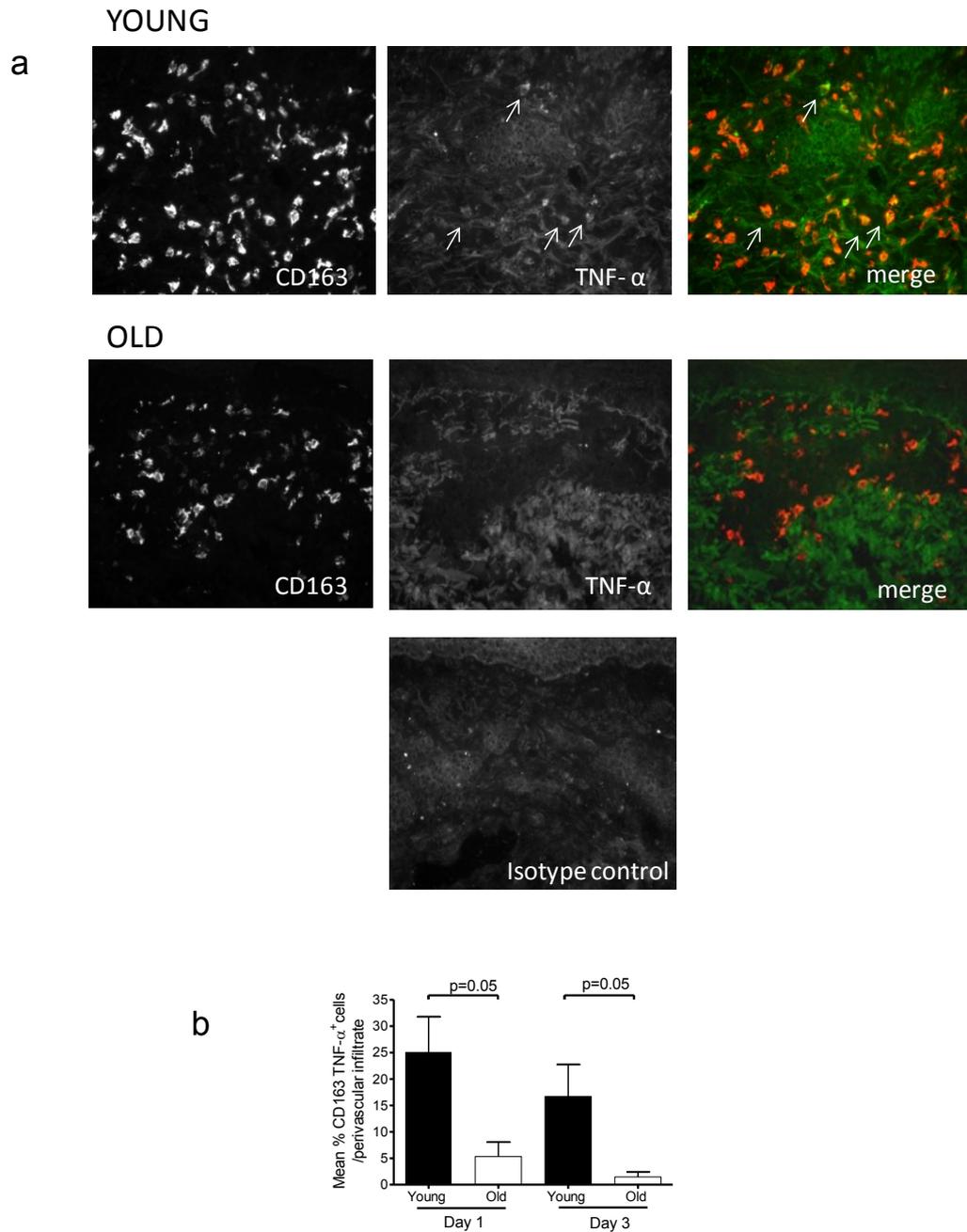


Figure 5-5: TNF- α expression is reduced in CD163⁺ macrophages in old skin

(a) Double immunofluorescence staining of representative biopsies from day 3 following candida injection shows perivascular infiltrate staining for TNF- α (middle panel grey level images, tinted green in merge) and CD163 (left panel

grey level images and tinted red in merge). The bottom panel shows staining with an isotype control. Original magnification x20. (b) Graph shows mean percentage of CD163⁺ macrophages producing TNF- α per perivascular infiltrate (n= 3 young, y old).

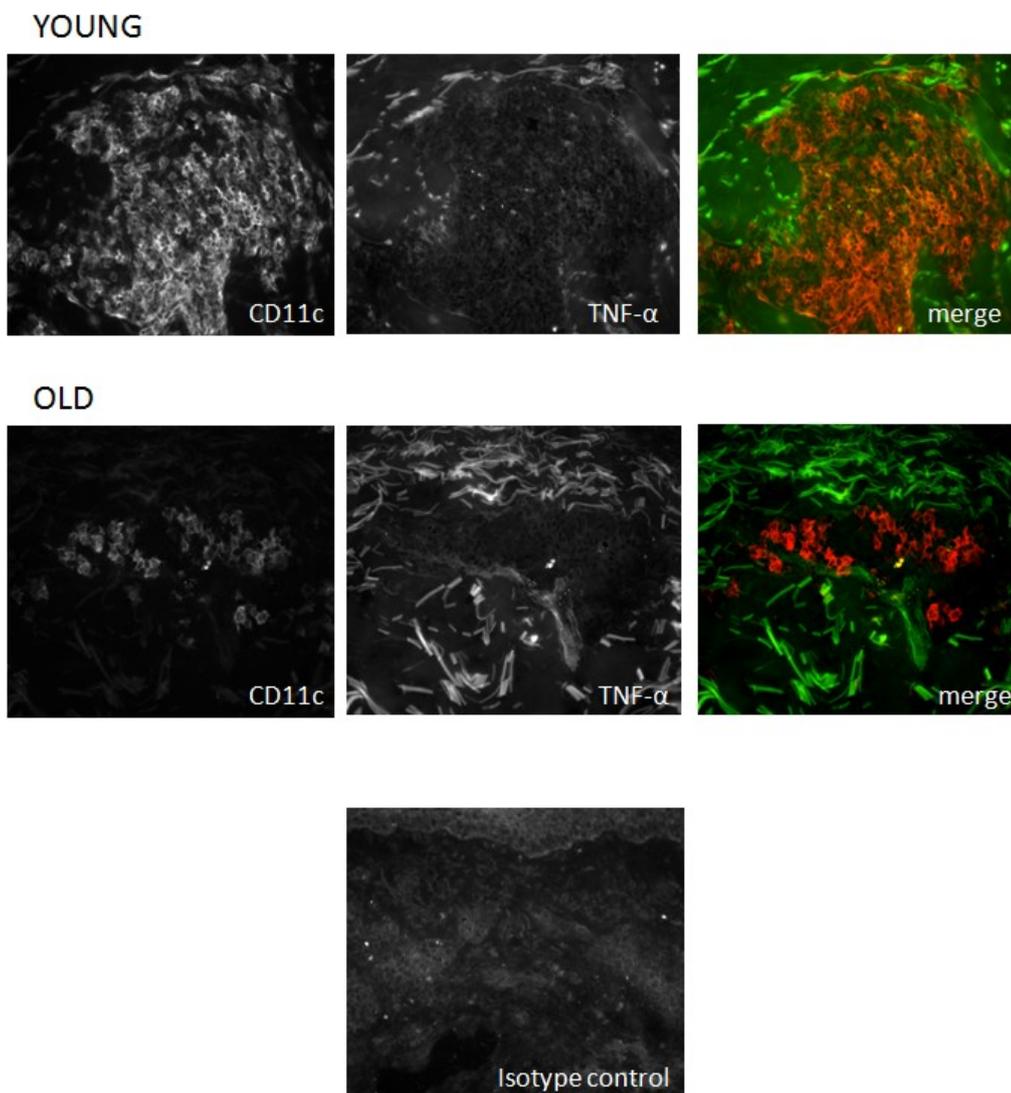


Figure 5-6: Perivascular cell cluster autofluorescence obscures detection of possible TNF- α expression by CD11c⁺ cells.

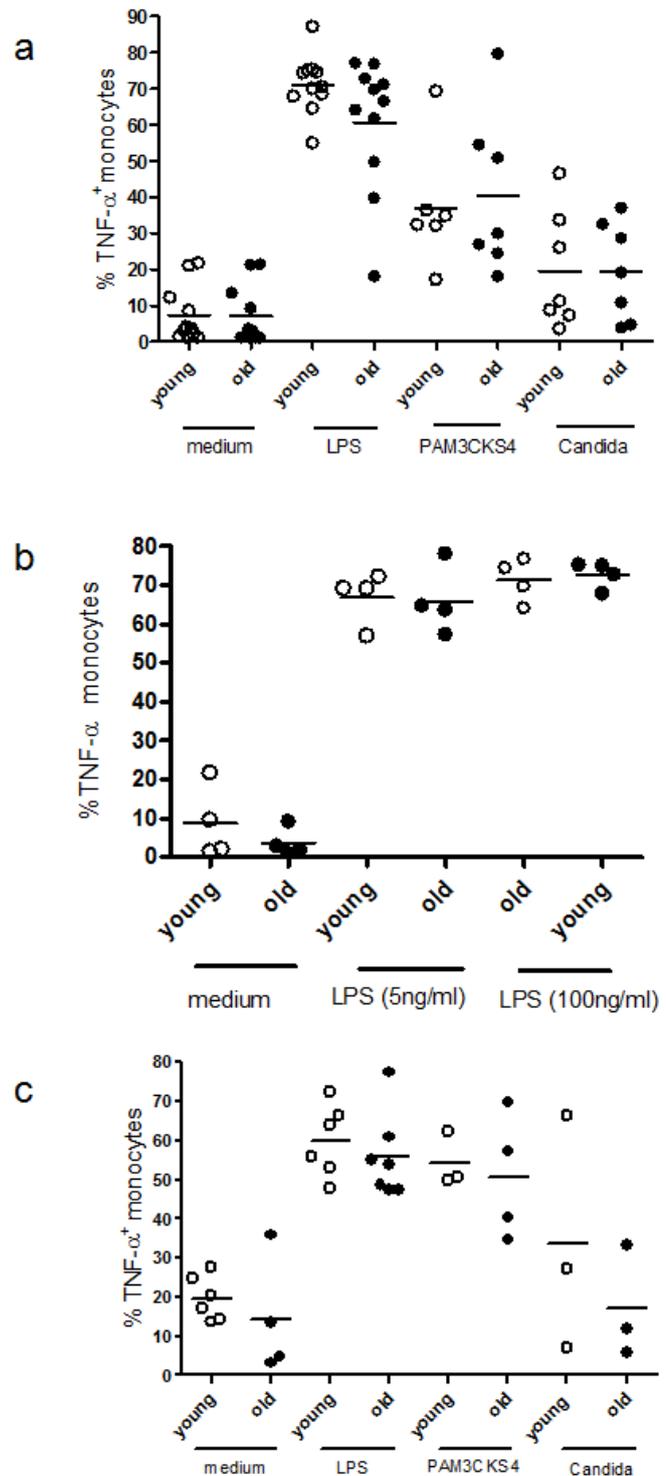
Double immunofluorescence staining of representative biopsies from day 3 following candida injection for CD11c (left panel grey level images, tinted red in merge) and TNF- α (middle panel black and grey level images white, tinted green in merge) CD11c⁺ DCs are located perivascularly in the dermis. Perivascular cells autofluoresce in the green channel as seen in the isotype control (bottom panel grey level images). Original magnification x20.

5.6. Dermal macrophages from old individuals can be induced to secrete TNF- α *in vitro*

The lack of TNF- α secretion by macrophages in old subjects could have been due to lack of activating signals for these cells in the aged skin microenvironment following antigen injection, or to a generalised defect in macrophage activation in old individuals *per se*. To clarify this, we investigated the capacity of macrophages that were isolated from the skin of old and young subjects 48 hrs after injection with candida, to secrete TNF- α upon challenge with the TLR 1/2 ligands Pam₃CSK4 and Candida or a TLR-4 ligand lipopolysaccharide (LPS) *in vitro*. Peripheral blood monocytes were examined in parallel. Peripheral blood CD14⁺ monocytes and cutaneous macrophages from both young and old donors secreted significantly more TNF- α in response to LPS, Pam₃CSK4 and Candida compared to unstimulated controls *in vitro* (Figure 5-7a). This was also true when we stimulated peripheral blood monocytes with a lower concentration of LPS (5ng/ml vs 100ng/ml; Figure 5-7b). However, in contrast to the low TNF- α production of old macrophages *in vivo*, cutaneous macrophages from old and young subjects secreted similar levels of TNF- α in response to LPS, Pam₃CSK4 stimulation *in vitro* (Figure 5-7c). Thus once removed from the skin environment macrophages can synthesize TNF- α suggesting that the cutaneous immune defect in old individuals may be reversible.

Figure 5-7: Macrophages isolated from the skin of old donors secrete TNF- α in response to TLR signals *in vitro*.

Cells removed from skin suction blisters 48 hours after candida injection were stimulated with LPS (100ng/ml; Sigma), Pam₃CSK4 (1 μ g/ml), Candida (10 μ g/ml) or medium as a control for 4hrs in the presence of Golgi Stop and then stained for CD14, CD163 and TNF- α . PBMC were stimulated in parallel. (a) Graph shows percentage of TNF- α -secreting CD14⁺ cells following *in vitro* stimulation of PBMC with LPS, Pam₃CSK4 and Candida (n=11 young and old for LPS; n=6 young, n=7 old for Pam₃CSK4; n=7 young, 7 old for Candida). (b) Graph shows percentage of CD14⁺ cells producing TNF- α following *in vitro* stimulation of blister cells with LPS, Pam₃CSK4 and Candida (n= 6 young, n=7 old for LPS; n=3 young and n=4 for old for Pam₃CSK4; n=3 young, 3 old for Candida). (c) Graph shows percentage of TNF- α -secreting CD14⁺ cells following *in vitro* stimulation of PBMC with LPS 5ng/ml or 100ng/ml.



5.7. Monocyte and DC transmigration into skin

Although T cell numbers are decreased after antigen challenge, macrophages identified by CD163 (this study) or CD68 and CD14 (unpublished observations, Dr Katie Lacy PhD thesis) are present in equal numbers in young and old skin. It has been shown that DC-SIGN/ICAM-2 interaction mediates dendritic cell trafficking (Geijtenbeek et al., 2000) and DC-SIGN is strongly expressed on CD163⁺ macrophages in both normal and inflamed skin (Zaba et al., 2007). ICAM-2 is constitutively expressed on endothelial cells and in contrast to E-selectin, VCAM-1 and ICAM-1, its expression is not dependent on the presence of TNF- α . (Silverman et al., 2001). We hypothesised that macrophages and DCs in young and old skin expressed similar levels of DC-SIGN and this is the way by which these cells enter the skin through the endothelium.

5.7.1. DC-SIGN expression on monocytes and DCs

We obtained young and old skin sections from biopsies of skin injected with candida as before and carried out double immunofluorescence staining for CD163 or CD11c and DC-SIGN (Figure 5-8). DC-SIGN was equally expressed on CD163⁺ cells in skin from young and old subjects at day 3 post candida injection (Figure 5-8a). CD11c⁺ DCs co-expressed DC-SIGN to a lesser degree than the CD163⁺ macrophages, though the pattern of staining was similar for young and old skin sections examined (Figure 5-8b).

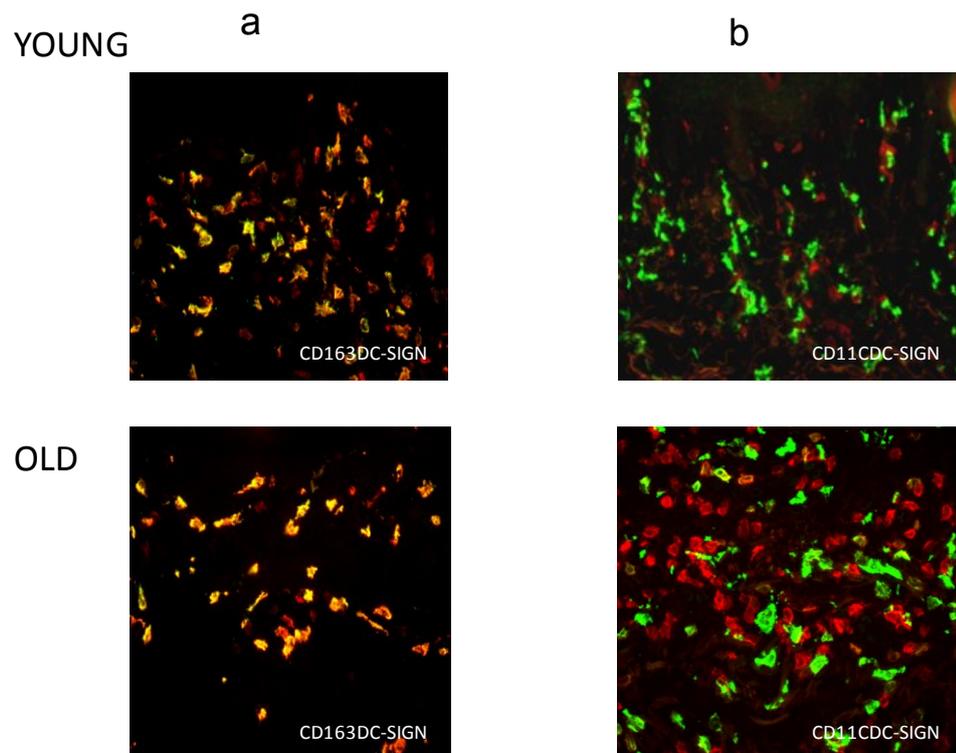


Figure 5-8: DC-SIGN expression by CD163⁺ macrophages and CD11c⁺ dendritic cells in skin not altered in old subjects.

Double immunofluorescence staining of representative biopsies from day 3 following candida injection shows merged image for (a) CD163⁺ (red) and DC-SIGN⁺(green) and (b) CD11c⁺ (red) and DC-SIGN⁺(green). Co-expression of DC-SIGN is reduced for CD11c⁺ compared to CD163⁺ cells. Representative experiments of n=3 young, 3 old experiments shown)

5.7.2. TLR activation leads to upregulation of DC-SIGN expression by peripheral monocytes and DCs

Lymphocytes express CLA which binds E-selectin on the dermal endothelium and leads to transmigration. CLA has also been shown to be important for monocyte transmigration into skin. However notwithstanding reduced E-selectin expression on dermal microvascular endothelium in old skin we have shown similar numbers of macrophages and DCs in young and old skin sections. Monocytes differentiate into macrophages and also give rise to inflammatory DCs when transminating into tissues and following TLR activation (Krutzik et al., 2005; Auffray et al., 2009). We

therefore hypothesised that in view of reduced E-selectin expression in old skin, monocytes and monocyte-derived cells use an alternative pathway to transmigrate into skin and therefore upregulate DC-SIGN in preference to CLA. We obtained PBMC from 3 young and 3 old volunteers and carried out staining for CLA, DC-SIGN, CD14 and CD11c directly *ex-vivo* and after 24 and 72 hrs of TLR stimulation with LPS (TLR4), Pam₃CSK₄ (TLR2/1) and Candida (TLR2/4). The monocytes were identified by their typical scatter characteristics on the forward side scatter. They expressed higher levels of CD14 compared to dendritic cells (Figure 5-9).

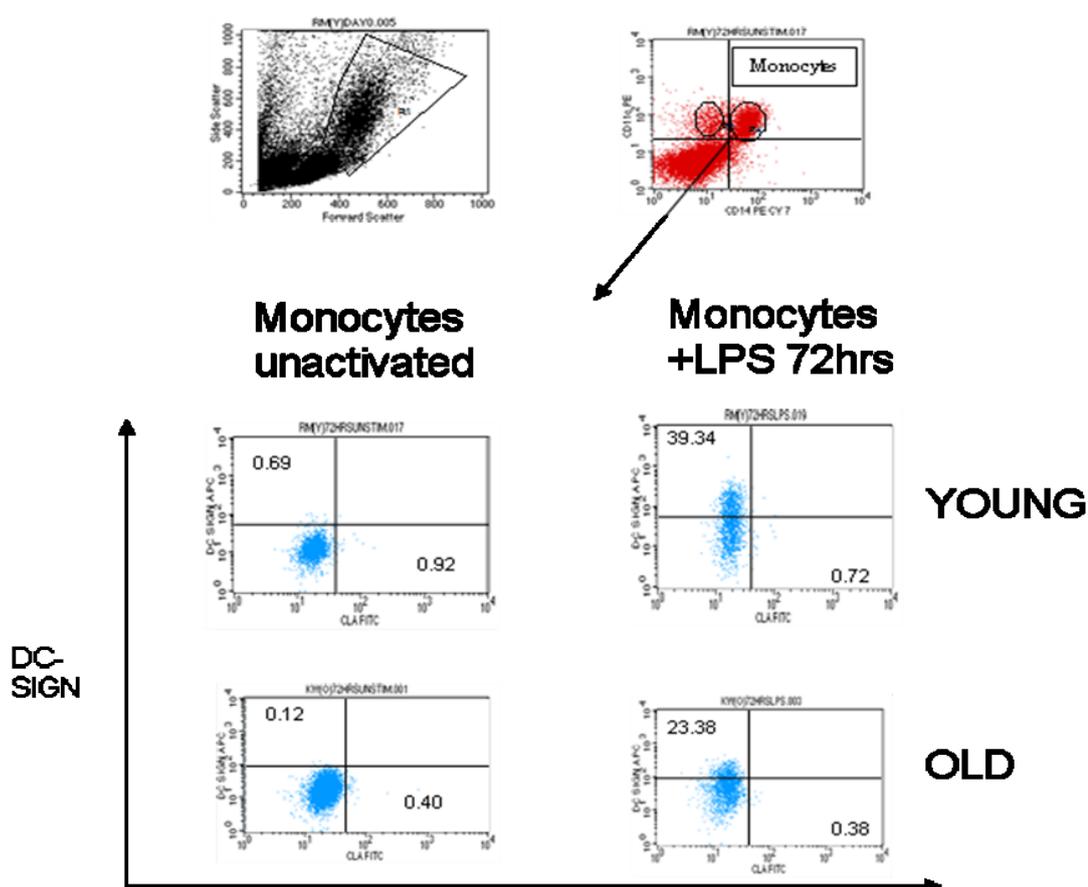


Figure 5-9: TLR stimulation with LPS upregulates DC-SIGN but not CLA expression on circulating monocytes from young and old subjects *in vitro*.

PBMC's were obtained from n=3 young, 3 old and activated with TLR ligands for 24 and 72hrs and labeled with specific antibodies. R1, monocytes gated based on large cell size (FSC) and high side scatter (SSC), R4, CD14 and CD11c double positive cells. DC-SIGN vs CLA expression (R1+R4) for young vs old and LPS activated vs unactivated monocytes.

We found a significant increase in the proportion of CD14⁺ cells expressing DC-SIGN post 72 hrs activation with all TLR ligands tested compared to control. The proportion of CD14⁺CLA⁺ monocytes was not significantly different after TLR activation compared to control (Figure 5-10a/b). CLA expression has also been observed to decrease on PBMCs in culture medium and this might also partly account for the gradual reduction in its expression from that on blood monocytes and DCs directly *ex-vivo* (Figure 5-10, Figure 5-11 and Figure 5-12). These results suggest a selective upregulation of DC-SIGN as opposed to CLA upregulation after TLR activation and imply that monocytes are able to infiltrate skin in old subjects equally well because they are using the DC-SIGN-ICAM2 interaction for migration.

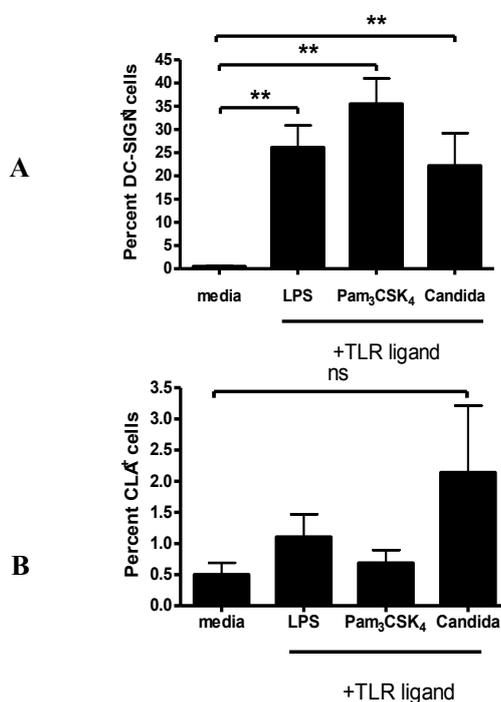


Figure 5-10: Monocytes from young and old subjects upregulate DC-SIGN and CLA expression similarly upon TLR stimulation

a) Young and old monocytes upregulate DC-SIGN after 72hrs of TLR (LPS) stimulation. b) TLR stimulation using TLR4 (LPS), TLR2/1(Pam₃CSK₄ and Candida) leads to significant DC-SIGN upregulation compared to medium alone but no significant CLA upregulation at 72hrs. Data are shown as the mean+SEM of 6 independent experiments.

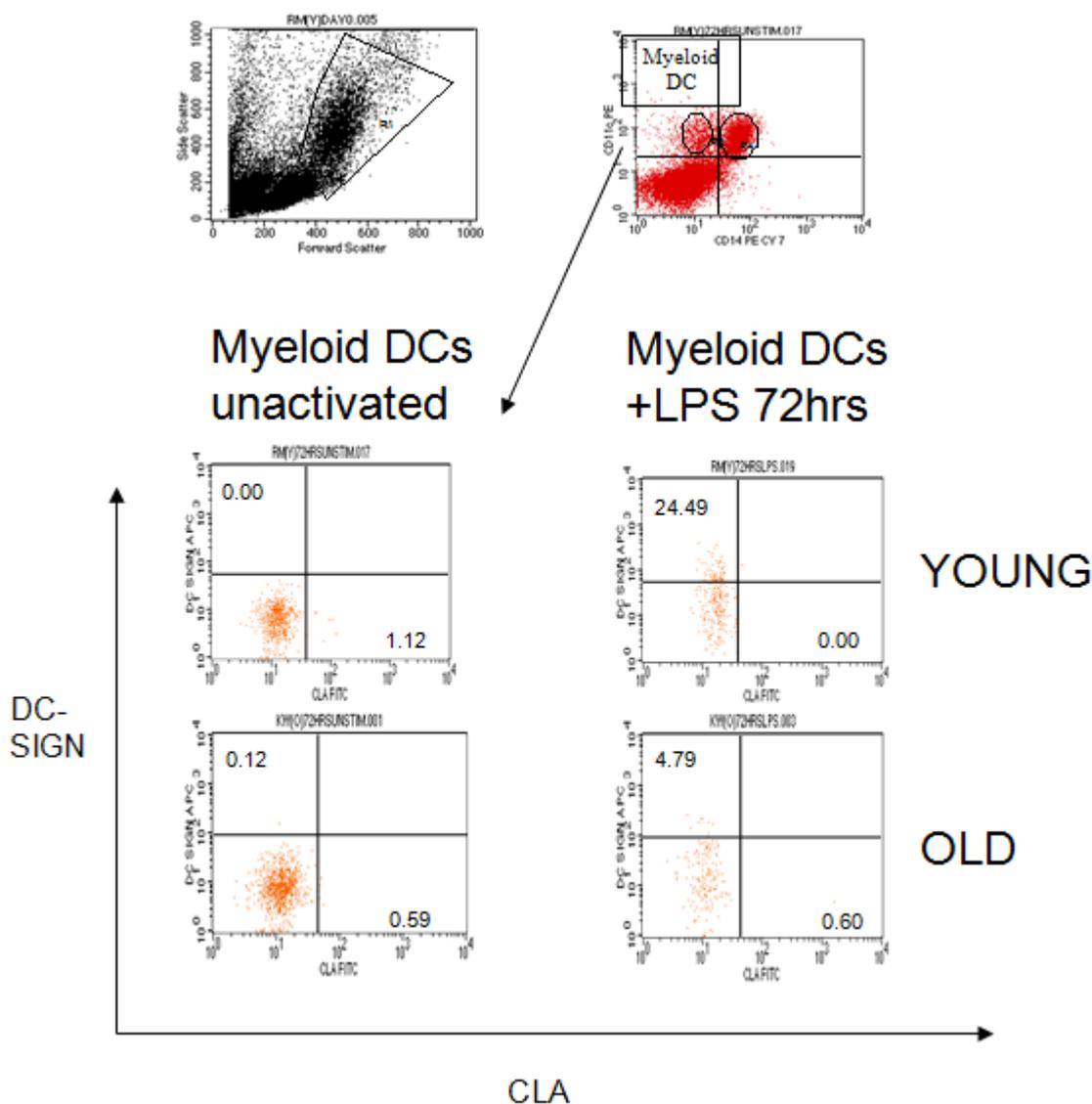


Figure 5-11: Old circulating myeloid DCs show reduced upregulation of DC-SIGN on TLR stimulation.

PBMC's were obtained from n=3 young, 3 old and activated with TLR ligands for 24 and 72hrs and labeled with specific antibodies. R1, monocytes gated based on large cell size (FSC) and high side scatter (SSC), R5, CD14-negative and CD11c positive cells. DC-SIGN vs CLA expression (R1+R5) for young vs old and LPS activated vs unactivated monocytes.

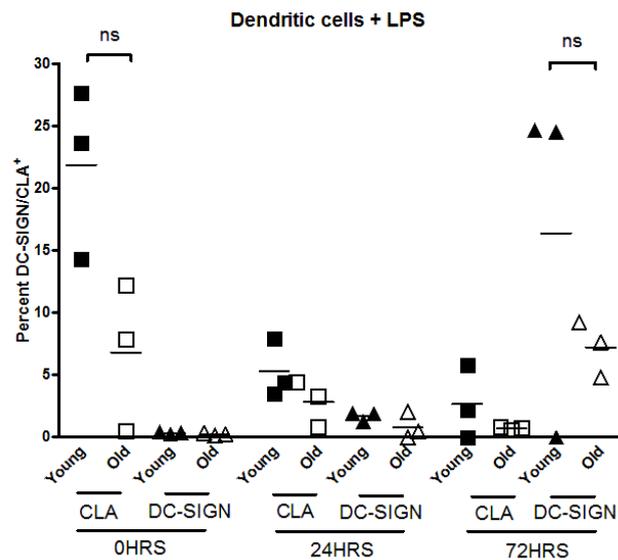


Figure 5-12: Old peripheral blood DCs show reduced capacity to upregulate DC-SIGN expression after 72 hours of LPS stimulation in vitro.

Similar data was obtained with *Candida* and Pam3CSK4 stimulation (not shown). Circulating myeloid DCs show high CLA expression at 0hrs (22% mean in young) compared to monocytes (2% mean in young as shown in Fig. 5.10).

5.8. Discussion

In this chapter we have used the candida antigen skin test model to investigate the mechanism for reduced endothelial activation and T cell infiltration in old skin. A secondary immune response relies on cells of the innate immune system working in a direct way to contain invading microbes and in an indirect way, through cytokine release, to induce adaptive T cell responses. We have shown in chapter 4 that skin endothelium requires TNF- α and IFN- γ for activation. Using immunohistology and cytokine analysis of blister fluid from the candida skin model we were able to determine that old skin lacks expression of TNF- α and IFN- γ early on post antigen injection, suggesting a possible defect in innate immunity in ageing skin.

A previous study using the mantoux test as a skin model in healthy young individuals showed that TNF- α is detectable at 6 hours and peaks at 72 hours post antigen injection. CD68⁺ cells were found to be the main cell type expressing TNF- α during

the mantoux reaction (Chu et al., 1992). CD68 is expressed by both macrophages and DCs in healthy skin (Zaba et al., 2007). We therefore hypothesised that the lack of TNF- α in old skin might be caused by either a reduced number of macrophages and/or DCs in old skin or by defective functioning of these cells.

Based on recent characterisation of novel phenotypic markers for cutaneous macrophages and DCs (Zaba et al., 2007) we used CD163 to identify macrophages and CD11c to distinguish DCs. Single staining for these markers on skin sections from normal or candida injected young and old skin showed similar numbers of both macrophages and dendritic cells. The discrepancy between the obviously reduced numbers of T cells found in old skin as opposed to the normal numbers of macrophages and dendritic cells is likely due to the different trafficking requirements. It has been shown that DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking (Geijtenbeek et al., 2000) and DC-SIGN is strongly expressed on CD163⁺ macrophages in both normal and inflamed skin (Zaba et al., 2007) ICAM-2 is constitutively expressed on endothelial cells and in contrast to E-selectin, VCAM-1 and ICAM-1, its expression is not dependent on the presence of TNF- α (Silverman et al., 2001). The DC-SIGN-ICAM-2 interaction accounts for 41% of transendothelial migration of DCs across mouse endothelium in the steady state. It was also shown that blocking of CD18 (β 2 integrins) had no effect on transendothelial migration of DCs (Wethmar et al., 2006). Therefore in the absence of TNF- α to upregulate the expression of selectins and integrin adhesion molecules on the endothelium, DCs and macrophages can still migrate into the skin, and this explains why similar numbers of these innate immune cells are present in young and old skin. Furthermore we show, using extracellular staining for DC-SIGN and CLA on circulating monocytes in young and old subjects that macrophages and DCs upregulate DC-SIGN in preference to CLA when they are stimulated with TLR ligands including LPS, Pam₃CSK4 and Candida antigen *in vitro*. Therefore we have shown that despite an equal number of innate immune cells in young and old skin, there is reduced TNF- α expression in old skin during a secondary memory cutaneous immune response to candida injection. We therefore next investigated if there could be a defect in cutaneous macrophage or DC function.

Studies carried out in mice have shown that when alveolar macrophages from old mice are stimulated *in vitro* with LPS they are unable to upregulate the expression of cyto-protective enzymes such as hemeoxygenase-1 (Ito et al., 2009). Another study showed reduced production of TNF- α by macrophages from old mice following candida antigen stimulation *in vitro* (Murciano et al., 2006). Studies on monocytes from whole blood have shown reduced production of TNF- α after stimulation with LPS in old humans (Gon et al., 1996) and mice (Effros et al., 1991). In this study we sought to characterise macrophage and DC function both *in vivo* by looking at TNF- α *in situ* expression by these cells in young and old skin sections and *in vitro* by directly activating macrophages derived from young and old skin with TLR agonists and assessing TNF- α production. We found that *in vivo* expression of TNF- α was drastically reduced in CD163⁺ macrophages in skin from old subjects injected with candida. However once these cells were removed from the skin environment and stimulated with LPS, Pam₃CSK4 and Candida antigen *in vitro* both old and young macrophages were able to produce TNF- α to a similar extent.

An important unanswered question is why macrophages in the old skin are not triggered to secrete TNF- α . Previous studies have shown that there is an age associated defect in human TLR1 and 2 function that results in a significant defect in TNF- α secretion after ligation of these receptors (van Duin et al., 2007). Since candida activates TLR1 and 2, this may explain the cutaneous defect in macrophage-derived TNF- α that we have observed. It has also been shown that there is a defect in TLR4 function and expression and function during ageing (Renshaw et al., 2002; van den Biggelaar et al., 2004) although this has not been confirmed in other studies (van Duin et al., 2007). However, we found that both peripheral blood monocytes and isolated cutaneous macrophages from donors of both age groups synthesized similar levels of TNF- α after TLR 1/2 and TLR-4 stimulation *in vitro*. We conclude therefore that macrophages in old humans are not inherently defective in terms of TNF- α synthesis but do not rule the possibility that other functions in these cells may be altered during ageing.

TNF- α has also been shown to upregulate expression of endothelial adhesion molecules on cutaneous lymphatics in mouse models and that this is an essential step to enable DC migration to lymph nodes (Johnson et al., 2006). This would mean that TNF- α is required very early on in the development of a secondary immune response to start the process of antigen presentation to T cells in the lymph node.

Another factor to consider is the lack of IFN- γ in the skin of old subjects injected intradermally with candida. For macrophages to become activated and fully functional, these cells must also interact with cytokines such as IFN- γ in addition to TLR ligands. Previous studies have shown that IFN- γ is mainly secreted by T cells during DTH responses (Chu et al., 1992). The lack of IFN- γ in the skin in our studies may reflect the decreased infiltration of T cells into the site of antigen challenge. Activation by IFN- γ leads to the expression of several genes that regulate macrophage biology including expression of MHC Class II genes (Celada et al., 1989; Cullell-Young et al., 2001; Gonalons et al., 1998), which are crucial for presenting antigens to T lymphocytes. It has been shown that mice deficient in IFN- γ receptor show profoundly altered responses to LPS suggesting that IFN- γ has an important role in making macrophages more responsive to LPS (Kamijo et al., 1993). It is possible that the lack of T cell signals such as IFN- γ early in the DTH response in old subjects may prevent the cascade of events required for its amplification. Alternatively, macrophages in the skin of old individuals may become modified through the interaction with other resident cells, such as regulatory T cells that have been shown to inhibit their capacity to synthesize TNF- α (Tiemessen et al., 2007). Both of these hypotheses require further investigation.

In conclusion we have shown for the first time a defect in a key innate immune pathway of the delayed type hypersensitivity response in old skin. This is the first study to show that lack of TNF- α production by CD163⁺ macrophages within old skin leads to impaired cutaneous secondary immune responses to antigen with ageing. The observation that Imiquimod, a TLR7 agonist which is applied topically is useful in the treatment of non-melanoma skin cancer in the elderly, and infections such as viral warts (Beutner et al., 1999) further suggests a possible role of the innate

immune system in the mechanism of sub-optimal secondary cutaneous immunity in this age group. Similarly this study highlights the need for novel immunotherapeutic agents for preventing/treating skin cancer and infections, aimed at improving pro-inflammatory cytokine secretion by macrophages within old skin.

Chapter 6. Regulatory T cells and their role in secondary cutaneous immune responses

6.1. Introduction

In the previous chapter we have shown that cutaneous CD163⁺ macrophages fail to express TNF- α *in situ* during a secondary cutaneous immune response in old subjects. However they are capable of TNF- α synthesis and release when exposed to TLR agonists *in vitro*. Therefore we hypothesised that a factor in the skin microenvironment in old subjects is contributing to altered macrophage function. We know that CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) have suppressive effects on the adaptive immune system and on CD4⁺ cells in particular (Sakaguchi, 2005; Shevach et al., 2006). It has also recently been shown that CD4⁺CD25⁺FoxP3⁺ Tregs are able to directly promote the alternative activation of monocytes/macrophages (Tiemessen et al., 2007). We hypothesised therefore that Tregs might play a role in old skin immunosuppression by suppressing/altering dermal macrophage function in old individuals.

Little is known about the kinetics of Treg accumulation during the cutaneous delayed type hypersensitivity response. Our group has previously characterised the clinical and histological characteristics of the CD4⁺ T cell response to the Mantoux test (MT) in young skin (Reed et al., 2004; Orteu et al., 1998). We demonstrated that the histological response to the MT is characterised by dermal perivascular infiltrates consisting of predominantly CD4⁺ T cells which peak in T cell number at day 7 post-injection of the MT. We also show that the peak of antigen-specific CD4⁺ IFN- γ ⁺ T cells in the MT occurs at Day 7 with a median of 30% CD4⁺IFN- γ ⁺ T cells present in suction blisters. The peak of the clinical response occurs at day 3 post injection of antigen (Reed et al., 2004). Most studies on CD4⁺CD25^{hi}Foxp3⁺ T cells in humans have been performed using peripheral blood populations and apart from a few notable exceptions (Ruprecht et al., 2005; van Amelsfort et al., 2004; Uhlig et al., 2006; Miyara et al., 2006) there is very little data on the behaviour of these cells at

sites of immune responses *in vivo*. In mice vaccinated with BCG and then infected with *Mycobacterium tuberculosis* by the aerosol route, it was shown that there is an increase in the size of the Treg compartment in lung tissue which is in proportion to the increase in other T lymphocyte subsets (Jaron et al., 2008). In order to determine whether Tregs are involved in the alteration of function of macrophages in skin from old subjects it was necessary to first fully characterise their presence, function and numbers during a secondary memory cutaneous immune response in humans *in vivo*.

6.2. Accumulation of CD4⁺ Foxp3⁺ T cells during a secondary response to antigen *in vivo*

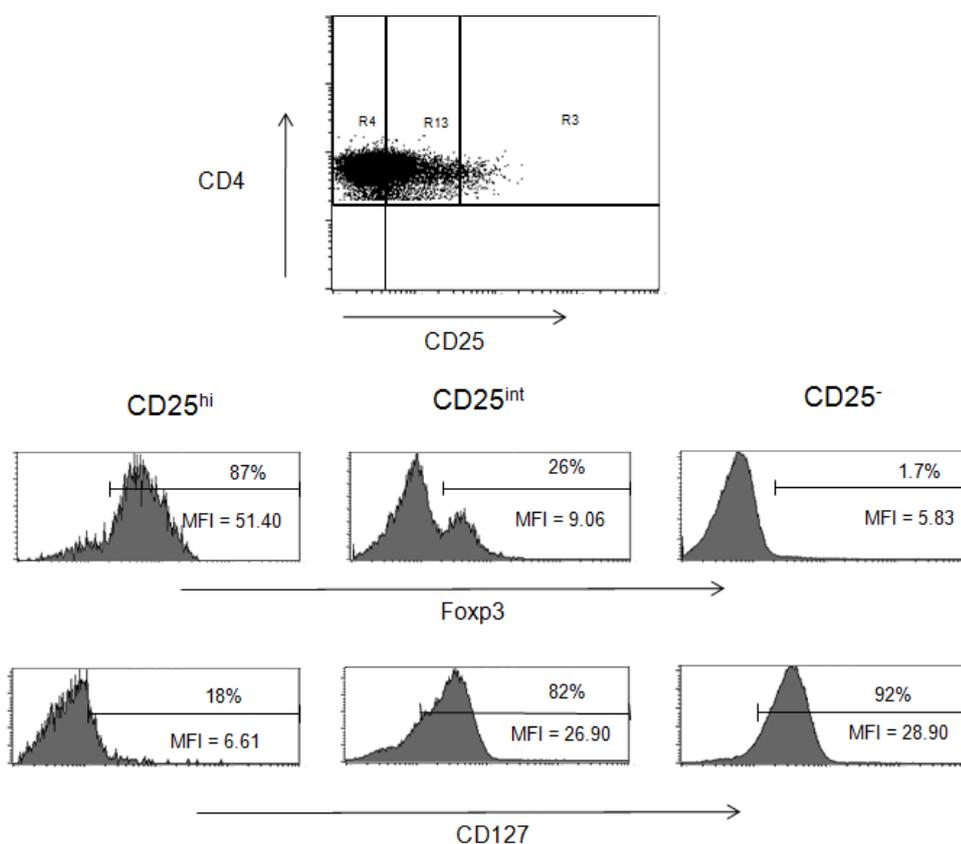


Figure 6-1: Treg gating and phenotype.

PBMCs were stained with CD4, CD25, CD127 and Foxp3 as described. Subsets are defined by the expression of CD25 as CD25⁻, CD25^{int} and CD25^{hi}. Expression of Foxp3 and CD127 by each subset is shown in the histograms.

Tregs are identified by expression of CD4, high expression of Foxp3 and CD25 and low expression of CD127. Figure 6-1 shows the gating strategy we used based on these three markers to distinguish nTregs from other CD4⁺ T cells.

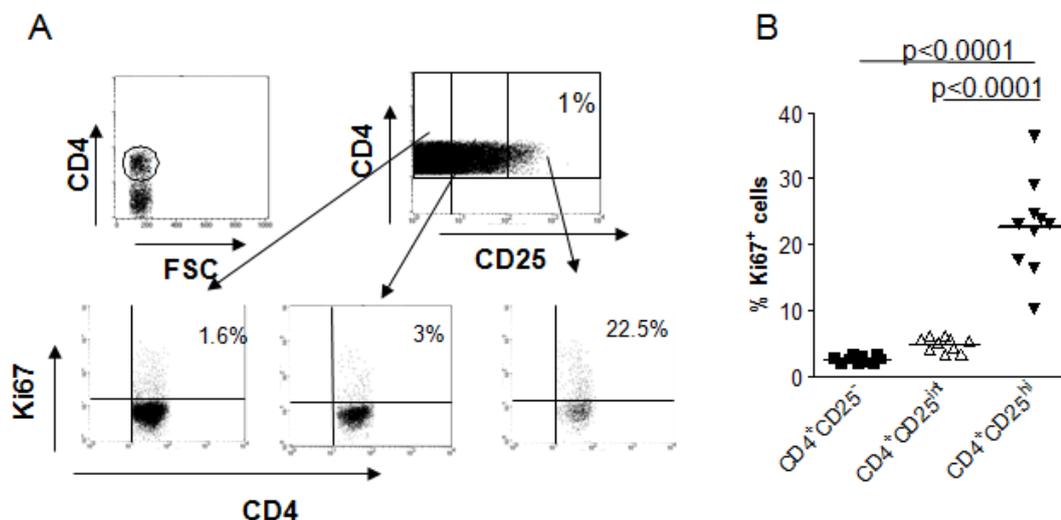


Figure 6-2: Human CD4⁺CD25^{hi} regulatory cells turnover rapidly *in vivo*.

Freshly isolated PBMC from healthy individuals were stained for CD4/CD25/CD127/Foxp3 and Ki67 following the standard protocol. Regulatory T cells were identified as CD25^{hi}, Foxp3⁺ and CD127^{lo} as in Figure 1). (A) Dot plots show expression of Ki67 on CD4⁺CD25^{hi}, CD4⁺CD25^{int} and CD4⁺CD25⁻ cells from a representative sample. Numbers denote the percentage of cells expressing Ki67, relative to the control gate set with an irrelevant antibody. FSC, forward scatter (B) Cumulative data showing percentage of Ki67⁺ cells in each subset. Each symbol represents a different individual (n=10 per group) and the mean percentage is shown as a horizontal line. Significance was determined by paired t-test

Our group has previously shown a high level of proliferation in circulating CD4⁺CD25^{hi} Tregs (Vukmanovic-Stejic et al., 2006). This was confirmed using Ki67, a nuclear protein expressed by proliferating cells in all phases of the active cell cycle (Gerdes et al., 1984). PBMCs were obtained from 10 healthy young volunteers and stained for CD4/CD25/CD127/Foxp3 and Ki67 as described in previous chapters. It was found that in freshly isolated peripheral blood mononuclear populations, in all individuals tested, significantly higher proportions of

$CD4^+CD25^+CD127^{lo}FoxP3^+$ T cells expressed Ki67 than $CD4^+CD25^{int}$ and $CD4^+CD25^-$ T cell populations (mean \pm SEM $22.8 \pm 2.2\%$ compared to $5 \pm 0.3\%$ and $2.6 \pm 0.2\%$ respectively (Figure 6-2 A/B).

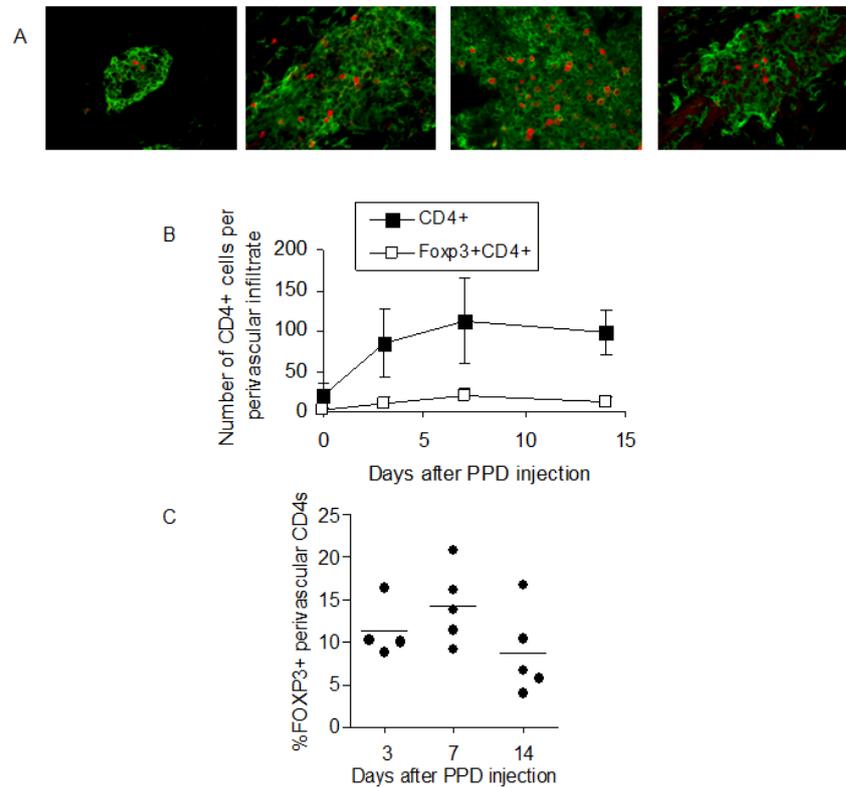


Figure 6-3: Accumulation of $CD4^+Foxp3^+$ T cells during a secondary response to antigen *in vivo*.

(A) Double immuno-fluorescence staining of representative biopsies (days 0, 3, 7 and 14, $n=5$ per time point) shows $CD4^+$ (green) and $Foxp3^+$ (red) cells in a perivascular lymphocytic infiltrate (original magnification, $\times 400$). The 5 largest perivascular infiltrates present in the upper and mid dermis were selected for analysis. Cell numbers were expressed as the mean absolute number of cells counted within the frame. (B) Number of total $CD4^+$ cells (black squares) and number of $Foxp3^+CD4^+$ cells (white squares) in perivascular infiltrates following PPD injection. (C) Percentage of $CD4^+$ cells expressing $Foxp3$ per perivascular infiltrate counted. Each symbol represents an average of 5 perivascular infiltrates counted for each individual ($n=5-7$ per time point). Data are mean \pm SEM.

We examined tissue biopsies taken throughout the course of the MT, for the expression of $CD4$ and $Foxp3$ and enumerated double positive cells in the

perivascular infiltrates. Figure 6-3A shows the expression of CD4 and Foxp3 in representative cellular infiltrates at day 0 (control skin), 3, 7 and 14 post PPD injection. Cumulative data from 5 individuals per time point is presented in Figure 6-3B showing a significant increase in the number of CD4⁺ and CD4⁺Foxp3⁺ T cells on day 7 (P=0.04, one way Anova). This increase in the percentage of CD4⁺Foxp3⁺ cells coincides with the increase in total CD4⁺ T cell numbers and also with peak cellular proliferation (p=0.01, one way Anova). However, when the corresponding increase in CD4⁺ T cell numbers is taken into account and the data expressed as the proportion of CD4⁺ T cells expressing Foxp3, this value remains fairly constant during the response (between 8-15% of CD4⁺ cells, Figure 3C). We concluded that both memory and Foxp3⁺ (putative regulatory) T cells therefore accumulate at the same relative rate after antigenic stimulation *in vivo*.

6.3. Accumulation and proliferation of CD4⁺ T cells during a secondary response to antigen *in vivo*

The increase in antigen-specific CD4⁺ T cells observed in the skin during the MT may not only result from the recruitment of T cells from the blood, but also from the localised proliferation of activated T cells in the skin. Evidence in mice, however, suggests that increase in numbers of antigen-specific T cells in non-lymphoid tissues is predominantly mediated by proliferation in lymphoid tissues followed by migration to non-lymphoid organs (Reinhardt et al., 2003; Roman et al., 2002; Ely et al., 2003; Harris et al., 2002; Ostler et al., 2001).

Tissue biopsies obtained from the site of antigen injection at different times were co-stained with Ki67 and CD4 (Figure 6-4A). The five largest perivascular infiltrates per section were photographed and counted, and data expressed as mean absolute cell number per frame. Very few proliferating cells were observed in normal skin (day 0), and the perivascular infiltrates were very small or absent. At day 7, the time of maximal CD4⁺ T cell accumulation, the number of CD4⁺ T cells expressing Ki67 is significantly increased (Figure 6-4B) and >20% of CD4⁺ T cells were in cycle (Figure 6-4A/C). This indicates that the increase in PPD specific CD4⁺ T cells in the

skin after antigenic challenge occurs in part through their extensive local proliferation

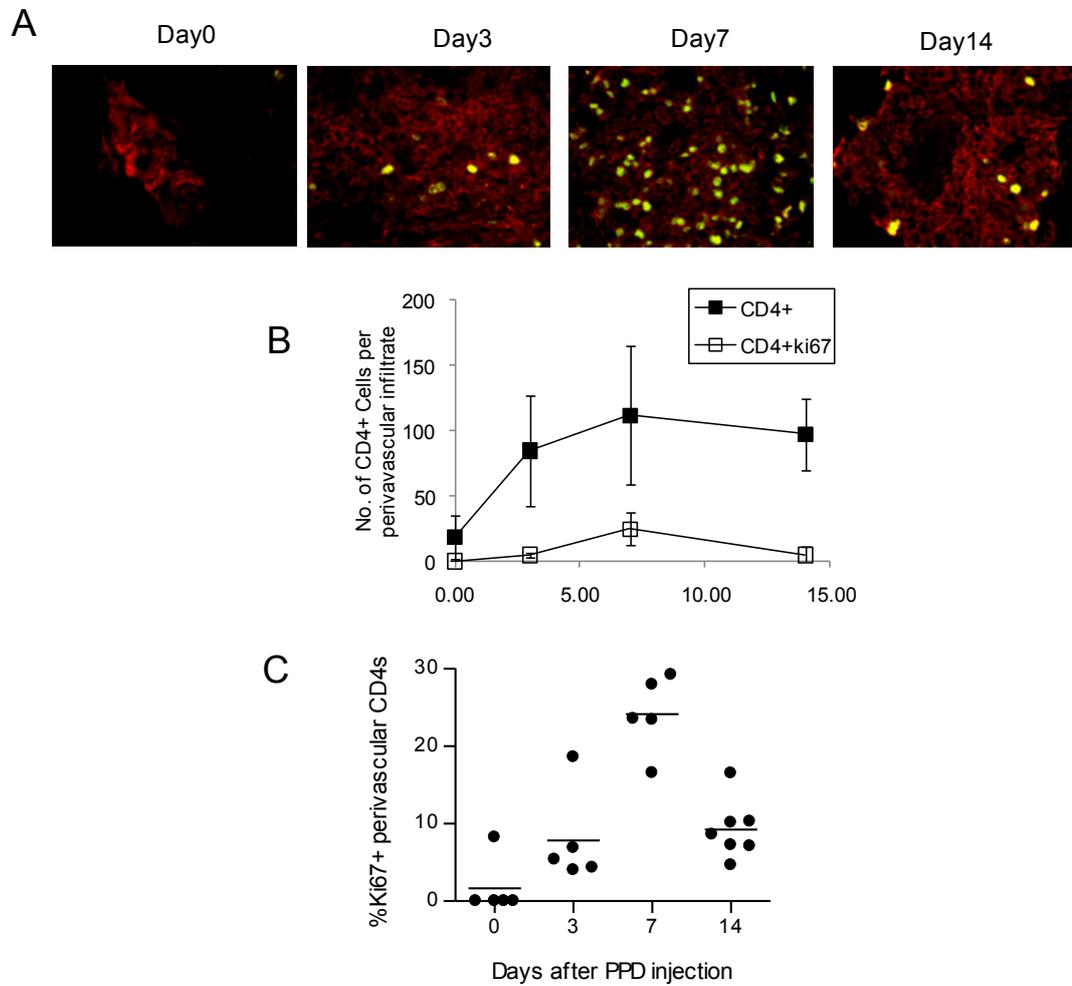


Figure 6-4: CD4⁺ T cell proliferation at the site of the MT

(A) Double immuno-fluorescence staining of representative biopsies from day 0, 3, 7 and 14 following MT induction. Ki67 is green, CD4 red. Original magnification was x400. (B) Number of total CD4⁺ cells (black squares) and number of Ki67⁺CD4⁺ cells (white squares) in perivascular infiltrates following PPD injection. (C) Percentage of CD4⁺ cells expressing Ki67 found per perivascular infiltrate in each donor. Each circle represents an average of perivascular infiltrates counted for each individual (n=5-7 per time point, horizontal line indicates the mean).

6.4. Transient expression of Foxp3 expression induced by activation of CD4⁺ T cells *in vitro*

Previous studies have found that human CD4⁺CD25⁻ effector T cells can upregulate Foxp3 transiently, following activation (Walker et al., 2005; Allan et al., 2005; Gavin et al., 2006). It was possible therefore that the Foxp3 expressing CD4⁺ T cells that were found in the skin after antigen challenge were activated memory T cell populations that were induced to express this marker temporarily. To clarify this we first investigated the relationship between Foxp3 expression and proliferation in CD4⁺CD25⁻Foxp3⁻ T cells after activation *in vitro* (Figure 6-5). This work was carried out in collaboration with Dr Nicola Booth (PhD student). These cells were isolated from peripheral blood, labelled with CFSE and then activated with anti-CD3/anti-CD28 coated beads. The expression of CD25, Foxp3 and Ki67 was determined on days 0, 3, 5 and 7. By day 3, approximately 80% of cells are CD25⁺ and 35% of CD4⁺ cells express Foxp3. All Foxp3⁺ cells are Ki67⁺ and ~80% of the activated CD4⁺ T cell population had lost the CFSE label by day 3 (Figure 5). Most cells lost Foxp3 expression (but remained CD25⁺ and Ki67⁺) by day 7 indicating that the induction of Foxp3 expression in CD4⁺ T cells after activation was transient (Figure 6-5).

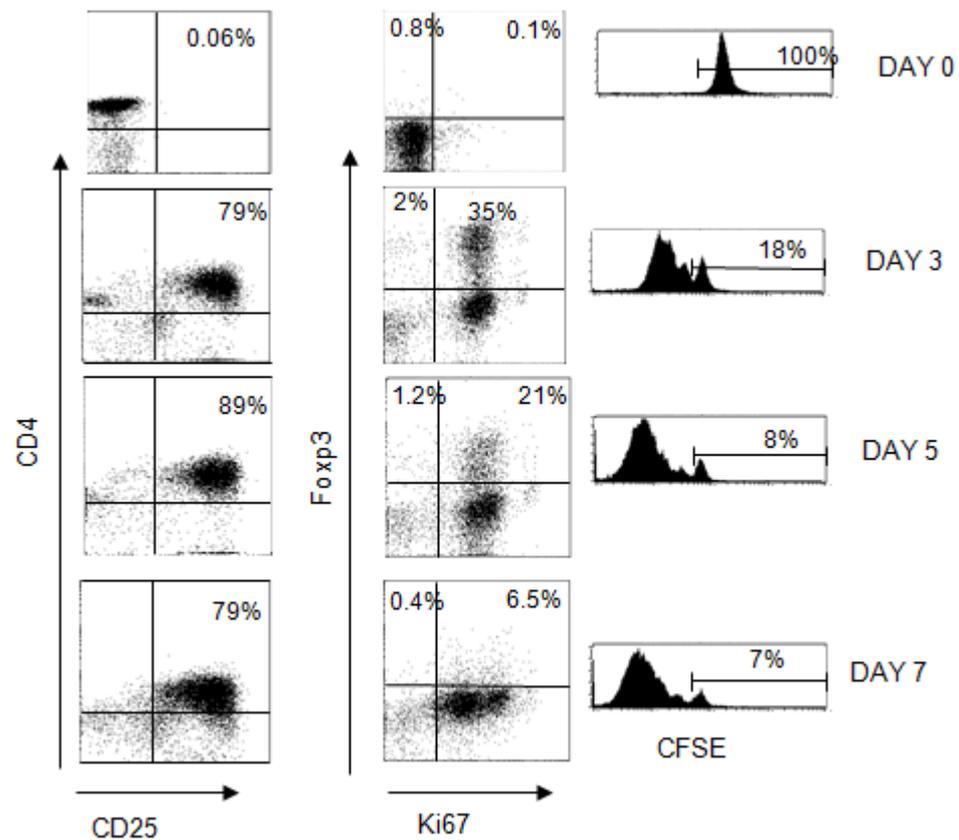


Figure 6-5: Memory T cells induced to express Foxp3 transiently in humans by stimulation.

CD4⁺CD25⁻ (responder) T cells were isolated from PBMC using MACS and labeled with CFSE. Cells were stimulated with magnetic beads coated with antibodies to CD3 and CD28 for 7 days. Samples were removed at regular intervals and stained for the expression of CD25, Foxp3 and Ki67. Numbers in dot plots indicate the percentage of cells in each quadrant. Dilution of the CFSE signal indicates proliferation of the cells. Data shown is representative of 3 independent experiments.

6.5. Foxp3⁺ T cells at the site of MT proliferate in parallel to effector T cells and have a regulatory T cell phenotype

To characterize the Foxp3⁺ T cells in the skin more extensively, we induced skin suction blisters at the site of the MT response on day 7 following PPD injection. This time point was chosen as it coincided with the peak CD4⁺ T cell infiltration and proliferation. Cells isolated from the blisters were stained for the co-expression of

Foxp3 with CD25, CD127, CD39 and CD27 as all these markers have been shown to identify nTregs (Ruprecht et al., 2005; Borsellino et al., 2007; Deaglio et al., 2007; Liu et al., 2006; Seddiki et al., 2006) (Figure 6-6, histograms right panel). Percentage of Foxp3⁺ cells in day 7 blisters was very similar to that observed in histology (range 7.1 to 22.2%, mean 14.5 ± 2 , n=6) (Figure 6-6, dot plot left panel). The majority of Foxp3 expressing CD4⁺ T cells were CD25⁺ ($78.5 \pm 7.5\%$, n=3) and expressed low levels of CD127 (mean and SEM, $10 \pm 2.7\%$ n=5). Furthermore these cells were uniformly CD27⁺, a hallmark of regulatory T cells at sites of immune activation *in vivo* (Ruprecht et al., 2005). Finally, the Foxp3 expressing T cells in skin also express CD39, another marker for nTregs (mean 83.6 ± 4) (Borsellino et al., 2007; Deaglio et al., 2007). In contrast, the CD4⁺Foxp3⁻ T cells that were found in the same samples were largely CD25 and CD39 negative but with high expression of CD127.

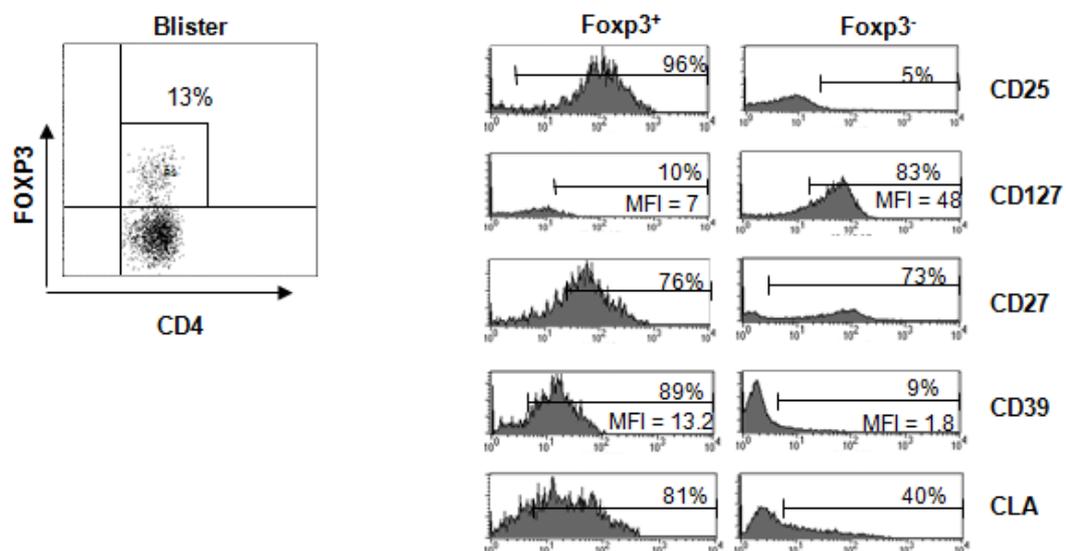


Figure 6-6: Foxp3⁺ T cells isolated from the MT have a Treg phenotype.

Blister cells were isolated on day 7 following MT induction and stained for Foxp3, CD127, CD25, CD27 and CD39. Representative staining (n=6) is shown. Dot plot indicates gating strategy. Histograms show expression of surface molecules on CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ subsets as indicated. MFI and % positive cells are indicated as appropriate.

6.6. Foxp3⁺ T cells at the site of MT have regulatory T cell function

Technical limitations, namely very small numbers of leucocytes collected from blisters precluded purification of the putative regulatory population from the skin. For day 7 blisters in 30 subjects that have been investigated, a mean of 295,000 cells were obtained (range 40-900,000 leucocytes, of which 5-50% were CD4⁺ T cells). Therefore the estimated mean number of CD4⁺ CD25⁺CD127^{lo} cells that are present is in the region of 2,000-25,000 cells that we are unable to isolate with current technology. However, nTregs, unlike effector cells do not secrete IL-2 and IFN- γ after recent stimulation *in vitro* (Pandiyan et al., 2007; Scott-Browne et al., 2007; Levings et al., 2001; Tiemessen et al., 2007). We therefore investigated the capacity of Foxp3⁺ and Foxp3⁻ CD4⁺T cells that were isolated from suction blisters to produce effector cytokines following re-stimulation with antigen *in vitro*. Cells isolated from day 7 blisters were stimulated with PPD for 16 hours, and stained for CD4, Foxp3, and IFN- γ or IL-2. Following re-stimulation, significantly greater numbers of CD4⁺Foxp3⁻ cells synthesized IFN- γ compared to CD4⁺Foxp3⁺ T cell population (n=6, for IFN- γ p=0.015, Wilcoxon paired test, Figure 6-7, left panel). Similar data was obtained for IL-2 in 4 separate donors (Figure 6-7, right panel, p=0.06). Thus although we were not able to assess suppressive function directly, we find that the CD4⁺Foxp3⁺ cells that are found in the skin after antigenic challenge are not recently activated effector CD4⁺ T cells and are clearly distinct, phenotypically and functionally from the CD4⁺Foxp3⁻ population.

6.7. Foxp3⁺ T cells at the site of MT proliferate in parallel to effector T cells

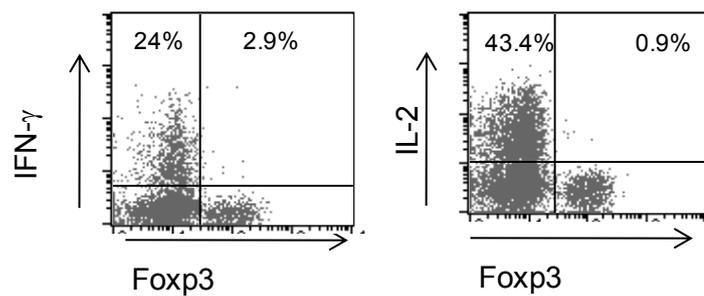


Figure 6-7: Foxp3⁺ T cells isolated from MT show Treg function.

Blister cells were isolated on day 7 following MT induction, stimulated with PPD for 15 hours in the presence of brefeldin A and stained for intracellular expression of cytokines and Foxp3. Percentage of Foxp3⁺ and Foxp3⁻ cells secreting cytokine is indicated. Dot plots are representative of 4-6 independent experiments.

To determine if the Foxp3⁺ cells found in the skin represent a regulatory population or activated CD4⁺ T cells that express Foxp3 transiently we double stained the MT skin sections for Ki67 and Foxp3 expression (Figure 6-8A). In contrast to the CD4⁺ T cells that were activated *in vitro*, where all Foxp3⁺ are also Ki67⁺ (Figure 6-6, middle panel), in the skin only a proportion of Foxp3⁺CD4⁺ cells express Ki67 (14-33%, at all times following PPD injection, mean 23.2%). The kinetics of Ki67 expression in Foxp3⁺ (putative Tregs) and CD4⁺Foxp3⁻ (effector cells) are very similar (Figure 6-8B).

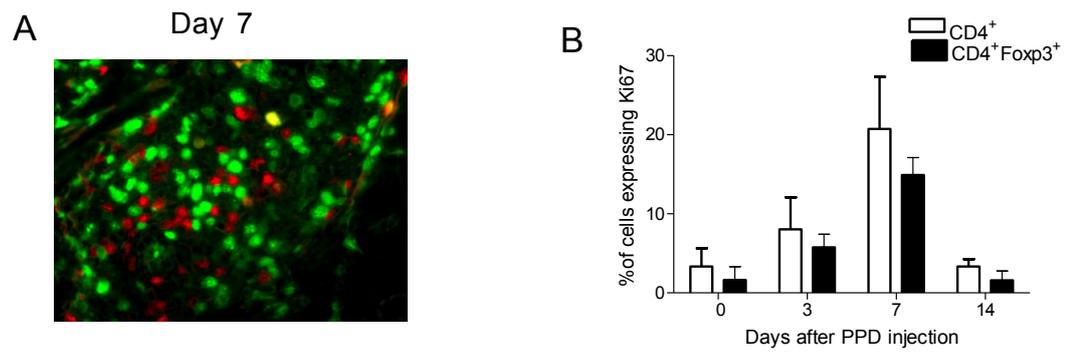


Figure 6-8: Foxp3⁺ T cells have a Treg phenotype and proliferate at the site of an immune response.

(A) Double immunofluorescence staining of Ki67(green)and Foxp3(red) in a representative day 7 skin section (original magnification, x400). Arrows indicate cells staining positive for both Ki67 and Foxp3 (yellow). (B) Graph shows percentage of CD4 cells expressing Ki67 (white bars) and percentage of Foxp3⁺ cells expressing Ki67 (black bars). Graph shows mean±SD, n=5 per time point.

Finally, to determine whether or not the cycling Foxp3⁺ cells (Ki67⁺) also exhibit a Treg phenotype, we compared the expression of CD127 on Foxp3⁺Ki67⁺ and Foxp3⁺Ki67⁻ T cells (Figure 6-9). Both cycling and non-cycling Foxp3 expressing populations expressed low levels of CD127 indicating that they were regulatory T cells (mean ± SEM: 8 ± 2.3 and 10.5 ± 3.2, n=6). In contrast, CD127 was highly expressed both in Foxp3⁻Ki67⁺ and Foxp3⁻Ki67⁻ populations (mean ± SEM: 55.7 ± 7 and 80.1 ± 2, respectively n=6) highlighting the fact that CD127 expression is a good marker to distinguish between Tregs and non-regulatory T cells. Therefore, Foxp3⁺ cells that accumulate during the course of MT have multiple phenotypic and functional characteristics of naturally occurring regulatory T cells and a significant proportion of these are proliferating in parallel with the corresponding effector T cells.

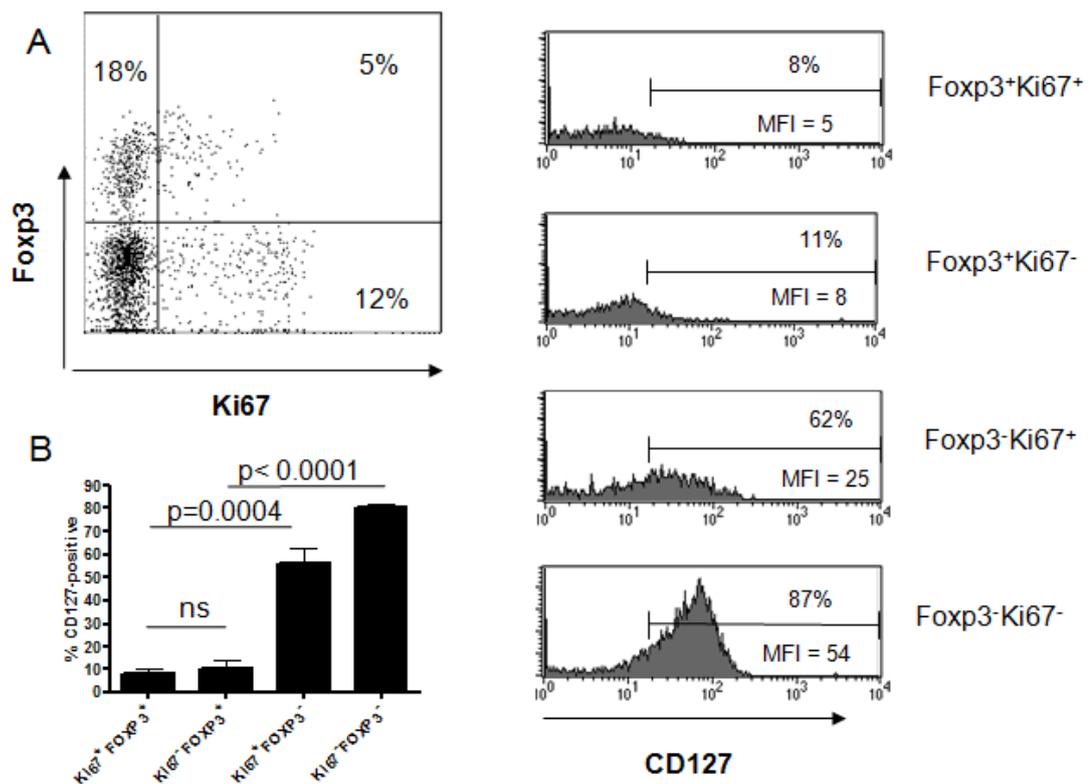


Figure 6-9: Proliferating Foxp3⁺ T cells in the skin express low levels of CD127.

Blister cells were isolated on day 7 following MT induction and stained for CD4, Foxp3, CD127 and Ki67. Representative staining (n=6) is shown. (A) Dot plot indicates gating strategy. The percentage of cells in each quadrant is indicated. Histograms show expression of CD127 on CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ subsets divided by Ki67 expression. Numbers indicate the percentage of cells expressing CD127. (B) Cumulative data comparing Foxp3⁺Ki67⁺ and Foxp3⁺Ki67⁻ subsets. P-values were calculated using a paired t-test. Data are mean +/-SEM.

6.8. Increased representation of CD4⁺Foxp3⁺ regulatory T cells (Tregs) in the skin of old humans

Previous studies have shown that CD4⁺Foxp3⁺ Tregs can inhibit TNF- α secretion by macrophages (Tiemessen et al., 2007; Taams et al., 2005). We therefore investigated whether the decreased macrophage production of TNF- α in old subjects was associated with increased proportions of these cells in the skin. In the data presented above we showed that cutaneous CD4⁺Foxp3⁺ T cells expressed typical phenotypic

and functional characteristics of regulatory cells (Vukmanovic-Stejic et al., 2008). There was a significant increase in the proportion of these cells in the skin of old subjects compared to the young group either before, or after injection of antigen (Figure 6-10 A,B). This data suggests that the over-representation of Tregs in the skin may explain in part the decreased TNF- α secretion by cutaneous macrophages in old humans.

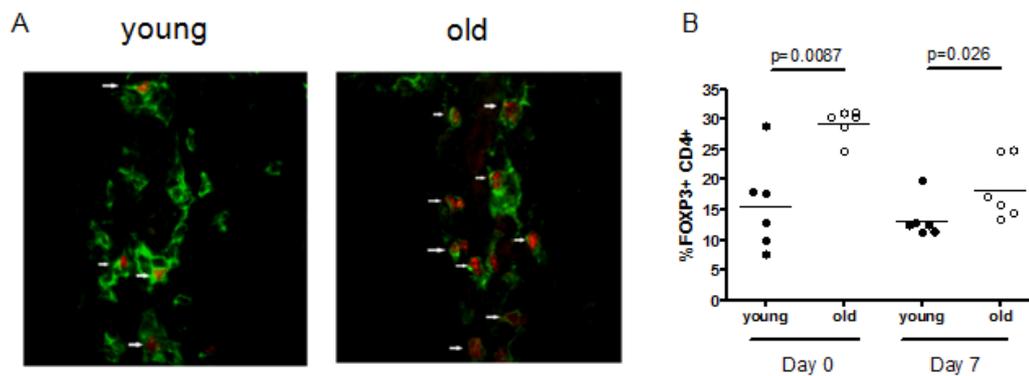


Figure 6-10: Numbers of CD4⁺Foxp3⁺ Tregs are increased in the skin of old subjects.

(A) Double immuno-fluorescence staining of representative biopsies from young and old normal skin; CD4⁺ (green) and Foxp3⁺ (red) cells in a perivascular lymphocytic infiltrate. CD4⁺Foxp3⁺ cells are indicated by a white arrow. (B) Percentage of CD4⁺ cells expressing Foxp3 per perivascular infiltrate counted. Five largest perivascular infiltrates present in the upper and mid dermis were selected for analysis for each subject. Each symbol represents an average of 5 perivascular infiltrates counted for each individual (n= 5-7 subjects per time point, line indicates the mean).

6.9. Discussion

We have clearly shown that cells expressing Foxp3 in human skin during a secondary memory cutaneous immune response have a proliferation rate that equals that of nTregs in blood (~10-25% of cells are in cycle at any one time) and also exhibit the functional characteristics of nTregs. Overall our results indicate that the accumulation of Tregs closely parallels the expansion of CD4⁺ effector T cells at the site of a controlled immune response. This intimate relationship between both

populations suggests that memory T cell proliferation is closely controlled by CD4⁺CD25^{hi} Tregs at the peripheral sites of immune reactivity. Moreover Tregs have recently been implicated in the regulation of macrophage phenotype and function and we applied our characterisation of nTregs in the mantoux model to the candida skin test model in young and old skin to ascertain if differences in nTreg numbers could explain the lack of TNF- α expression by macrophages in old skin as shown in Chapter 5.

In humans, Foxp3 expression does not always correlate with regulatory activity as it is induced on recently activated CD4⁺ effector populations (Walker et al., 2005). The question therefore arises as to whether the CD4⁺Foxp3⁺ T cells that we observed in the skin are actually Tregs or recently activated effector cells. CD4⁺Foxp3⁺ cells isolated from skin blisters expressed all the phenotypic hallmarks associated with natural regulatory T cells (CD25^{hi} CD39^{hi} CD127^{lo}) irrespective of whether or not they were in cell cycle. In contrast Foxp3⁻ T cells were CD25^{lo}, CD39^{lo} and mainly CD127^{hi}. Furthermore, previous studies have shown that in inflamed tissues, high expression of CD27 can be used to distinguish regulatory T cells from activated CD25⁺CD4⁺ effectors (Ruprecht et al., 2005) and we found that Foxp3⁺ cells isolated from the skin showed uniformly high expression of CD27. Finally, CD4⁺Foxp3⁺ cells isolated from skin blisters did not produce any cytokines associated with effector T cells (IL-2, IFN- γ). In contrast, CD4⁺Foxp3⁻ population synthesized IL-2 and IFN- γ following Ag-specific re-stimulation *in vitro*. This is in agreement with a widely accepted view that Foxp3⁺ regulatory T cells do not produce IL-2 or IFN- γ (Tiemessen et al., 2007; Pandiyan et al., 2007; Scott-Browne et al., 2007; Levings et al., 2001). Taken together these results strongly support the view that the Foxp3⁺ T cells that are found in the skin after secondary antigenic challenge are a regulatory T cell population, however we were not able to test regulatory function directly due to limitations in cell numbers that were obtained from suction blisters.

The origin of the Tregs in the skin is not clear. High expression of skin homing receptors CLA and CCR4 on peripheral blood Foxp3⁺ cells (Hirahara et al., 2006) and on Foxp3⁺ cells recovered from skin after antigenic challenge suggests that some

of the Tregs in the skin may be recruited from the blood (Hirahara et al., 2006; Chong et al., 2004). Furthermore, we and others (Clark and Kupper, 2007) have demonstrated that $CD4^+CD25^{hi}Foxp3^+$ Tregs are present in normal skin and it is possible that the increase in numbers of these cells at the site of antigenic challenge may reflect the proliferation of skin-resident cells. A third but not mutually exclusive possibility is that some $CD4^+CD25^{hi}Foxp3^+$ Tregs may be derived from responsive memory $CD4^+$ T cell populations during the immune response in the skin *in vivo* (Akbar et al., 2007).

In Chapter 5 we showed that $CD163^+$ macrophages in skin from old skin subjects show drastically reduced expression of $TNF-\alpha$. It is interesting that the number of $CD4^+FoxP3^+$ Tregs is increased in old skin sections compared to young, both at baseline and throughout the course of the response to candida skin test, since Tregs have been recently implicated in the control of monocyte/macrophage phenotype (Tiemessen et al., 2007). Monocytes/macrophages are involved in both the initiation and resolution of an inflammatory immune response. The resolution phase of an immune response is characterised by alternatively-activated macrophages (AAMs) which produce mainly anti-inflammatory cytokines (Mosser and Edwards, 2008). The excessive presence of the latter type of macrophage could cause damage to the host because this could prevent an appropriate immune response leading to hampered cutaneous tumour and infection immunity. Treg-modulated monocytes have up-regulated CD206 and CD163 expression, increased phagocytic activity, increased CCL18 and IL-1Ra production, decreased HLA-DR expression, and down-regulated proinflammatory cytokine/chemokine production (Tiemessen et al., 2007). Recent *in vitro* and *in vivo* studies have shown that macrophages in tissues can undergo phenotypic switches from M1 to M2 macrophages and vice versa (Lumeng et al., 2007; Arnold et al., 2007; Porcheray et al., 2005). Therefore when entering tissues, monocytes/macrophages will not only be affected by the local microenvironment (cytokines, chemokines, growth factors, and tissue cells) but also by the presence of activated effector T cells and Tregs. For example, on this basis, it can be hypothesised that the increased presence of Tregs in skin from old subjects skews newly recruited monocytes towards an AAM phenotype. This would mimic the

situation previously described at tumour sites (Curiel et al., 2004; Woo et al., 2001) where the increased presence of Tregs together with the tolerogenic milieu skews newly recruited monocytes toward an AAM phenotype. Indeed the presence of M2-like tumour-associated macrophages with low proinflammatory and high phagocytic capacity have been described in various human and mouse tumours (Sica et al., 2006; Van Ginderachter et al., 2006). Thus Tregs may hamper effective tumour immunity not only by inhibiting CD4⁺ and CD8⁺ T cell responses but also by steering monocytes/macrophages toward an alternatively activated phenotype and function. Tregs may therefore promote the induction of AAM/macrophages, which will help to maintain tissue homeostasis and prevent local tissue damage but may also contribute to hampered antitumor immunity. This newly discovered ability of Tregs may help us to understand many disease processes and may also provide a novel tool to manipulate local immune responses.

Squamous cell carcinomas (SCCs) of the skin are sun-induced skin cancers that are particularly numerous in patients on T cell immunosuppression and become more prevalent with increasing age. Clark et al have recently reported that approximately 50% of the T cells infiltrating SCCs were FoxP3⁺ Tregs. The blood vessels in the SCC tumour itself were also found to express less E-selectin than surrounding unaffected skin and there were few CLA⁺ T cells infiltrating the tumour. The authors concluded that the SCC was evading the immune response by downregulating E-selectin expression thus preventing uptake of CLA⁺ immunosurveillance cells, and the SCC is also recruiting immunosuppressive Tregs. They also found that when tumours were treated with Imiquimod (a TLR7 agonist applied in topical cream formulation), they contained a decreased percentage of Treg cells and showed induction of E-selectin on tumour vessels together with recruitment of CLA⁺ CD8⁺ T cells, and histological evidence of tumour regression. SCCs treated *in vitro* with Imiquimod also expressed vascular E-selectin. They therefore found that the TLR7 agonist neutralized both of these strategies, supporting its use in SCCs and other tumours with similar immune defects (Clark et al., 2008). We have also found reduced expression of E-selectin as well as increased nTreg numbers during the course of a secondary memory cutaneous immune response in skin from old subjects.

It is possible that the initial problem in old skin is Treg induced immunosuppression which induces an AAM phenotype which allows the SCC to develop and that the TLR7 agonist used in this study reverses this process by directly activating the suppressed macrophages in old skin. This is currently under investigation.

In summary we have shown that regulatory T cells and effector memory T cells proliferate in parallel during a secondary memory cutaneous immune response in vivo. In the skin microenvironment from old subjects, the presence of increased numbers of Tregs might be skewing the monocytes present into an AAM phenotype thus favouring less production of TNF- α by CD163⁺ macrophages which leads to reduced endothelial activation and reduced entry of effector memory T cells into the dermis in old subjects.

Chapter 7. Summary and future directions

There is an increased incidence of cutaneous malignancy and infection with advancing age in humans (Diffey and Langtry, 2005; Laube, 2004). In addition, the incidence and severity of contact allergic eczema is decreased with ageing (Balato et al., 2008; Piaserico et al., 2004). Both of these facts suggest a reduction in skin immunity with age. The central observation of this study is that the cutaneous DTH response to challenge by bacterial, fungal and viral recall antigens injected into the skin was defective in older humans. The cutaneous DTH response to antigen is widely considered to be a manifestation of memory T cell responsiveness *in vivo*. An unexpected observation of this study was that the defect in the response to cutaneous antigenic challenge was not in the memory T cell compartment, but in the innate conditioning of the skin after antigen stimulation.

Cells of the innate immune system in the skin sense danger signals after antigen injection and respond appropriately with the release of pro-inflammatory mediators which leads to conditioning of the skin so that a memory T cell response can develop. This is the first study to show that lack of TNF- α production by CD163⁺ macrophages in old skin leads to impaired cutaneous secondary immune responses to antigen with ageing. Furthermore we show low level expression of E-selectin, ICAM-1 and VCAM-1 on dermal endothelium secondary to decreased TNF- α in the old skin environment. This in turn leads to the reduced infiltration of antigen specific CD4⁺ T cells that we found both on histology and in blister fluid of secondary cutaneous immune responses in old subjects. Hence the majority of old individuals are unable to respond effectively to recall antigen despite prior exposure.

Although T cell numbers are decreased after antigen challenge, macrophages identified by CD163 (this study) or CD68 and CD14 (unpublished data) are present in equal numbers in young and old skin. This is likely due to the different trafficking requirements and preferential reliance on DC-SIGN/ICAM-2 interactions that mediate dendritic cell trafficking (Geijtenbeek et al., 2000) and is strongly expressed on CD163⁺ macrophages in both normal and inflamed skin (Zaba et al., 2007). We

have also shown that high levels of DC-SIGN are expressed on activated peripheral blood monocytes. ICAM-2 is constitutively expressed on endothelial cells and in contrast to E-selectin, VCAM-1 and ICAM-1, its expression is not dependent on the presence of TNF- α (Silverman et al., 2001). Activated monocytes may therefore enter the skin through an alternative pathway of transmigration through the endothelium compared to memory T cells. Furthermore, significant numbers of CD163⁺ macrophages are present in the skin of both young and old subjects even without injection of antigen therefore the recruitment of circulating myeloid cells at the very early stages of the response may not be a limiting step in DTH responses of old humans.

The dominant role of TNF- α in regulating leucocyte migration via the activation of endothelial cells is supported by studies using murine models of contact hypersensitivity. It was shown that selectively blocking TNF- α significantly inhibited the up-regulation of ICAM-1 and VCAM-1 expression and the development of ear swelling in response to contact allergen, but blocking of IL-1 α and IL-1 β had no such effect (McHale et al., 1999). Furthermore, it was found that anti-TNF- α therapy in humans decreases the expression of adhesion molecules E-selectin, ICAM-1 and VCAM-1 in various assays, leading to reduced trafficking of leucocytes (Paleolog et al., 1998). This decreased endothelial activation is associated with an increase in the frequency of skin and soft tissue infections in anti-TNF- α treated rheumatoid arthritis patients compared to those receiving traditional disease-modifying anti-rheumatic drugs (DMARDs) (Dixon et al., 2006).

The need for appropriate leucocyte migration for immunosurveillance and the prevention of disease is further underscored by the use of anti- α 4 β 1 integrin monoclonal antibody (mAb) that binds to VLA-4, the main homing molecule involved in lymphocyte migration to inflamed brain in rodent models of multiple sclerosis (Engelhardt et al., 1995). This mAb is thought to work by blocking the ability of VLA-4 to bind to its endothelial counter-receptor, VCAM-1. In so doing it led to reduction of the inflammatory infiltrate in brain tissue and blocked clinical paralysis in various animal models of multiple sclerosis (Deloire et al., 2004;

Yednock et al., 1992; Theien et al., 2003). However, the use of Natalizumab, a humanised monoclonal antibody to $\alpha 4\beta 1$ integrin, for treatment of multiple sclerosis led to the development of severe opportunistic brain infections (Steinman, 2005). Thus inhibiting leucocyte migration in humans can lead to severe pathological consequences.

The initiation of an inflammatory immune response depends on the ability of local antigen presenting cells to recognise danger signals in response to tissue injury, microbial infection and other changes of homeostasis (such as tumour cells or contact allergens;(Matzinger, 2007). The macrophages play a critical role in this respect through their ability to recognise danger signals via their TLRs and other pattern-recognition receptors which leads to the local production of pro-inflammatory cytokines (Gordon, 2007). This study and others have shown that the macrophage is the main source of TNF- α production during a DTH in the skin (Chu et al., 1992). We used CD163 and CD11c markers to distinguish between cutaneous macrophages and dermal dendritic cells respectively and found that TNF- α is abundantly expressed by CD163⁺ macrophages in young skin during a DTH response but not in old. Moreover we found no significant difference in the numbers of CD163⁺ macrophages in normal or antigen injected young and old skin, pointing to functional impairment in macrophages in old skin accounting for sub-optimal immunity rather than reduced recruitment or fewer resident skin macrophages.

Although our finding of reduced TNF- α expression by cutaneous macrophages is clear-cut it could be argued that the lack of T cells early on in the response is also responsible for reduced cytokine release and endothelial activation. This question is difficult to establish in our human model, however a study carried out in a murine model of contact hypersensitivity elegantly clarifies this issue. McHale et al showed that if T lymphocyte migration into skin was blocked by intraperitoneal injection of mAb against CD11a and VLA-4 integrins at 1 hour prior to cutaneous challenge with oxazolone, there was no down-regulation of ICAM-1 and VCAM-1 expression at 4 hours compared to control suggesting that initial cytokine release required for proper endothelial activation happened as normal despite lack of T cells infiltrating the

response. However similar blockade of T cell migration at 24 hours led to a significant downregulation of ICAM-1 and VCAM-1 compared to control suggesting that at this later stage of the immune response T cell cytokine release is essential for continued expression of endothelial adhesion molecules (McHale et al., 1999). Therefore T cell signals are needed to maintain expression of endothelial adhesion molecules.

The defect in macrophage triggering to secrete TNF- α in the skin, raises the question of whether there is a defect in endothelial activation during immune responses in other organs during ageing as the potential decrease in T cell immunosurveillance, may contribute to the reported increase in susceptibility of old individuals to a wide range of infections (Yoshikawa, 2000; Nicholson et al., 1997; Schmader, 2001).

An important unanswered question is why macrophages in the old skin are not triggered to secrete TNF- α . Previous studies have shown that there is an age associated defect in human TLR1 and 2 function that results in a significant defect in TNF- α secretion after ligation of these receptors (van Duin et al., 2007). Since candida activates TLR1 and 2, this may explain the cutaneous defect in macrophage-derived TNF- α that we have observed. It has also been shown that there is a defect in TLR4 function and expression and function during ageing (Renshaw et al., 2002; van den Biggelaar et al., 2004) although this has not been confirmed in other studies (van Duin and Shaw, 2007). However, we found that both peripheral blood monocytes and isolated cutaneous macrophages from donors of both age groups synthesized similar levels of TNF- α after TLR 1/2 and TLR-4 stimulation *in vitro*. We conclude therefore that cutaneous macrophages in old humans are not inherently defective in terms of TNF- α synthesis but do not rule the possibility that other functions in these cells may be altered during ageing. This is currently under investigation.

In addition to the need for TLR signalling for macrophage activation, these cells must also interact with cytokines such as IFN- γ to become fully functional. Previous studies have shown that IFN- γ is mainly secreted by T cells during DTH responses (Chu et al., 1992). The lack of IFN- γ in the skin in our studies may reflect the

decreased infiltration of T cells into the site of antigen challenge. Activation by IFN- γ leads to the expression of several genes that regulate macrophage biology including expression of MHC Class II genes (Celada and Maki, 1991; Cullell-Young et al., 2001; Gonalons et al., 1998), which are crucial for presenting antigens to T lymphocytes. It is possible that the lack of T cell signals such as IFN- γ early in the DTH response in old subjects may prevent the cascade of events required for its amplification.

An alternative possibility is that macrophages in the skin of old individuals may be inhibited functionally *in situ*. It has been shown that human CD4⁺Foxp3⁺ Tregs are potent inhibitor of macrophage activation and TNF- α secretion by these cells (Tiemessen et al., 2007; Taams et al., 2005). In addition, in a previous study we showed that there is a significant increase in functional circulating Tregs in old individuals (Vukmanovic-Stejic et al., 2006) which is in agreement with the observation that functional Tregs accumulate in tissues of aged mice (Lages et al., 2008). We now show that there are significantly higher proportions of CD4⁺Foxp3⁺ T cells in the skin of old volunteers either before or 7 days after injection of candida antigens. Although circumstantial, this suggests that the accumulation of Tregs in the skin may be one possible mechanism for the observed decrease of TNF- α secretion by cutaneous macrophages in old individuals. This is currently under investigation.

The innate immune system has evolved to allow humans to live together with commensal microorganisms whilst at the same time keeping the growth of microbes under tight control. However the activation of the innate immune system has side-effects ie tissue damage to the host caused by inflammation. The reduced activation of the innate immune system in old skin could be an attempt to protect against a state of chronic inflammation that would result if the immune system was targeting debris that accumulates with age in the skin, in a similar way to increasing debris deposited in the blood vessel wall (atherosclerosis leading to heart attacks and strokes) and retina (lipofuscin pigments leading to age related macular degeneration) with age (Richards et al., 2007). This implies that tuning down but not turning off innate surveillance may delay the ageing of tissues and prevent or attenuate inflammatory

disorders. It is clear that a balance must be achieved within the local innate immune system in specific organs such as the skin in the old between too much inflammation and too little protection against infection. We postulate that this imbalance in certain old individuals may lead to the increased frequency of infections and cutaneous tumours that are found in this age group.

There are various examples in the body where an attenuation of the immune response is desirable and are worth examining as they could parallel our observations in ageing skin immunity; the gut immune system can distinguish between harmless commensal microorganisms and dangerous pathogens, and attenuates its response to the former to avoid dangerous chronic inflammation. Dendritic cells (DCs) are crucial to intestinal immune regulation because of their roles in inducing protective immunity against pathogens while maintaining tolerance to commensal bacteria. The mechanisms that maintain this hyporesponsiveness are just beginning to be understood. It has been shown that DCs migrating from the rat intestine in lymph are hyporesponsive to LPS stimulation, thus possibly preventing harmful immune responses being induced to commensal flora. Furthermore it seems like hyporesponsiveness might only be restricted to LPS and not to other molecular associated molecular patterns (MAMP) (Cerovic et al., 2009). In fact these DCs were able to react to all TLR ligands they were subjected to *in vitro* apart from TLR4. Therefore the gut innate immune system or the local gut microenvironment in healthy young individuals is able to finetune itself so as to respond to certain bacterial elements only and thus prevent chronic longterm inflammation. In a similar way the skin microenvironment might place a selective block on responses of the skin innate immune system to prevent chronic inflammation.

The DTH response to recall antigen in the skin has been used as a marker of systemic immunity (Takahashi et al., 2003; Almeida et al., 2001). Our results indicate that older subjects have decreased ability to mount a clinical response to recall antigen challenge in the skin, which is also associated with a decreased antigen-specific CD4⁺ T cell accumulation. However this study demonstrates for the first time that in the old volunteer cohort this does not equate to decreased immunity, as the numbers

of antigen-specific T cells in peripheral blood and their ability to respond to antigen is not different in subjects in both age groups. This points to a skin specific rather than a general decline in cell-mediated immunity. This has particular relevance in the context of Mantoux skin testing in the elderly. In the developed world tuberculosis is more prevalent in the elderly population than other age groups. The Mantoux test is used widely to assess for both immunity to and potential infection with tuberculosis. However our study clearly demonstrates that mantoux skin testing in the elderly is not ideal for assessing presence of immunity to tuberculosis in the old.

The clinical relevance of our findings of a suppressed innate immune system locally within the skin of old subjects is highlighted by the observation that Imiquimod, a TLR7 agonist which is applied topically is useful in the treatment of non-melanoma skin cancer in the elderly, and infections such as viral warts (Beutner et al., 1999). This study links lack of macrophage-derived TNF- α in response to antigen challenge with ineffective cutaneous immunity in the old population. Given the shift towards an ageing population and the high prevalence of non-melanoma skin cancer in this age-group, we propose that new strategies aimed at inducing macrophage-derived TNF- α should be considered both for prevention and early therapeutic intervention in non-melanoma skin cancers and cutaneous infections in old individuals.

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