Investigating strategies to boost cutaneous varicella zoster virus-specific immune responses in ageing humans

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I, Neil Pradip Patel, 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.

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ABSTRACT

The age-related decline in the immune system, known as immunosenescence, predisposes old individuals to increased mortality and morbidity from infectious diseases (such as shingles, caused by reactivation of varicella zoster virus, VZV) and is associated with impaired vaccine responses. While multiple age-related changes have been identified in circulating memory T cells, little is known about the effects of ageing on memory T cells within peripheral tissues.

T cells within human skin were characterised, and surprisingly neither their numbers, differentiation markers or effector functions were altered during ageing. VZV-specific $CD4^+$ T cells were more frequent in the skin than in the blood, and their frequency in the skin did not decline with age. Increased PD-1 expression on T cells and an increased frequency of regulatory T cells in ageing skin consolidated evidence that old skin represents an inhibitory microenvironment.

Vaccination of old individuals with the shingles vaccine Zostavax® did not alter the total number of T cells, or frequency of VZV-specific T cells, in the skin but did boost the frequency of circulating VZV-specific T cells. This was associated with a heightened delayed type hypersensitivity (DTH) response to VZV antigen, and with an upregulation of genes involved in T cell migration and activation. It is proposed here that Zostavax® prevents shingles by enhancing the recruitment of circulating VZV-specific memory T cells to sensory nerves during episodes of silent VZV reactivation.

Increased early expression of p38 MAPK-associated pro-inflammatory genes has been observed in VZV- or saline-challenged old skin and was associated with poor DTH responses to VZV antigen. In an experimental medicine study, pre-treatment of ageing individuals with the p38 MAPK inhibitor losmapimod effectively restored robust VZV-specific DTH responses. Ageing of the population necessitates improved vaccination strategies, and p38 MAPK inhibition prior to vaccine administration presents a potential therapeutic opportunity to achieve this.

IMPACT STATEMENT

The human population is rapidly ageing, with the United Nations predicting the number of people aged over 60 will more than double to 2.1 billion in 2050 and more than triple to 3.1 billion in 2100. A multi-faceted decline in the ageing immune system, known as *immunosenescence*, renders old people more susceptible to cancer and infectious diseases, such as shingles, influenza and pneumonia. The weakened immune system in the old is compounded by the fact that ageing people respond poorly to vaccination. Concurrently, old people are more prone to developing increased inflammation in the body, which can contribute to the onset of arthritis, heart disease and dementia. This excessive inflammation in old age, known as *inflammaging*, has also been associated with poor vaccine responses.

Shingles is a painful and debilitating skin disease caused by reactivation of varicella zoster virus (VZV), the same virus that causes chickenpox. In the UK there are over 50,000 cases of shingles in the over 70s each year, and the lifetime risk of developing shingles is 1 in 4. Shingles is more frequent, more severe, and more likely to lead to chronic pain (post-herpetic neuralgia) in the old – yet it remains unknown why VZV reactivates in certain individuals. In work funded by the skin disease charities Dermatrust and the British Skin Foundation, we used a novel research strategy to investigate how the shingles vaccine Zostavax® boosts skin immunity. We induced immune responses to VZV in the skin of ageing volunteers before and after vaccination, and by taking skin biopsies over the course of 7 days we were able to delineate the kinetics of the skin immune response at the cellular level. This is a research technique that could be adopted by vaccinologists researching the mechanism of action of other vaccines.

Increased inflammation was observed in ageing skin and this was associated with impaired skin immunity to VZV. In an experimental medicine project funded by an MRC Grand Challenge grant in collaboration with GlaxoSmithKline, we pre-treated ageing volunteers with an anti-inflammatory drug (the p38 MAP kinase inhibitor, losmapimod) for 4 days, and this led to an enhancement in their skin immunity to VZV. This suggests that blocking inflammation may be a strategy for boosting cutaneous immunity during ageing. We plan to extend this concept to a forthcoming trial investigating whether losmapimod enhances immune responses to the influenza vaccine in ageing individuals. The implications of this work are

potentially far-reaching with regards to optimising vaccination strategy and enhancing the health of older people.

During this work we engaged the public by regularly hosting “Ageing Immunity” Open Days at UCL, where we showcased our immunology research, educated the public with talks and interactive sessions, and offered ageing individuals the opportunity to volunteer for immunology research studies.

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ABBREVIATIONS

AMPK	5' adenosine monophosphate-activated protein kinase
AP-1	Activator protein 1
APC	Antigen-presenting cell
Blimp1	B lymphocyte-induced maturation protein-1
BSA	Bovine serum albumin
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte antigen
CMI	Cell-mediated immunity
CMV	Cytomegalovirus
COX	Cyclooxygenase
CRP	C-reactive protein
CTCL	Cutaneous T cell lymphoma
CTL	Cytotoxic lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAPI	4′,6-diamidino-2-phenylindole
DC	Dendritic cell
DDC	Dermal dendritic cell
DEG	Differentially expressed genes
DMSO	Dimethyl sulfoxide
DTH	Delayed type hypersensitivity
EBV	Epstein-Barr virus
EI	Erythema index
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked ImmunoSpot
Eomes	Eomesodermin

FABP	Fatty-acid-binding protein
FBS	Fetal bovine serum
FDR	False discovery rate
FFA	Free fatty acids
FMO	Fluorescence minus one
Foxp3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
gE	Glycoprotein E
H2AX	H2A histone family, member X
HBSS	Hanks' Balanced Salt Solution
HIV	Human immunodeficiency virus
Hobit	Homolog of Blimp1 in T cells
HSV	Herpes simplex virus
ICAM-1	Intercellular adhesion molecule 1
ICOS	Inducible T-cell costimulator
ICOSL	Inducible T-cell costimulator ligand
IFN	Interferon
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
KLF2	Krüppel-like Factor 2
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LAG-3	Lymphocyte-activation gene 3
Lck	Lymphocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex

mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor κB
NK cell	Natural killer cell
NSAID	Non-steroidal anti-inflammatory drug
P38 MAPK	p38 mitogen-activated protein kinase
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD-1	Programmed cell death 1
PFU	Plaque-forming unit
PGE2	Prostaglandin E2
PHN	Post-herpetic neuralgia
PMA	Phorbol 12-myristate 13-acetate
PPD	Purified protein derivative
PRR	Pathogen recognition receptor
RCF	Responder cell frequency
ROS	Reactive oxygen species
S1P	Sphingosine 1-phosphate
$S1P_1$	Sphingosine 1-phosphate receptor
SASP	Senescence-associated secretory phenotype
SEB	Staphylococcal enterotoxin B
SGC	Satellite glial cell
SLO	Secondary lymphoid organ
SPS	Shingles Prevention Study
T-bet	T-box expressed in T cells
T_{CM}	Central memory T cell

TCR	T cell receptor
T_{EM}	Effector memory T cell
T_{EMRA}	Effector memory T cell re-expressing CD45RA
Tfh	T follicular helper cell
TGF-β	Transforming growth factor beta
Th	T helper cell
TIM-3	T-cell immunoglobulin and mucin domain 3
TLR	Toll-like receptor
T_{MM}	Migratory memory T cell
TNF	Tumour necrosis factor
Treg	Regulatory T cell
T_{RM}	Tissue-resident memory T cell
T_{SCM}	Stem cell memory T cell
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen-4
VZV	Varicella zoster virus
ZAP-70	Zeta-chain-associated protein kinase 70

1 INTRODUCTION

Ageing is accompanied by a multi-faceted decline in virtually all components of the immune system, a process known as immunosenescence[1,2]. As a consequence, aged humans are more susceptible to morbidity and mortality from infectious diseases and cancer[1–4], and lose the ability to respond effectively to vaccination[5,6]. Old individuals are more susceptible to infections that are novel[7], previously encountered[8], or reactivated from a latent state – such as shingles, due to reactivated varicella zoster virus (VZV)[9,10]. Meanwhile, hyperresponsiveness of the innate immune system leads to increased inflammation in tissues, predisposing the ageing individual to osteoarthritis, atherosclerotic disease and neurodegenerative disease[11]. The human population is ageing rapidly, such that the United Nations predicts the number of people aged over 60 will more than double from 962 million in 2017 to 2.1 billion in 2050 and more than triple to 3.1 billion in 2100 [12]. The projected increase in morbidity and mortality from age-related infectious diseases, coupled with the lack of effective measures to prevent them, raises an interest in investigating the mechanisms underlying immunosenescence – ultimately in order to inform strategies to boost immunity during old age.

1.1 T Cell Biology

1.1.1 T cell development

Haematopoietic stem cells in the bone marrow give rise to lymphoid progenitors, some of which migrate via the blood to the thymus, where they differentiate and proliferate intensely, producing large numbers of thymocytes. Thymocytes undergo gene rearrangement of their T cell receptors (TCR), generating a colossal breadth of antigen specificity across the naïve T cell population. Rigorous positive and negative selection occur, ensuring the 2% of surviving thymocytes can recognise self-peptide:self-major histocompatibility complex (MHC) complexes and are self-tolerant. Mature T cells, single positive for either CD4 or CD8, are exported from the thymus into the peripheral circulation as naïve T cells, specific for a given epitope. $CD4^+$ T cells recognise peptide bound to MHC class II molecules, expressed by antigen presenting cells (APCs), while $CD8^+$ T cells recognise peptide bound to MHC class I molecules, expressed by all nucleated cells in the body. The thymus also generates a smaller population of

$CD4^+$ T cells termed natural regulatory T cells (nTregs) that express the transcription factor Forkhead Box p3 (Foxp3). These cells have high expression of CD25 and CTLA-4, and serve to maintain self-tolerance[13].

1.1.2 T cell activation

Dendritic cell activation

Dendritic cells (DCs) are specialised APCs. They take up antigen in peripheral tissues and migrate to secondary lymphoid organs (SLOs), where they present peptide:MHC class II complexes to naïve $CD4^+$ T cells. DCs sense and are activated by pathogen-associated molecular patterns (PAMPs), which they recognise using pathogen recognition receptors (PRRs), including multiple Toll-like receptors (TLRs). Activated DCs intensify their antigen-processing and upregulate the lymph node homing marker CCR7, facilitating their egress from the periphery and entry into the draining lymph node (LN). By the time it arrives in the LN, the mature DC has increased its expression of MHC molecules, adhesion molecules, the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2), and secretes the chemokine CCL18 which attracts naïve T cells[14].

Naïve T cell entry into lymph nodes

Naïve T cells, expressing the lymph node homing markers CCR7 and CD62L, access the SLOs from the blood, and pass through the T cell zones of lymph nodes or the white pulp of the spleen. There, they scan the peptide:MHC complexes on the surface of thousands of DCs every day, to enhance the probability of encountering their cognate antigen. If their specific antigen is not encountered, the naïve T cells re-enter the circulation via efferent lymphatics (or blood vessels in the case of the spleen). Egress from the SLO is controlled by the concentration gradient of the lipid molecule sphingosine 1-phosphate (S1P), recognised by the S1P receptor ($S1P_1$) on the surface of the T cell. The concentration of S1P is higher in the blood and lymph than in the SLO, encouraging the T cell to leave the SLO[15].

T cell activation

During their migration through the cortical region of the lymph node, naïve T cells bind transiently to each APC they encounter, aided by the interactions between cell-adhesion molecules on both cell surfaces, including LFA-1 on the T cell. In

the rare event of the naïve T cell recognising cognate antigen presented by the APC, the affinity between the two cells increases and the association may persist for several days, during which the T cell becomes activated, proliferates and differentiates into effector T cells.

Activation of naïve T cells requires the delivery of 3 signals to the cell. Signal 1 involves the interaction of the TCR with its specific peptide:MHC complex on the surface of an APC. This interaction is stabilised by co-receptors, including the CD3 complex, which contains immunoreceptor tyrosine-based activation motifs (ITAMs) that participate in signal transduction, and also the co-receptors CD4 or CD8, which bind MHC as previously mentioned. CD4 and CD8 recruit lymphocyte-specific protein tyrosine kinase (Lck) on their cytoplasmic tails to the TCR-CD3 complex, leading to phosphorylation of the ITAMs associated with this complex. This allows recruitment and phosphorylation of zeta-chain-associated protein kinase 70 (ZAP-70) and further downstream signalling events, eventually leading to induction of transcription factors, including nuclear factor of activated T-cells (NFAT), activator protein 1 (AP-1) and nuclear factor κB (NFκB), that promote IL-2 gene expression[16].

Signal 2 comprises co-stimulatory signals delivered by the APC, characteristically involving the binding of B7 molecules on the APC surface with CD28 on the T cell. This signal is essential for the optimal clonal expansion of naïve T cells. CD28 co-stimulation contributes to the production of IL-2 by the T cell, which acts in an autocrine manner to promote T cell survival, proliferation and differentiation. Additional co-stimulation may be provided by the following pairs of co-stimulatory receptors on T cells and ligands on APCs respectively: ICOS and ICOS ligand (ICOSL); CD27 and CD70; OX40 and OX40L; 4-1BB and 4-1BBL. CTLA-4 on T cells also binds B7 molecules, but this leads to an inhibitory signal as opposed to a co-stimulatory one. Antigen recognition in the absence of co-stimulation leads to T cell deletion or anergy[17].

Signal 3 relies on cytokine production by the APC, which directs the polarisation of the T cell into various subsets, including Th1, Th2, Th17, Th9, Th22, T follicular helper cells (Tfh) and induced regulatory T cells (iTregs)[18,19]. These subsets are defined based on the cytokines they secrete, and contribute different roles to the immune response, as shown in Table 1-1.

T cell subset	Characteristic cytokines	Cell type assisted / recruited	Physiological role
Th1	IFN-γ	Macrophages	Protect against intracellular bacteria
Th2	IL4, IL-5	Eosinophils, mast cells, plasma cells (producing IgE)	Protect against parasites
Th17	IL-17	Neutrophils	Protect against extracellular bacteria, fungi
Th9	IL-9		Protect against parasites
Th22	IL-22		Skin immunity
Tfh	IL-21	B cells	Antibody production
Induced Treg	TGF-β, IL-10		Self-tolerance

Table 1-1 Characteristics and functions of $CD4^+$ T cell subsets

Activation of $CD8^+$ T cells leads to their differentiation into cytotoxic lymphocytes (CTLs), which play a key role in killing host cells infected by intracellular pathogens, particularly viruses. Infected cells display viral antigens on their surface MHC class I molecules, which are recognised by CTLs and destroyed. $CD8^+$ T cell activation is generally assisted by $CD4^+$ effector T cells.

Effector T cell expansion and function

After intensely proliferating for several days, expanding up to 10^5-fold [20], activated T cells differentiate into effector T cells that can produce the molecules needed to perform their helper or cytotoxic functions. They increase their expression of the adhesion molecules LFA-1 and VLA-4 but lose expression of CD62L, facilitating their entry into peripheral sites of infection via inflamed endothelium. On encountering cognate antigen, effector T cells are able to perform their effector functions without requiring co-stimulation. Effector T cells bind to target cells via LFA-1 and CD2. If the TCRs recognise their specific antigen:MHC complexes, the interaction between the two cells is strengthened, and an immunological synapse is formed, through which the effector T cell releases its effector molecules. T cells secrete cytokines, which have a broad range of effects, and cytotoxic T cells also secrete cytotoxins (including perforin, granzymes,

granulysin and Fas ligand) which assist in the killing of infected cells. Once the infection has cleared, >95% of effector T cells die by apoptosis during the contraction phase, and the remaining T cells develop into long-lived memory T cells[21]. As an example of their longevity, virus-specific T cells have been detected at least 25 years after vaccination[22].

1.1.3 T cell memory

Memory subsets

Memory T cells are heterogeneous and have been divided into various subsets based on their phenotype, migration properties, effector functions and proliferative capacity. These subsets include central memory T cells (T_{CM}), stem cell memory T cells (T_{SCM}), effector memory T cells (T_{EM}), effector memory T cells re-expressing CD45RA (T_{EMRA}), and tissue resident memory T cells (T_{RM}) [23–25].

The characteristic phenotypic markers used to differentiate between these subsets include CCR7, CD62L, CD27 and CD45RA[25]. CCR7 and CD62L are both lymph node homing markers, facilitating the entry of the memory T cell into the lymph nodes to scan for cognate antigen. CD27 belongs to the TNF receptor family and acts as a co-stimulatory molecule to enhance T cell activation when it binds to its ligand CD70 on APCs. CD27 signalling promotes T cell survival and proliferation, and is required for the generation of memory T cells. CD45RA is the longest isoform of the leukocyte common antigen CD45, which is a tyrosine phosphatase that regulates the activation threshold of T cells.

T_{CM} have a $CCR7^+$ $CD62L^+$ $CD27^+$ $CD45RA^-$ phenotype and circulate between the lymph nodes and blood. They have high proliferative potential, and on stimulation produce high amounts of IL-2, but have low effector function.

T_{SCM} are a relatively rare subset with a $CCR7^+$ $CD62L^+$ $CD27^+$ $CD45RA^+$ phenotype that is typically associated with the naïve T cell compartment, as well as expressing CD95. They have high proliferative and self-renewal capabilities, with a multipotent ability to generate T_{CM}, T_{EM} and effector T cells, but have no effector function[26].

T_{EM} are $CCR7^-$ $CD62L^-$ $CD27^-$ $CD45RA^-$ and traffic between the blood and peripheral tissues. They display various adhesion molecules and chemokine receptors to facilitate access to inflamed tissues. They are less proliferative than T_{CM} but are potent immediate producers of effector cytokines such as IFN-γ.

T_{EMRA} are $CCR7^-$ $CD62L^-$ $CD27^-$ $CD45RA^+$ as well as $CD28^-$ and are present in the circulation. They are mostly present within the $CD8^+$ T cell compartment, and their frequency in blood is correlated with persistent cytomegalovirus (CMV) infection[27]. They are considered to be terminally differentiated and exhibit various features of senescence[28]. They exhibit a low capacity to proliferate or produce IL-2, but a high capacity for IFN-γ production.

T_{RM} remain confined to tissues and do not recirculate. They are $CCR7^-$ $CD62L^-$ but are harder to categorise using the above system, as they express their own set of unique phenotypic markers. This will be discussed below in the context of skin residency.

Factors determining effector and memory T cell differentiation

In view of the heterogeneous production of effector and memory T cells during an immune response, a number of different models have been proposed to explain the fate decisions made by activated naïve T cells[15,29,30]. The *effector-first* model proposes that the activated T cell proliferates and differentiates into effector T cells. These then subsequently die, or differentiate into memory T cells.

The *linear progression* or *decreasing potential* model suggests that repeated antigenic stimulation of activated T cells drives greater proliferation and passage through a stepwise differentiation programme. The consensus on the differentiation sequence appears to be T_N – T_{SCM} – T_{CM} – T_{EM} – T_{EMRA} and the endpoint is a terminal effector T cell that will eventually die.

The *asymmetric division* model suggests that a single activated precursor T cell divides unequally, such that the daughter cell closer to the APC becomes an effector T cell due to increased TCR signals through the immunological synapse, and the distal daughter cell becomes a memory T cell.

Similar to this is the *signal strength* model, whereby the strength of stimulatory signals early on during T cell activation determines the differentiation fate of the cell. Stronger signals drive clonal expansion and a more differentiated memory phenotype, but excessive signals lead to the formation of terminal effector cells (and eventual cell death). Thus, divergent cell fates are possible from a single precursor T cell. In reality there may be overlap between all of these models, and the same outcome is reached – the generation of a heterogeneous population of effector and memory T cells.

The factors that influence the differentiation of T cells into the various memory subsets remain a subject of debate. These factors include TCR signal strength, APC co-stimulation, proximity to APC at the time of T cell division, inflammatory cytokines, transcription factors and metabolic regulators[23]. The following are associated with differentiation of activated T cells into memory T cells as opposed to effector T cells:

- Reduced duration of antigen exposure and reduced inflammatory stimuli during T cell activation[29,31]
- Increased expression of IL-7Rα, CXCR3, CD27, CD62L, BCL-2 and reduced expression of KLRG1 and IL-2Rα [23,29]
- Increased expression of the transcription factors Id3, BCL-6, TCF-1, STAT3, Foxo1, Eomes and reduced expression of T-bet, Id2, Blimp1, Zeb2, STAT4 [15,23,29,31]
- Increased availability of the cytokines IL-10, IL-21, TGF-β and reduced availability of IL-2, IL-12, IFN-γ, IL-27 [23,31]
- Energy requirements derived from fatty acid oxidation and mitochondrial oxidative phosphorylation, and signalling through AMPK (with effector cells favouring glycolysis and mTOR signalling) [15,23,29]

Maintenance of T cell memory

Memory T cells persist long-term independently of antigen, and this is a dynamic process involving cell proliferation and death. The doubling time of a naïve T cell was estimated to be 1 year, a T_{CM} cell every 60 days, and a T_{EM} cell every 20 days[32]. The maintenance of memory T cells is dependent on the homeostatic cytokines IL-7 and IL-15 [33], with the former appearing more important for survival and the latter for proliferation[15].

1.2 Immunosenescence

1.2.1 The Innate Immune System

Immunosenescence affects both the innate and adaptive arms of the immune system. Alterations in the numbers, phenotype and function of various innate immune cell types – haematopoietic stem cells, neutrophils, monocytes, macrophages, dendritic cells, natural killer cells – and changes in TLR expression and signalling have been demonstrated in ageing humans[34–36]. Furthermore, the decline in innate immunity has been associated with poor clinical outcomes from infectious diseases[37,38].

1.2.2 The Adaptive Immune System

Ageing has been shown to have multiple profound effects on the adaptive immune system. A significant change is the reduced thymic output of naïve T cells[39] accompanied by decreased numbers of naïve T cells in blood and SLOs[40]. Age-related naïve T cell decline predominantly affects $CD8^+$ T cells, with $CD4^+$ T cells relatively preserved[41,42]. There is also a reduction in the diversity of the naïve T cell repertoire by a factor of 2 to 5 between the ages of 30 and 70, as well as a major inequality in naïve T cell clonal sizes, suggesting a disturbance in homeostatic proliferation[43].

Memory T cells first generated in youth are considered to be strongly protective long-term, but memory T cell responses first derived in old age are profoundly impaired – suggesting the critical factor in memory persistence is the age of the naïve cell at the time of activation, and not the duration of time since memory induction[44,45]. With increasing age, naïve T cells develop shortened telomeres, altered TCR signalling, and incomplete differentiation towards memory T cells on activation[46,47]. The age-related decline in the naïve T cell compartment is considered to be a factor in the increased susceptibility to novel, previously unencountered pathogens in the old.

The loss of naïve T cells is accompanied by the accumulation of highly-differentiated antigen-experienced effector memory T cells in ageing individuals infected with CMV, who undergo a massive clonal expansion in CMV-specific $CD8^+$ T cells[48,49]. Late-differentiated T_{EMRA} cells demonstrate reduced expression of the co-stimulatory molecules CD28 and CD27; they take on characteristics of senescence with shortened telomeres, reduced telomerase activity, increased

DNA damage as visualised by γH2AX, and increased expression of KLRG1 and CD57; and they manifest reduced IL-2 production and impaired proliferation[28,50]. The effector functions of these cells are nevertheless preserved, with high production of cytokines and high cytotoxic activity[28,50,51]. $CD8^+$ T_{EMRA} senescent cells take on innate-like features of natural killer (NK) cells, pointing to a convergence of innate and adaptive immunity with increasing age[52]. Shortened telomere length in aged individuals has been associated with a higher mortality rate from infectious disease[53], providing evidence for a link between cellular senescence and clinical outcomes from age-related disease.

Intracellular signalling downstream of the TCR has been shown to be disrupted in late-differentiated T cells in ageing humans[54]. The early phases of the TCR signalling cascade are prominently affected; an alteration in Lck activation is particularly notable with increasing age[54]. The classical and alternative p38 mitogen-activated protein kinase (p38 MAPK) pathways were recently shown to be lacking, and increased p38 MAPK activation occurred due to a novel intrasensory pathway driven by low-nutrient and DNA damage response signalling[55]. Inhibition of p38 MAPK in $CD4^+$ T_{EMRA} cells restored telomerase activity and survival after TCR activation[56,57]. Senescent $CD8^+$ T cells undergo several metabolic changes, including reliance on glycolysis for energy, mitochondrial dysfunction, reduced mitochondrial biogenesis, increased production of reactive oxygen species (ROS), impaired autophagy, and failure to activate mammalian target of rapamycin (mTOR)[51]. Blocking p38 MAPK reversed many of these metabolic derangements[58], demonstrating the close relationship between metabolic and senescence pathways.

Persistent antigenic challenge in the form of chronic viral infection can lead to exhaustion of T cells in aged individuals – such viral infections include HIV, Epstein-Barr virus, hepatitis B and C. Exhaustion involves the progressive loss of T cell function, leading to deletion of the cell. Functions that are lost include the production of IL-2 and other cytokines, proliferative capacity and cytotoxicity. Exhausted cells are characterised by the accumulation of inhibitory receptors including PD-1, CTLA-4, TIM3, LAG3, BIM and Blimp1 [28,44].

PD-1 is a surface immune checkpoint receptor found on T cells and other immune cells (including B cells, monocytes and DCs). Its expression is transient on activated T cells and sustained on exhausted T cells. Engagement of PD-1 with

its ligands PDL-1 and PDL-2 leads to the transduction of an inhibitory signal, giving rise to diminished T cell proliferation and cytokine production. These result in the promotion of tolerance and inhibition of immune responses[59].

Thus, in summary, with ageing numerous changes to the adaptive immune system may occur including decline of naïve T cells, accumulation of terminally-differentiated senescent T cells, cell signalling and metabolic disturbances, and functional exhaustion – all of which may contribute to immune impairment in the old.

1.2.3 Inflammaging

Ageing is associated with a persistent low-grade inflammatory state, termed *inflammaging*, and this is characterised by increased levels of IL-6, TNF-α and CRP[60]. The cause of this heightened inflammation is unconfirmed but multiple putative sources have been proposed. One potential source is the accumulation of senescent immune and stromal cells that take on a senescence-associated secretory phenotype (SASP)[61], producing increased cytokines, chemokines, growth factors and proteases[62]. Other contributory sources during ageing include increased intestinal permeability and changes in the gut microbiota, persistent viral antigens stimulating the innate immune system, accumulating self-debris (including damaged cellular and organelle components, free radicals, fatty acids), circulating mitochondrial products serving as DAMPS, increased adiposity, increased activation of the coagulation system, and defects in the complement pathway[62,63]. Chronic inflammation is proposed to induce tissue damage either by the actions of infiltrating immune cells that release reactive molecules, or by the action of inflammatory cytokines inducing phenotypic changes in nearby cells[63,64]. Inflammaging is a significant risk factor for mortality and morbidity in the elderly, and is implicated in the pathogenesis of various chronic diseases including diabetes mellitus type II, ischaemic heart disease, osteoporosis and Alzheimer's disease[62,64]. Evidence for the detrimental effect of inflammaging on immune function includes the down-regulation of CD28 expression on $CD4^+$ T cells by TNF-α [65], telomerase inhibition and telomere erosion in $CD8^+$ T cells by IFN-α [66], and observations that chronic inflammatory diseases are associated with shortening of telomeres in peripheral blood leukocytes[51].

1.3 The Skin Immune System

The majority of immunosenescence studies have focused on changes occurring in the circulation. However, pathogens are typically encountered and engaged by the immune system at the body's various interfaces with the external environment i.e. the skin and the mucosal surfaces of the respiratory tract, gut or urogenital tract. In recent years, attention has shifted to characterising the local immune system in these peripheral tissues, as it is recognised that these peripheral sites play a critical and active role in immune surveillance and defence.

The skin, as the outermost layer of the body with an extensive surface area of approximately 1.7 m^2, is the first line of defence against a multitude of external pathogens. It consists of two layers: above is the epidermis, a thin layer (approximately 0.1mm thick) of stratified squamous epithelium, composed of four strata of keratinocytes in progressive stages of differentiation. The surface of the epidermis is densely populated by diverse commensal microbiota, which have been recognised to contribute to host immunity through competition with pathogenic microorganisms, enhancement of host innate immune responses, and priming of host adaptive immunity[67,68].

Below the epidermis is the dermis, a thicker layer (up to 3-4 mm depending on body site) consisting of a matrix of connective tissue produced by fibroblasts, and structures such as blood vessels, lymphatics, nerves, sweat glands and pilosebaceous units. Aside from presenting a mechanical barrier to physical and microbial insults, the skin contains a complex network of innate and adaptive immune cells with surveillance and effector functions (Figure 1-1), which in healthy adults provides a robust defence against pathogenic invasion[69]. The functions of these cells are discussed in turn below. Importantly, the skin is a tissue whose immune system is highly amenable to scientific investigation, in view of its accessibility and relative ease of sampling.

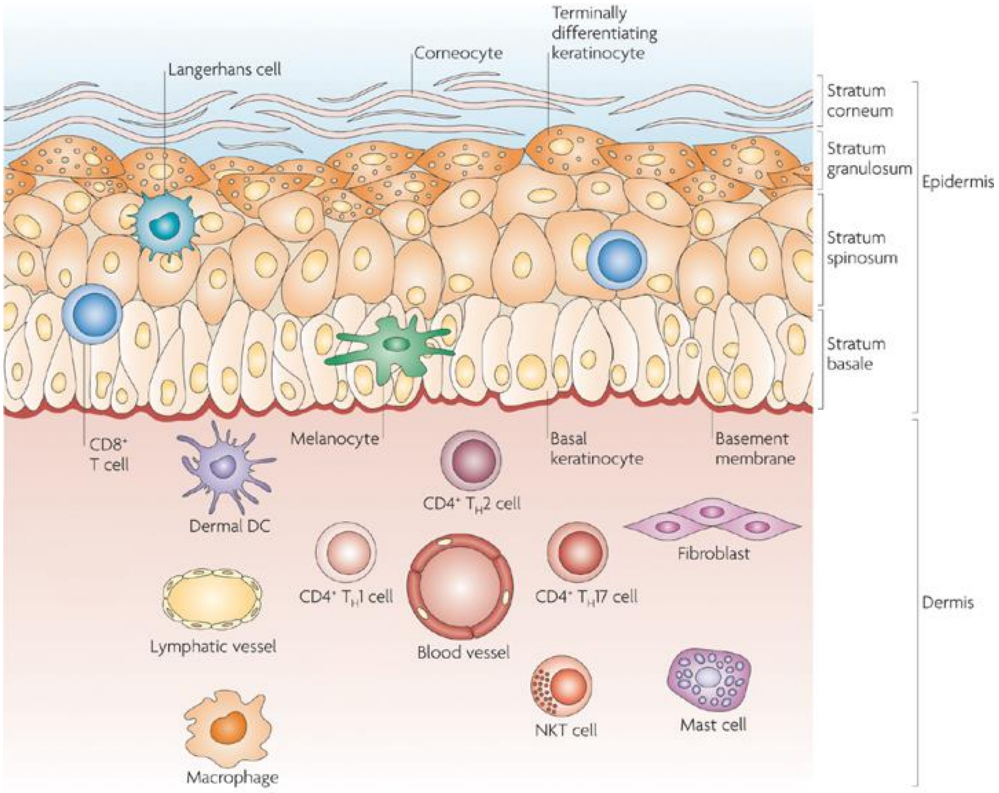


Figure 1-1 Immune cells within the skin

Diagram demonstrating the anatomical relationship between the epidermis and dermis, and the array of immune cells contained within these layers (adapted from Nestle et al[70])

1.3.1 Epithelial cell defence

Keratinocytes contribute to the innate immune response in the skin. They recognise PAMPS through their PRRs, and in response to pathogenic stimuli they produce pro-inflammatory cytokines, chemokines, and anti-microbial peptides[70,71]. Keratinocytes may also contribute to the adaptive immune response. They were shown to have the ability to process and present antigen to antigen-specific $CD4^+$ and $CD8^+$ memory T cells in vitro, leading to cytokine secretion and target cell lysis respectively[72]. Thus, keratinocytes are multifunctional cells that are crucial for host defence, with important roles in maintenance of the epidermal barrier, early sensing of pathogens and release of innate immune mediators, as well as priming of the adaptive immune response.

1.3.2 Innate immune cells

The Epidermis

Langerhans cells are immature, dendritic, antigen-presenting cells that reside in the epidermis. As the outermost immune sentinels in the skin, they extend dendrites to the stratum corneum to probe for foreign antigens[73]. During a skin infection, they become activated through PRRs, take up antigen, undergo maturation and migrate to the skin-draining lymph nodes to present antigen to naïve T cells. Migration is dependent on signals from TNF-α and IL-1β [74]. Studies have shown that Langerhans cells maintain immune tolerance in normal skin, but in the presence of infection stimulate effector T cell responses[75,76].

The Dermis

The key innate immune cells in the dermis are dermal dendritic cells (DDCs) and monocytes/macrophages. Other cell types include mast cells and innate lymphoid cells, which are understood to contribute to skin inflammatory responses but which have been poorly characterised to date[77]. DDCs take up and process antigen, and after migration to the skin-draining lymph nodes, present antigen to naïve or memory $CD4^+$ or $CD8^+$ T cells[78]. Activated DDCs also contribute to immune responses by producing pro-inflammatory cytokines[70]. Skin resident macrophages within the dermis remain in situ and scan the dermis for antigen. Additionally, circulating monocytes pass through the skin, assisting with antigen recognition and transport to the draining lymph node. The various subsets of monocytes and macrophages in the skin may be pro-inflammatory, anti-inflammatory, or assist with wound healing[77].

1.3.3 T cells

Besides central memory and effector memory T cells, it is now recognised that there is a third major population of memory T cells in the body, termed tissue resident memory T cells (T_{RM})[79]. These have been identified in the skin, mucosal surfaces (including gut, lungs, genital tract), non-barrier internal organs (including brain, liver and kidney), exocrine glands (pancreas, salivary glands) and secondary lymphoid organs[24,80–82]. These cells do not recirculate and are therefore absent from blood. T_{RM} have been shown to be phenotypically and transcriptionally distinct from T_{CM} and T_{EM} and therefore represent an altogether separate population of cells[83–85].

It has been estimated that there are 2 x 10^{10} T cells in human skin[86], more than double those present in the blood, and the majority of these (80-90%) are T_{RM} [87]. Skin T_{RM} express CD69, are negative for CCR7, and approximately 50% are positive for CCR8 [87–89]. CD69 is a surface receptor which is considered to block the egress of T_{RM} from peripheral tissue by inhibiting $S1P_1$ [90,91]. The minority of T cells in the skin that recirculate between skin and blood are $CD69^-$, and have been divided into a population termed skin tropic central memory T cells, which are $CCR7^+$ $CD62L^+$ and have access to the lymph nodes, and another population called migratory memory T cells (T_{MM}), which are $CCR7^+$ $CD62L^-$ and may have access to skin draining lymph nodes but not other lymph nodes[87].

As well as CD69, another surface marker, CD103, has been found to be expressed by T_{RM}. CD103 is an integrin that binds to E-cadherin which is expressed on keratinocytes[92], and this interaction may be relevant for the retention of T_{RM} in the skin[93]. Of the $CD69^+$ T_{RM} in human epidermis, 70% were $CD103^+$, while only 40% of dermal T_{RM} were positive for this marker[87]. The ratio of $CD4^+$ to $CD8^+$ T cells was found to be approximately 3:1 in human epidermis and 6:1 in dermis[87], compared to 1.8:1 in the blood[94]. Thus, the predominant T cell type in the skin is a dermal $CD4^+CD69^+CD103^-$ T_{RM}. Dermal $CD4^+$ T cells tend to be arranged in clusters around blood vessels in the upper dermis[86].

Transcriptional analysis of T_{RM} in various tissues showed downregulation of the transcription factors KLF2, T-bet and Eomes, and of the homing receptors $S1P_1$, CD62L and CX3CR1, and upregulation of the transcription factors Hobit and Blimp1, the chemokine receptor CXCR6 and the collagen-binding integrin CD49a – all of which may be relevant for the retention of T_{RM} in peripheral tissue sites[83,84,95,96]. Additionally, CD49a positivity was shown to denote a subpopulation of $CD8^+$ skin T_{RM} that produced IFN-γ, perforin and granzyme on stimulation[97]. T_{RM} express IL-2, IFN-γ, TNF-α, IL-17 more highly than circulating T_{EM}, and this was also true for the inhibitory cytokine IL-10 [24,95]. Furthermore, T_{RM} express the inhibitory receptors PD-1, CTLA-4 and LAG3 [24,95]. These transcriptional data suggest T_{RM} have both protective and regulatory roles.

Cutaneous T_{RM} are generated after skin infections and are antigen-specific to the causative pathogen[98]. Accordingly, skin T_{RM} were found to exhibit a diverse TCR repertoire[86]. Neonatal skin was shown to have very few T_{RM}, reflecting minimal previous cutaneous exposure to pathogens[87]. During a primary skin infection,

DCs from the skin take up antigen, migrate to the draining lymph node and present antigen to naïve T cells, upon which these T cells become activated, differentiate into effector cells and undergo clonal expansion. DCs originating from the skin, expressing skin-derived vitamin D3 and prostaglandin E2 (PGE2), imprint these effector T cells to express CLA, CCR4, CCR8 and CCR10 [99,100], and the effector T cells migrate via the circulation to the inflamed tissue. Here, activated endothelium expressing E-selectin, CCL17, CCL1, CCL27 and ICAM-1 – the respective ligands for CLA, CCR4, CCR8, CCR10 and LFA-1 expressed on the surface of skin-homing T cells – assists effector T cells in entering the skin where they can perform their various effector functions[101].

Once the skin infection has cleared, a proportion of KLRG1$^-$ effector T cells differentiate into T_{RM} [83], with upregulation of CD69 and CD103 occurring within weeks[87]. Local signalling by cytokines including IL-15, TGF-β, Il-33 and TNF-α have been implicated in the formation of T_{RM} [83,102,103]. Concurrently, clonal populations of T_{CM} are generated in LNs with identical antigen-specificity to the clonal populations of T_{RM} generated in the skin, indicating that T_{CM} and T_{RM} are derived from a common naïve T cell precursor[104]. Following a localised skin infection, long-lasting T_{RM} are generated throughout the entire skin surface, but with the highest concentration at the initial site of infection[105].

These T_{RM} now sustain a stable, long-lived population within the skin and are protective against future encounters with the same pathogen[79,105–107]. Long-term maintenance of T_{RM} appears to be antigen-independent[103]. Transcriptional analysis of skin T_{RM} suggested these cells were poised to respond to danger signals and were in a more activated state than their recirculating counterparts[85]. Human patients with alemtuzumab-treated cutaneous T cell lymphoma (CTCL), whose circulating T cells are depleted but have intact skin $T_{RM,}$ have a low incidence of skin infections, highlighting the protective role of this latter T cell population[108]. The specificity of T_{RM} located at each tissue location relates to the pathogens previously encountered at that site. For example, skin contains VZV-, HSV- and Candida-specific T cells, lung has influenza-specific T cells, hepatitis B-specific T cells may be found in the liver, and EBV-specific T cells are located in the spleen and tonsils[24].

Following secondary skin infection, DCs can directly present antigen to skin T_{RM}, which proliferate in situ, ultimately resulting in the generation of increased

numbers of T_{RM} [105,109,110]. Local inflammation facilitates the recruitment of bystander T cells and other immune cells from the circulation in a non-specific manner[71,111]. Antigen-specific T_{CM} are also recruited from the draining LN, which then home to the site of infection to contribute to the immune response, and a proportion of these cells will subsequently differentiate into long-lived T_{RM}, adding to the existing T_{RM} population[71,105,109–111]. Secondary antigen encounter in the skin massively increased the formation of vaccinia-specific $CD8^+$ skin T_{RM} in mice by 50-100 fold[112], although another study in mice found that inflammation alone in the absence of antigen was sufficient to generate HSV-specific $CD8^+$ skin T_{RM} [113]. Although both T_{RM} and T_{CM} contribute to the secondary immune response, T_{RM} have been shown to dominate the local T cell response and are more protective against re-infection than T_{CM}[87,109,110].

More work is needed to investigate the mechanisms through which T_{RM} perform their protective functions. While 95% of skin T_{RM} were shown to be Th1 polarised[86], as a whole the skin T_{RM} population is heterogeneous, and production of IFN-γ, TNF-α, IL-9, Il-13, IL-17 among others has been demonstrated alone or in combination[81]. There is some evidence that cytotoxic T_{RM} can migrate towards virally-infected cells[114,115] and perform cell lysis[116]. In animal models, reactivated CD8 T_{RM} rapidly conditioned the local environment by inducing broadly active antiviral and antibacterial genes[117], and secreting cytokines that triggered brisk adaptive and innate immune responses, including local humoral responses, maturation of local dendritic cells, and activation of natural killer cells[118,119]. This rapid response induced an antiviral state that was inhospitable to an antigenically unrelated virus[118].

$CD8^+$ skin T_{RM} expressed high fatty-acid-binding proteins 4 and 5 (FABP4 and FABP5), which are cytoplasmic proteins that mediate cellular uptake and intracellular transport of free fatty acids (FFA), and demonstrated increased uptake of extracellular FFA[120]. T_{RM} relied on FABP4/FABP5 for survival and protection against viral infection[120]. The maintenance and protective capability of skin T_{RM} may be dependent on oxidative metabolism of exogenous FFAs[120], and this might be a crucial metabolic adaptation for survival in the epidermis, a lipid-rich but nutrient-poor environment[121].

Overall, therefore, it appears that T_{RM} are well-poised as immune sentinels at the interface between the skin and the environment, and on pathogen detection their

actions are broad - they rapidly proliferate, activate and recruit other immune cells to the site of infection, and release cytokines and perform cytotoxic activity against infected cells.

Regulatory T cells

$CD4^{+}Foxp3^{+}CD25^{hi}$ Tregs constitute approximately 10% of the T cell population in the skin[122,123]. The predominant expression of CD25, CTLA-4 and ICOS, as well as CD45RO, indicated an activated memory Treg phenotype, and the lack of CCR7 expression suggested that these cells were resident in the skin[124]. Tregs in circulation express high levels of the skin homing markers CLA, CCR4 and CCR6, indicating that the skin is a major trafficking site for these cells[125]. As a major interface with the environment, the skin is constantly exposed to a high antigenic load, and it is speculated that the role of the cutaneous Treg population is to dampen excessive immune responses, including to innocuous non-self antigens, auto-antigens, and non-pathogenic commensal microbiota[125,126]. Mice in which Tregs were unable to migrate to the skin developed severe cutaneous inflammation, demonstrating the importance of maintaining immune tolerance at this site[127].

1.4 The Delayed Type Hypersensitivity Response

The delayed type hypersensitivity (DTH) response is a useful model for studying in vivo immune responses in the skin. This involves intradermal injection of a recall antigen into the forearm skin, allowing for direct visualisation and assessment of the clinical response. The clinical response is a visible indicator of the degree of inflammation at the antigen-challenged site and takes into account erythema, palpability and diameter of the swelling. The presence of erythema reflects localised dilatation of skin capillaries and attendant increased blood flow, while the palpable swelling is due to fluid (containing cells and protein) escaping from these leaky capillaries causing local oedema. The clinical response evolves over days – first becoming apparent by 24 hours, then peaking at 48-72 hours before tailing off over the next several days. The skin at the DTH site can be sampled at a chosen time-point, with analysis of the various immune cells present.

The DTH response is a memory $CD4^+$ T cell-mediated immune response[128–130]. A robust DTH response requires successful integration of the innate and adaptive arms of the immune system. Following intradermal injection of antigen, the presence of tissue damage and pathogen-derived antigen stimulates a variety of skin-residing cells with innate immune functions to produce pro-inflammatory cytokines to initiate the inflammatory process[70]. These cells include epidermal keratinocytes and Langerhans cells as well as dermal macrophages and DCs[70]. The production of TNF-α, IL-1 and IL-6 by macrophages activates local endothelium, triggering endothelial cell expression of the adhesion molecules E-selectin, ICAM-1 and VCAM-1 that allows further recruitment of leukocytes from the circulation[131–135]. IFN-γ production by T cells also contributes to endothelial activation via upregulation of ICAM-1[136–139]. Modelling of the kinetics of the DTH to tuberculin purified protein derivative (PPD) revealed a brisk cellular influx at the site of injection, with the majority of cells being neutrophils at 6 hours, macrophages at 24 hours, and T cells 48 hours and beyond[128]. The peak of the T cell response was shown to occur at 7 days[128].

Preparations of injectable recall antigens exist for various pathogens. The first was the tuberculin skin test, first employed by Von Pirquet in 1909 to demonstrate past exposure to tuberculosis[140]. Our group has extensively studied the DTH response to VZV skin test antigen[141] as an in vivo measure of cell-mediated

immunity to VZV[135,142,143]. Greater than 90% of the UK adult population are seropositive for VZV[144], meaning the skin test is suitable as a recall antigen for the majority of research participants. The VZV skin test has been shown to be a reliable indicator of immunity to VZV[145,146], and predicts the risk of developing shingles[147] and post-herpetic neuralgia[148].

Previous work by our group has shown that after skin challenge with VZV antigen, VZV-specific $CD4^+$ T cells, mainly of a central memory ($CD27^+CD45RA^-$) phenotype, accumulated at the challenge site and this was due in part to local proliferation[142]. The number of infiltrating $CD4^+$ T cells at the peak of the cellular response (Day 7) correlated with the magnitude of the clinical response (Day 3), highlighting the importance of early inflammation in the subsequent recruitment of T cell numbers to the challenge site[142]. Accumulation of $CD4^+Foxp3^+$ Tregs occurred in parallel and in proportion to $CD4^+$ T cell effectors after VZV[142] and PPD[149] skin challenge. The proportion of Tregs in perivascular immune cell infiltrates inversely correlated with the degree of clinical response, providing indirect evidence for their inhibitory effect on the cutaneous memory immune response in vivo[142].

1.5 Age-related changes in the Skin Immune System

Clinical evidence for the impairment of the skin immune system in old humans is provided by the increased frequency of skin infection[150], skin cancer[151], autoimmune skin disease[152] and the attenuation of DTH responses[135,153] in this age group. Skin infections that are more prevalent in the old are due to a range of bacterial (eg. *Staphylococcus aureus* and beta-haemolytic Streptococci), viral (eg. VZV) and fungal (eg. *Candida albicans*) pathogens[150].

Physiological changes in elderly skin, such as thinning of the epidermis, reduced water retention in the stratum corneum, and diminished blood flow to the skin lead to impaired barrier function and prolonged healing[150], increasing the likelihood of invasion by pathogens. In addition to these structural changes, the skin immune system also appears susceptible to the effects of ageing. Evidence for age-related changes in the skin immune system is discussed below.

1.5.1 The innate immune system

Toll-like receptors

TLRs are expressed by multiple cell types in the skin, including keratinocytes, Langerhans cells, DDCs, macrophages, endothelial cells and fibroblasts[154]. Some evidence exists for the impairment of TLR function during ageing[155]. In monocytes, stimulation of the TLR1/2 heterodimer and TLR7 resulted in reduced cytokine production in the old; TLR1 but not TLR2 expression was reduced in these old individuals[156]. In DCs, stimulation via TLR4, TLR7 and TLR9 led to impaired cytokine production in the old[157]. Diminished activation of innate immune cells via TLRs may therefore lead to the insufficient priming of adaptive immune responses in the skin.

Antigen-presenting cells

Several studies have demonstrated age-related changes affecting the various populations of APCs in the body, but only limited data exists relating to the skin specifically. Langerhans cells were shown to be reduced in number in the epidermis of old compared to young individuals[158,159]. Furthermore, after exposure to TNF-α, their capacity to migrate out of the skin was impaired in old humans, and this was considered to be due to inadequate IL-1β signalling[158,159]. During inflammation or injury, the Langerhans cell population in the skin is thought to be replenished by circulating monocyte precursors. However, no age-

related defect was demonstrated in the phenotype and function of monocyte-derived Langerhans cells[160]. Thus, it appears that there is an age-related impairment in the Langerhans cell population derived from local precursors in normal human skin, but not in the monocyte-derived Langerhans cell population in inflamed skin.

Data on DDCs is lacking, but studies have found that the number of circulating myeloid DCs does not change in the old[161,162]. The phenotype of DCs appears to be comparable between young and old, as measured by surface co-stimulatory and antigen-presenting molecules[163]. Furthermore, in vitro stimulation of DCs resulted in the expression of comparable levels of surface activation markers, indicating no age-related decline in the ability of these cells to undergo maturation[161,164]. However, there does seem to be an age-related impairment of DCs in their ability to perform phagocytosis and micropinocytosis of antigens, as well as in their migration capability[163]. Cytokine-secreting ability may also be dysregulated in the old. Secretion of pro-inflammatory cytokines by DCs was increased in the resting state in the old, but there was reduced secretion when DCs were activated by a variety of stimuli[157,163,165,166]. Finally, DCs from old individuals were significantly impaired in their abilities to activate $CD8^+$ T cells, induce T cell proliferation, and stimulate secretion of IL-12 and IFN-γ by T cells[166].

Similarly, there is a lack of data on dermal macrophages, and also a lack of human immunosenescence studies on macrophages in general. Mice studies have pointed to age-related defects in TLR expression, signal transduction and downstream cytokine production by macrophages, as well as impaired phagocytic and antigen-presenting properties[167,168].

1.5.2 The adaptive immune system

Skin resident memory T cells

Data on the effects of ageing on the skin T_{RM} population are lacking, and investigating this will be the focus of the work here. A previous study by the Akbar group demonstrated significant telomere shortening in antigen-specific $CD4^+$ T cells during the DTH response to PPD, and this was associated with the inhibition of telomerase[169]. This may suggest that T cells in the skin are particularly prone to premature replicative senescence.

Regulatory T cells

The frequency of Tregs is higher in old skin and blood compared to young[135,170,171] - possibly due to greater resistance to apoptosis in the old[172]. nTregs appear to increase with age, whereas iTregs seem to decline in number[173]. The proportion of circulating Tregs expressing the skin homing marker CCR4 is higher in the old, suggesting a mechanism for circulating Tregs in the old to preferentially home to and lodge in the skin[174].

The phenotype of Tregs from old and young humans is comparable, with similar levels of expression of Foxp3, CD25, CTLA-4 and GITR[171]. Studies have shown that Tregs from old and young humans are equivalent in their ability to suppress proliferation and production of IFN-γ by $CD4^+$ T cells[170]. The expression of co-stimulatory molecules on DCs was reduced in aged compared to young mice, and this was shown to be due to the greater suppressive effect of aged Tregs[175]. However, Tregs from old mice were less capable of inhibiting the production of IL-2 [173] and IL-17 [176] by T cells. Overall these data suggest that ageing is accompanied by an increase in Tregs, which appear to remain as suppressive in the old than the young.

1.5.3 Impaired DTH responses in ageing skin

The DTH clinical response is diminished in old individuals in response to mycobacterial, fungal and viral antigens[135,143,153] (Figure 1-2 and Figure 1-3) . The cellular response, assessed both by total as well as antigen-specific $CD4^+$ T cells, is also diminished in the old compared to the young – this has been demonstrated for VZV, candida and PPD[135,143].

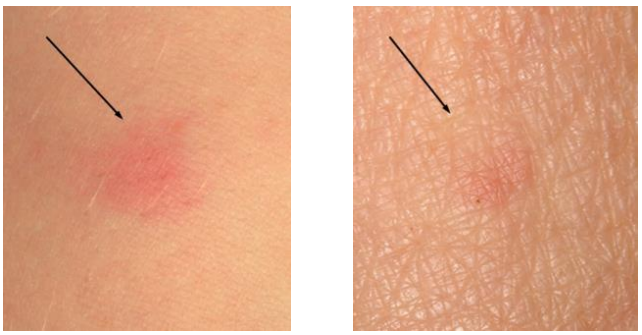


Figure 1-2 Typical clinical response to VZV antigen challenge in a young (left) and old (right) individual[135]

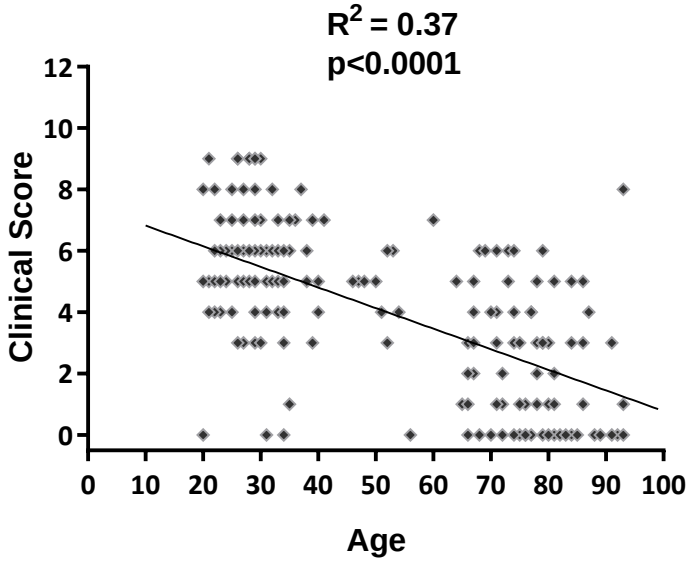


Figure 1-3 The effect of age on clinical response to VZV skin antigen challenge[143]

Graph showing Day 3 clinical score plotted against age. Linear regression analysis line, R^2 value and significance value are shown (n=185).

In experiments performed by the Akbar group, it was shown that following intradermal injection of VZV antigen, young individuals had a strong transcriptional response in the skin at 72 hours in comparison to unchallenged, normal skin. The top 30 overexpressed genes in the young were also

overexpressed in the old (compared to normal skin), but to a much lesser degree in the old than the young. Genes that were expressed in the young but not the old were associated with T cell and dendritic cell activation; immunological, inflammatory and antiviral signalling pathways; pathways induced by type I interferons and IFN-γ signalling[143]. These transcriptional data lend support to the histological findings of impaired T cell accumulation and proliferation at the site of the DTH response in the old[143].

The mechanisms underlying the impaired DTH responses in the old remain unclear. Conceivably, a defect in any one (or more) of the following processes would attenuate the DTH response:

1. The taking up of recall antigen by skin-resident DCs, DC activation and induction of local inflammation, antigen presentation to antigen-specific skin T_{RM}.
2. Antigen-specific T_{RM} activation and proliferation, execution of effector functions, recruitment of other immune cells to response site.
3. Recruitment of circulating memory T cells via dermal endothelium into the skin, and differentiation into effector cells.

Diminished DTH T cell responses to candida antigen in the old at the 24 and 72 hour time points were associated with reduced endothelial activation and decreased TNF-α production by dermal macrophages[135]. TNF-α production by dermal macrophages ex vivo was not impaired in the old, suggesting environmental factors in the skin may impair innate immune responses[135]. Our study of DTH responses to VZV showed impaired endothelial activation and reduced accumulation of DCs at the challenge site in the old, at 24 hours and beyond[143]. These studies indicate impaired cellular recruitment via the endothelium is central to impaired DTH responses in the old.

1.6 Varicella Zoster Virus Biology

1.6.1 Varicella pathogenesis

VZV is an exclusively human alpha-herpesvirus. Primary infection, which affects the vast majority of the population during childhood, causes chickenpox (varicella). Primary VZV is acquired via the respiratory mucosa, leading to invasion of the tonsillar epithelium, followed by viremia; next, infected lymphocytes transport virus to keratinocytes in the skin. Innate immunity temporarily inhibits VZV replication in the skin but is then overcome, leading to the development of a characteristic, generalised, vesicular rash. The incubation period is 10-21 days. Chickenpox (and shingles) skin lesions both contain high concentrations of infectious virus and are the source of transmission to susceptible individuals[177,178].

Primary VZV infection induces VZV-specific T cells and VZV-specific antibody. VZV-specific T cells are required to clear primary infection and also prevent viral reactivation. Cell-to-cell spread of VZV in the host, which avoids extracellular circulation of virions, may explain why T cell-mediated immunity is more critical for protection than specific antibody.

During the course of primary infection, VZV particles reach cranial nerve ganglia and peripheral sensory nerve dorsal root ganglia, and this is thought to be either via retrograde axonal transport or via haematogenous spread inside T cells[177]. Latent infection is thus established and persists for life[179]. Human autopsy studies have shown that VZV genomes (~ 2-9 copies per cell) are present in approximately 4% of sensory neurons during latency[180–183].

1.6.2 Herpes zoster pathogenesis

Occasionally, VZV reactivates from its latent state – the virus multiplies and spreads within the ganglion, infecting many additional neurons and supporting cells, which induces intense inflammation and neuronal necrosis[184,185]. Virions travel via anterograde axonal transport and access the area of skin (dermatome) innervated by the sensory nerve, leading to the symptoms of shingles infection (herpes zoster)[177,184].

The trigger for reactivation is unknown, but shingles is more frequent in the elderly, in whom there is an impairment in the degree of VZV-specific cell-mediated immunity (CMI)[184,186–189] but no decline in titres of VZV-specific

antibody[186,190]. Approximately 50,000 cases of shingles occur annually in those aged 70 and over in the UK; the estimated lifetime risk of developing shingles is 1 in 4 [191,192]. Post-herpetic neuralgia (chronic nerve pain) is more likely to accompany shingles infection in the aged population and also more likely to be severe[178,189,193].

Similarly, immunocompromised patients with HIV infection, Hodgkin's lymphoma, organ transplant recipients, and patients receiving immunosuppressant therapy, all of whom have impaired CMI to VZV but intact VZV-specific antibody levels, are at heightened risk of VZV reactivation[184,187]. Contrastingly, patients with X-linked agammaglobulinaemia with intact CMI to VZV are not at greater risk of shingles[184]. These observations demonstrate that CMI is critical in host defence against VZV reactivation whereas humoral responses do not have a significant role.

While VZV-CMI is correlated with immune protection against reactivation, the mechanisms of this protection have not been elucidated. The role of VZV-specific T cells and whether they can directly limit viral reactivation is unknown[177]. Ganglia latently infected with herpes simplex virus (HSV) have been shown to harbour HSV-specific resident T cells (predominantly $CD8^+$)[194], but in contrast, ganglia latently infected with VZV do not harbour VZV-specific resident T cells. This is despite the fact that neurons latently infected with VZV have been shown to express several VZV proteins (despite doubts about the specificity of the immunostaining), thereby presenting a theoretical opportunity for the immune system to recognise latent infection and contain it[184,195,196]. However, constitutive expression of MHC class I is low on neurons[197,198] and furthermore, VZV has been shown in vitro to be responsible for the inhibition of MHC class I expression on fibroblasts (and T cells)[199–201], pointing towards a potential mechanism of immune evasion during latency. In addition, VZV impairs IFN-γ-induced MHC class II expression on infected cells, suggesting a potential mechanism for evasion of $CD4^+$ T cell-mediated immune surveillance[202]. This raises the possibility that virus latency is regulated by an innate immune response involving cytokines or chemokines[203].

Psychological stress around the time of space missions is associated with a depression in CMI to various antigens[204], and CMV and EBV have been shown to reactivate in astronauts undergoing spaceflight[205–207]. Evidence for silent

reactivation of VZV, associated with a temporary depression in immunity, comes from a fascinating study in 3 astronauts, in 2 of whom VZV DNA was repeatedly detected in the saliva during a 13-day spaceflight as well as for the first 5 days after landing back on earth[208]. After landing, salivary samples were cultured and live virus was detected in the samples of these 2 astronauts from Day 2 only and no other days[208]. VZV DNA and infectious virus can be found in the saliva of patients suffering with acute shingles[209]. It is postulated that VZV reaches the saliva following reactivation in the geniculate ganglion and transaxonal transport to the nasopharynx[208]. Interestingly, VZV IgG levels were raised in these 2 individuals at 10 days before the space mission compared to baseline (2-3 months prior), and returned to baseline 2 weeks after landing[208]. The third astronaut had suffered with shingles 10 years previously and no changes were detected in his samples during the study, suggesting a boosting of his VZV-specific immunity had occurred as a result of previous infection[208].

The lack of ganglion-resident T cells means it is unlikely that VZV reactivation is continually actively prevented by T cell interactions with neurons[9]. Innate immune mechanisms probably suppress viral reactivation under normal circumstances, and the silent reactivation of VZV during spaceflight suggests that these innate mechanisms are interrupted during psychological stress. Once reactivated, the virus was presumably contained by the astronauts' functional T cell responses, preventing the full-blown clinical manifestation of shingles. Some astronauts have suffered with shingles[207], however, likely reflecting global dampening of innate and adaptive immunity during spaceflight[210].

In the general population, it is considered likely that intermittent VZV reactivation occurs in the absence of overt shingles skin lesions, with active virus replication and spread within the ganglion presumably stimulating recruited VZV-specific T cell responses to terminate the propagation of virus[9,189]. This is likely to explain both episodes of silent reactivation detectable only by the resulting boost in VZV-specific immunity[211,212], as well as the clinical phenomenon zoster sine herpete[184,213], where VZV-induced dermatomal pain occurs in the absence of a skin rash[195]. This capacity to contain, rather than prevent, viral reactivation is required for the lifespan of the individual and is the target of the shingles vaccine[189].

Steain et al obtained ganglia from a 93-year-old male patient who had died from pneumonia and dementia, and who was suffering with acute shingles at the time of death[196]. Intense staining for the VZV protein IE63 was demonstrated in the ganglion in which VZV had reactivated[196]. This ganglion was densely infiltrated with $CD4^+$ and $CD8^+$ T cells, and many of the latter were positive for granzyme B[196]. A large number of $CD3^-$ granzyme B^+ cells were present and these were proposed to be NK cells[196]. There was marked upregulation of MHC classes I and II on satellite glial cells (SGCs) but not on neurons (which typically do not express MHC molecules), suggesting that SGCs play an important role in the direction of the immune response during shingles[196]. SGCs completely encircle sensory neurons, providing protection[214], barrier function[215], nutritional support and displaying properties of APCs[216]. However, SGCs decline in number with increasing age[215], thereby possibly contributing to the greater risk of uncontrolled VZV viral reactivation in the ganglion.

VZV infection of ganglia in an explant ganglion model led to increased CXCL10 production in vitro; this chemokine was also found to be elevated in a naturally infected ganglion during acute shingles infection. In the latter, this was associated with the presence of lymphocytes expressing CXCR3, the receptor for CXCL10, implicating this chemokine as a driver of T cell recruitment during shingles infection[217].

Combined, these findings demonstrate that during VZV reactivation in ganglia, intense expression of VZV protein may attract a robust T cell response, and in view of the lack of ganglion-resident T cells during latency, these infiltrating T cells are likely to be recruited from other sites, such as the circulation. In support of this, acute shingles infection was associated with an increase in circulating VZV-specific $CD4^+$ T cells, with a striking shift in cytokine profile to IFN-γ single positive T cells compared to healthy controls[218]. The age-related decline in circulating VZV-specific $CD4^+$ T cells would likely impair the circulating VZV-specific T cell immune response and would be consistent with the greater propensity for VZV reactivation in older individuals.

1.7 Vaccination

Unfortunately, few therapeutic interventions currently exist to boost immunity in old individuals. The principal current strategy is to vaccinate the elderly against the serious infections from which they are most likely to suffer, but this is limited by whether a vaccine exists for each specific disease. The current routine immunisation schedule for older adults in the UK recommends vaccinating against *S. pneumoniae* at 65 years old, influenza (annually) after 65 years of age, and shingles after the age of 70 [219]. Due to senescence of the immune system, however, vaccination responses are significantly diminished in old compared to young adults[220–222] – and none of the three vaccines mentioned above confer satisfactory protection in the ageing population, although they do mitigate the effects of the disease[223].

The detailed mechanisms of action of vaccines are largely unknown. Most vaccines mediate their efficacy through the induction of specific antibodies, which must be present at the site of pathogen replication[224]. For the vaccines against shingles and tuberculosis, protection correlates with $CD4^+$ T cell responses[224,225]. Protective mechanisms are likely to be complex and rely on multiple arms of the immune system. For example, $CD4^+$ T cells are required for the generation of most antibody responses, by providing B cell help and cytokine production[224,225]. It is generally recognised that an ideal vaccine should induce a long-lasting, large and diverse antigen-specific T and B cell memory repertoire, as well as a robust specific antibody response[221,226].

The influenza vaccine provides poorer clinical protection in old compared to young individuals[227,228]. The standard correlate of protection for influenza vaccination is the specific antibody level (measured by haemagglutination inhibition), but immunological studies have shown that old individuals produce impaired antibody responses[228,229] as well as $CD8^+$ T cell-mediated responses[229,230] to this vaccine.

A number of changes in the ageing immune system may account for the impairment of vaccine responses in the old. The loss of naïve T cell numbers and diversity, and the accumulation of dominant clones of late-differentiated memory T cells, for example specific to CMV, restricts the expansion of pathogen-specific naïve cells in response to a vaccine[223,226]. With increased age there is an expansion of $CD28^-$ memory T cells, which manifest impaired activation and

proliferative capacity. Loss of CD28 in $CD4^+$ T cells is also accompanied by a loss of CD40L, compromising B cell activation and antibody responses[220,221,231]. A higher proportion of $CD8^+$ $CD28^-$ T cells in the old has been associated with impaired antibody responses to the influenza vaccine[222,232]. Additionally, telomere length was positively correlated with B cell and influenza M1-specific $CD8^+$ T cell responses after influenza vaccination in older adults[233], suggesting that reduced lymphocyte replicative capacity impairs vaccination responses in the old. Furthermore, the duration of T cell[234] and antibody responses[235] are impaired in the old.

The B cell compartment is also adversely affected by ageing, impairing vaccination-induced specific antibody responses in the old. Similar to the T cell compartment, naïve B cell numbers decrease, and effector B cells with limited repertoire diversity accumulate in old age[220,223,236]. Age-related defects in isotype switching and somatic mutation lead to a reduced diversity of antibody responses, which are quantitatively decreased, less efficient and of lower avidity[220,237].

1.7.1 Zostavax® introduction

The live attenuated shingles vaccine Zostavax® has been available since 2006 and is currently offered to individuals in their 70s in the UK. The Shingles Prevention Study (SPS) showed this vaccine was 63.9% effective at reducing the incidence of shingles in the 60- to 69-year-old population, but this fell to 37.6% in the over 70s[238]. The increased incidence of shingles in older people is postulated to be due to an age-related decline in circulating VZV T-cell-mediated immunity[186], and there is evidence that the vaccine stimulates VZV-specific $CD4^+$ T cell responses in the circulation in old individuals[189,239]. The immunological effect of the vaccine on skin immunity is unknown. Given that shingles is a disease manifesting in the skin, it is of interest to consider how the vaccine modifies immune cell populations resident in the skin, and whether in vivo VZV-specific immune responses in the skin can be enhanced by the vaccine.

As of October 2017, a new non-live shingles vaccine Shingrix® has been licensed for use in the USA; preliminary studies have demonstrated a high efficacy which did not decline in older ages. This vaccine will be discussed in Chapter 4.

1.7.2 Review of the immune effects of Zostavax®

The Shingles Prevention Study[240] immunology substudy

The substudy demonstrated a boost in CMI responses at 6 weeks after vaccination, as measured by responder cell frequency and IFN-γ ELISPOT[239]. A separate study investigating the early T cell response to Zostavax® demonstrated the frequency of IFN-γ-producing T cells increased approximately tenfold to peak between 8 and 14 days post-vaccination, but a decline was already observed by Day 28 (but still threefold higher than before vaccination)[241]. Importantly, the boost in VZV-specific T cell-mediated immunity was similar to that developing after naturally occurring shingles infection[242]. The boost in CMI was observed for the 3-year duration of the immunology substudy, but declined over time[239]. The largest drop was seen between 6 weeks and 1 year, during which period the vaccine-induced boost declined from 85% to 42% as measured by RCF and from 120% to 60% as measured by ELISPOT[239]. The VZV-CMI responses to Zostavax® correlated with the clinical efficacy observed in the SPS[240], confirming the hypothesis that boosting VZV-CMI would result in clinical protection. Follow-up studies demonstrated that clinical efficacy of the vaccine declined with each additional year after vaccination, with no demonstrable clinical benefit after 8 years[240,243,244].

In addition, there was a boost in the antibody response at 6 weeks as measured by ELISA[239]. The boost in VZV antibody in vaccine recipients declined from 78% to 20% from week 6 to 1 year after vaccination, and fell 7%–15% per year thereafter[239]. There was a poor correlation between VZV-CMI and VZV antibody response, suggesting these classes of immune response are independent[239]. Despite this, fold-change in VZV antibody titres after vaccination of 50-59 year-olds was an excellent correlate of clinical protection, perhaps suggesting that the antibody response is an indirect marker of a robust and multi-faceted immune response[189,245].

The measures of CMI at 6 weeks were lower in the ≥70s compared to participants aged 60-69[239]. When age was assessed as a continuous variable, measures of CMI at 6 weeks declined by 3.5% - 3.8% for every additional year of age[239]. In contrast, there was no significant difference in VZV antibody titre between the 60-69 and ≥70 age groups at the 6-week time-point[239]. When age was assessed as

a continuous variable, antibody titres declined by 1% per additional year of age, at week 6[239].

Effect of Zostavax® on the quality of circulating T cell responses

A limited number of recent studies have examined the effect of Zostavax® vaccination on the breadth and functionality of VZV-specific T cell responses in individuals aged over 50 [246–248]. Laing et al showed that $CD4^+$ T cells responded to a median of 2.5 VZV proteins before vaccination, and the breadth of the response improved 4.2-fold at 1 month after vaccination to a median of 10.5 VZV proteins[246]. However, the breadth of responses returned to near baseline levels by 6 months[246]. The enhanced breadth of responses occurred in conjunction with an increase in magnitude of $CD4^+$ T cell responses as discussed previously[239,246]. These results were consistent with findings by Qi et al, who identified a diversification of the T-cell receptor (TCR) repertoire following vaccination[248]. Interestingly, vaccination preferentially favoured expansion of infrequent VZV antigen-reactive TCRs, with a smaller effect on dominant, in-vivo-selected T cell clones[248]. Data from this study also suggested that VZV vaccination not only increased the frequencies of existing memory VZV-reactive $CD4^+$ T cell clones but also activated naïve $CD4^+$ T cell clones, thereby increasing the richness of the VZV-specific memory $CD4^+$ T cell repertoire and potentially improving protective immunity to VZV[248].

Sei et al performed a comprehensive study into the functionality of ex vivo T cell responses following Zostavax® administration in 21 volunteers aged 55-65 years[247]. After vaccination, IFN-γ and IL-2 production were boosted following whole blood stimulation with each of 17 separate VZV antigens or VZV lysate. Additionally, vaccination led to a boost in the frequency of VZV-specific $CD4^+$ T cells producing IFN-γ by 8.6-fold, IL-2 5.5-fold, TNF-α 6.1-fold and CD154 (a T cell surface protein that regulates B cell responses by engaging CD40) 5.1-fold. Poly-functional T cells were defined as those expressing all four of these functions, and this subset constituted the highest frequency of memory VZV-specific $CD4^+$ T cells before and after vaccination. After vaccination, there was a boost in the frequency of poly-functional $CD4^+$ T cells in response to all antigens tested. IE63 induced the strongest poly-functional $CD4^+$ T cell responses[247].

On a per-cell basis, poly-functional $CD4^+$ T cells expressed markedly higher levels of IFN-γ, IL-2, and TNF-α than mono-functional T cells, both before and

after vaccination. Vaccination led to a boost in IFN-γ production by poly-functional $CD4^+$ T cells specific to IE63 and ORF-9 but not to other antigens[247]. Whereas $CD4^+$ T cell responses were observed in response to multiple antigens, only ORF-9 induced significant $CD8^+$ T cell responses. Following vaccination, ORF-9-specific poly-functional $CD8^+$ T cells produced ~10-fold higher levels of IFN-γ, TNF-α and IL-2 compared to mono-functional $CD8^+$ T cells[247].

This study suggests that poly-functional T cell responses contribute an important role in the boost in VZV-specific immunity due to Zostavax®. Poly-functional $CD4^+$ and $CD8^+$ T cells were predominantly found within the T_{CM} and T_{EM} compartments, indicating high proliferative potential. These cells have been shown to be important both in the control of infections such as *Leishmania major*[249] and HIV[250], as well as in vaccine responses to Vaccinia virus[251] and others.

1.8 Aims & Objectives

The aim of this project was to characterise skin T_{RM} in young and old individuals with a view to identifying potential defects in the latter group, and exploring means to reverse the age-related impairment in cutaneous antigen-specific immune responses.

The objectives were:

- Characterise skin T_{RM} in young and old individuals to identify defects in the latter group
- Assess how the shingles vaccine modifies the skin T_{RM} population in the old
- Investigate whether the shingles vaccine boosts the in vivo VZV-specific T cell response in old skin, and the underlying mechanisms involved
- Investigate whether modulating inflammatory pathways in old skin can lead to an enhanced VZV-specific cutaneous immune response

2 MATERIALS & METHODS

2.1 Recruitment and ethics

Healthy young (18−35 years) and old (>65 years) volunteers with a history of chickenpox infection were recruited to donate blood and skin biopsy samples. Young volunteers, predominantly personal contacts of our lab members, university students, and university staff, were recruited via word-of-mouth or via recruitment posters displayed around the university. Old volunteers were recruited from community organisations for older people, as well as from regularly held Divisional Public Engagement Days at the university open to older people from the local community.

My specific roles in the recruitment of volunteers were:

- Publicising our lab's research studies among the above groups of people
- Visiting and giving talks at community organisations for older people
- Organisation of Public Engagement Days and speaking at these events to inform the public of our work
- Designing recruitment posters
- Making amendments to the Participant Information Sheet (PIS)
- Seeking approval from the Research & Development Department at the university for amendments to posters and the PIS
- Being the point of contact for interested volunteers
- Consenting volunteers to participate in the study
- Taking blood and skin samples from volunteers
- Administering the intradermal VZV skin test and interpretation at 72 hours
- Administering the Zostavax® vaccine and informing the participants' General Practitioners
- Training the Research Nurse to perform the above tasks independently

All volunteers provided written, informed consent, and the study was conducted in accordance with the Declaration of Helsinki. Ethical approval for the study was granted by the National Research Ethics Service London Queen Square. Exclusion criteria for recruitment were:

- Active inflammatory skin disease
- Acquired or inherited immunodeficiency
- Immunosuppressant medication
- Chronic inflammatory diseases
- Diabetes mellitus
- Infection or vaccination within preceding 1 month
- Pregnancy or breastfeeding
- Bleeding disorders
- Cancer within the last 10 years (excluding nonmelanoma skin cancer)
- History of radiotherapy or chemotherapy
- History of keloid scarring

2.2 Clinical Procedures

2.2.1 Skin biopsy

The site used for skin biopsy was standardised as the sun-protected skin on the proximal, medial, volar forearm surface. The target site was cleaned with a swab impregnated with 70% isopropyl alcohol, and then locally infiltrated with 1% lidocaine for anaesthesia. Next the site was washed with Unisept 0.05% chlorhexidine gluconate solution (Medlock Medical, Oldham, UK). Once the volunteer had lost sensation of the target site, a Stiefel 5mm punch biopsy device (Schuco, Watford, UK) was used to produce the skin sample, and the wound was sutured. Aseptic technique was maintained throughout to reduce the risk of post-operative skin infection. The skin biopsy was collected into saline-soaked gauze to avoid dehydration of the sample, and visible subcutaneous fat was removed with a surgical scalpel before further processing.

2.2.2 DTH response

Clinical response to VZV challenge was assessed by intradermal injection into the proximal, volar surface of the forearm of 0.02ml VZV skin test antigen. This was obtained from The Research Foundation for Microbial Diseases of Osaka University (BIKEN), and is composed of VZV viral glycoprotein. Production of VZV skin test antigen involves infecting human MRC-5 cells (derived from human embryonic lung cells) with VZV, followed by centrifugation of the culture fluid and then heat inactivation of the supernatant[141,145]. The injection site was ringed with marker pen, and the clinical response was then assessed after 72 hours.

The response was graded according to 3 parameters: diameter of skin induration, palpability, and erythema. These parameters were assigned individual scores (Table 2-1), which were then summed to produce an overall clinical score. This method of assessing the clinical response has previously been published by our group[252].

The erythema index (EI) was measured using the DermaSpectrometer (Cortex Technology, Hadsund, Denmark), a portable narrowband reflectance spectrophotometer[253]. This is placed directly on the skin and generates an EI based on the intensity of absorbed and reflected light[253]. The mean of 3 repeated readings taken from the volunteer's normal, uninjected skin was used to generate a background EI. Similarly, the mean of 3 readings taken from the injected site

was used to give a test site EI. Finally, the background EI was subtracted from the test site EI to produce a final EI result. This method takes into account the natural background variation in skin tone that exists between individuals.

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 Palpable	Marked	Very marked		
						TOTAL	

Table 2-1 Table for calculation of clinical score.

Example: at Day 3 the test site has an erythema index of 4, induration size of 7mm, and is easily palpable. This adds up to an overall clinical score of 5.

2.2.3 Shingles vaccination

The shingles vaccine (trade name Zostavax®) was obtained from Sanofi-Pasteur MSD (Maidenhead, UK). This is a live, attenuated vaccine, each dose comprising at least 19,400 plaque-forming units of VZV (Oka/Merck strain). After reconstitution, the 0.65ml dose was administered subcutaneously into the deltoid region.

2.3 Sample acquisition and preparation

2.3.1 Peripheral blood mononuclear cells (PBMCs) isolation

PBMCs were isolated by density centrifugation. Venous blood was collected in Vacuette lithium heparin tubes (Greiner Bio-One, Stonehouse, UK) and diluted 1:1 with Hanks' Balanced Salt Solution (HBSS - Sigma-Aldrich, St. Louis, USA). 15ml of Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) was added per 50ml conical centrifuge tube, and 25ml of the blood/HBSS mix was layered slowly on top using a 25ml serological pipette, so as to produce 2 discrete layers. Centrifugation was performed at 800*g* for 20 minutes with no brake, leading to the formation of a thin layer of PBMCs above the layer of Ficoll-Paque Plus and below a layer of plasma. The PBMC layer was carefully removed using a Pasteur pipette, and transferred to a 50ml conical centrifuge tube. HBSS was added to make a total volume of 50ml, and the tube was centrifuged at 700*g* for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended in a further 50ml of HBSS. Centrifugation was performed at 300*g* for 10 minutes. The supernatant was discarded, and the pellet was re-suspended in complete medium – RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine (all Sigma-Aldrich) – ready for counting and use in experiments.

2.3.2 Cell counting

To count cell numbers, 10µl of cell suspension was removed and mixed thoroughly with 10µl of trypan blue in a well of a 96-well round bottom plate. 10µl of the resulting suspension was transferred to each chamber of an improved Neubauer haemocytometer, and cells were then viewed using a microscope. Dead cells that stained blue were ignored, and live cells were counted using a tally counter. The average count for each of the 2 chambers was used to reduce error. The total cell number was calculated using the following formula:

$$\text{Total cells} = \text{Counted cells} \times 10000^{\dagger} \times 2^{\ddagger} \times \text{Total volume of cell suspension (ml)}$$

† 10000 is the scaling factor from the haemocytometer chamber volume to 1ml
‡ 2 is the dilution factor to account for the addition of trypan blue

2.3.3 Freezing and thawing PBMCs

PBMCs that were not required on the day of blood sampling were frozen for future use. The cell suspension was centrifuged at 700*g* for 5 minutes, the supernatant discarded, and the pellet resuspended in FBS. 20% dimethyl sulfoxide (DMSO) in FBS was added in a 1:1 ratio. The final concentration of cells ranged from 5 to 10 million per ml, and 1ml of cell suspension was added per Nunc cryogenic vial (Thermo Scientific, Waltham, USA). Cryogenic vials were placed into a Nalgene Mr Frosty (Thermo Scientific) containing isopropyl alcohol, and then transferred to a -80°C freezer for slow freezing.

When needed, frozen PBMCs were thawed by briefly warming in a 37°C water bath, followed by rapid and repeated addition of pre-warmed (37°C) complete medium to the cryogenic vial and transfer of all thawed contents to a 15ml conical tube. The cell suspension was centrifuged at 700*g* for 5 minutes and then resuspended in fresh complete medium before counting and use in experiments.

2.3.4 Skin biopsies - frozen sectioning for immunofluorescence

Excess moisture was removed from the skin biopsy by drying in paper towel. Each sample was then placed in a 12x12x20mm Shandon Peel-A-Way Disposable Embedding Mold (Thermo Scientific, Runcorn, UK) half-filled with Cryo-M-Bed optimal cutting temperature compound (Bright Instruments, Huntingdon, UK). The sample was oriented so that the epidermis lay vertical, and then immediately snap-frozen in a Dewar flask of liquid nitrogen, followed by storage at -80°C.

Subsequently, frozen tissue blocks were cut into 6µm sections on polylysine-coated slides, using a microtome-cryostat (Thermo Shandon, Runcorn, UK). Sections were left to dry overnight at room temperature. The following day, sections were fixed in acetone for 10 minutes, followed by ethanol for 10 minutes, and then left to dry for 10 minutes. Slides were wrapped individually in polyvinyl chloride cling film and then stored at -80°C.

2.3.5 Skin biopsies - enzymatic extraction of leukocytes

2 x 5mm fresh skin biopsies per volunteer were obtained to achieve adequate cell numbers for flow cytometry experiments. The samples were washed by transferring between 4ml Bijou tubes containing phosphate-buffered saline (PBS) and shaking, to reduce blood contamination. The biopsies were then manually

dissected into fragments up to 1mm in size (all dimensions), using a surgical scalpel on a plastic Petri dish. A small volume of PBS was added during the dissection process to prevent dehydration of the sample. Tissue fragments from each separate biopsy were added to 1ml of RPMI containing 0.8 mg/ml collagenase IV (Life Technologies, Paisley, UK) and 20% FBS in a well of a 24-well plate, and left overnight in a humidified 5% CO_2 incubator at 37°C.

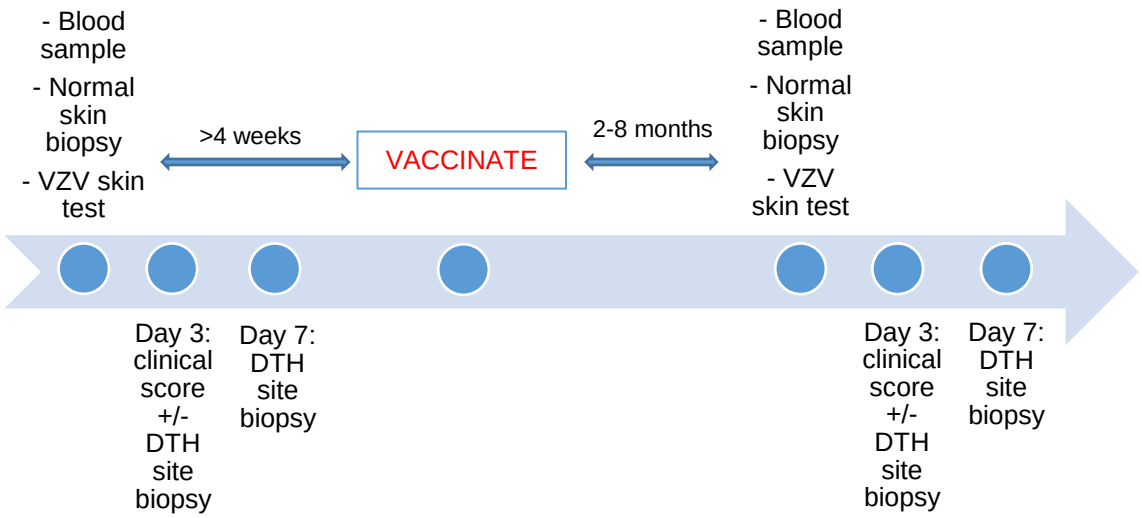
The following day, 5ml FBS was added to a 50ml conical centrifugation tube. A 100µm cell strainer (Corning, Corning, USA) was placed onto the tube, and the strainer moistened with PBS. The tissue fragments from the well plate were transferred with a Pasteur pipette onto the strainer, and a 5ml syringe plunger was used to pulverise the tissue. PBS was added regularly to keep the strainer moist and wash cells through. This was continued until there only remained a small clump of residual, agglutinated tissue on the strainer that could not be broken down further. The well plate was washed out with PBS and the contents added onto the strainer. Finally, the 50ml tube was centrifuged at 300*g* for 10 minutes, and the pellet was resuspended in complete medium, ready for counting and use in experiments. The number of skin-derived cells obtained from 2 biopsies was typically in the range of 1-3 $x10^5$.

2.4 Shingles vaccine study protocol

Volunteers participating in the shingles vaccine study were required to be aged 70 and over, in accordance with the target age range of the national immunisation programme for this vaccine. Potential participants who had already received the shingles vaccine (at their GP surgery) were excluded. The timeline of the study protocol is shown in Figure 2-1. Volunteers donated a 20ml blood sample, received the VZV skin test challenge, and had a skin biopsy taken from normal skin on the contralateral arm. PBMCs were isolated from the blood sample and frozen for further use. The clinical score at 72 hours was recorded, and those with a score of 4 or more were excluded from further participation, as they were deemed to have robust baseline VZV-specific skin immunity. Skin biopsies of the DTH test site were collected from volunteers at 3 days and 7 days after injection; each individual was used for only 1 time-point. Samples were frozen for use in immunofluorescence experiments.

After a minimum of 4 weeks, volunteers went on to receive the shingles vaccine. Following vaccination, volunteers proceeded to undergo repeat blood sampling, normal skin biopsy sampling, VZV skin challenge and clinical scoring, and skin biopsy sampling at the DTH test site. The time interval between vaccination and repeat VZV challenge ranged from 2 to 8 months.

Figure 2-1 Timeline of shingles study protocol



2.5 Losmapimod study protocol

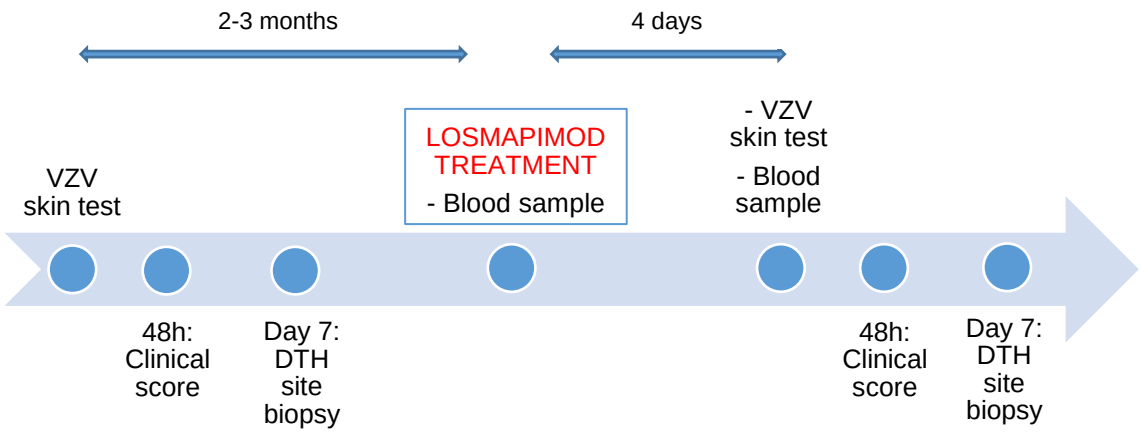
Additional exclusion criteria for this study were:

- History of liver disease
- Elevated alanine transaminase or bilirubin level >1.5 times upper limit of normal
- Prolonged QTc interval (abnormality on electrocardiogram)

The timeline for this study protocol is shown in Figure 2-2. Old volunteers received the VZV antigen skin test and 48 hours later the clinical score was graded, as in Table 2-1. Volunteers with a score of 4 or above were excluded from further participation. A 5mm skin biopsy was taken from the site of antigen challenge at 7 days after skin test injection, and frozen for subsequent immunohistological analysis.

Approximately 2 to 3 months later, volunteers received 15 mg of losmapimod twice daily for 4 days (provided by GlaxoSmithKline under a Medical Research Council Industrial Collaboration Agreement). The dose used in this study was chosen on the basis of the pharmacokinetic, pharmacodynamic, and safety profiles of losmapimod observed in GlaxoSmithKline phase I and II studies[143]. On day 4 of losmapimod treatment, VZV skin testing was repeated and clinical scores were recorded 48 hours later, as before. A 5mm skin biopsy at 7 days after injection was again taken and frozen for immunohistological analysis.

Figure 2-2 Timeline of losmapimod study protocol



2.6 Experimental Techniques

2.6.1 Immunofluorescence microscopy

Indirect immunofluorescence was used to enumerate cells and study their phenotypic markers in normal and VZV-injected skin biopsies. A maximum of 4 markers (including the nuclear stain DAPI) could be included per stain.

2.6.1.1 Staining protocol

Slides frozen at -80°C were thawed at room temperature for 5 minutes. The cling film was removed, and tissue sections were circled using a hydrophobic Dako pen (Dako, Ely, UK). The sections were rehydrated by submerging the slides in a PBS bath for 5 minutes. After this time, the slides were removed and excess PBS around the tissue sections was absorbed with paper towel. The hydrophobic rings were checked to be intact and dry. Incomplete rings were reinforced with hydrophobic pen once more. The slides were placed flat in a humid, dark chamber, and 2-3 drops of Protein Block, Serum-Free (Dako) were added for 20 minutes at room temperature. After this time, excess blocking solution was removed and the primary antibodies were added (having been diluted in PBS at the appropriate concentration - see Table 2-2) at a sufficient volume to ensure coverage of the entire section, typically approximately 100µl. This was followed by overnight incubation in the staining chamber at 4°C.

The following day, slides were washed twice in a PBS bath for 10 minutes each. Slides were removed individually and excess PBS was removed. Next, secondary antibody (diluted in PBS as below) was added at room temperature for 45 minutes. After this time, excess antibody was removed, and slides were washed twice in a PBS bath in the dark for 10 minutes each. Slides were then removed individually, excess PBS removed, and a drop of Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, USA) was applied before mounting a coverslip, which was secured in place using nail varnish. Slides were subsequently stored at -20°C.

Primary Antibody	Source	Concentration	Secondary Antibody	Source	Concentration
NORMAL SKIN					
Rat (IgG1) anti-CD4	AbD Serotec	1:100	Goat anti-rat IgG AF488	Life	1:250
Mouse (IgG1) anti-CD4	Santa Cruz	1:50	Goat anti-mouse IgG1 AF488	Life	1:400
Rabbit (IgG) anti-CD4	Abcam	1:200	Goat anti-rabbit IgG AF488	Invitrogen	1:800
Rat (IgG2b) anti-CD8	AbD Serotec	1:100	Goat anti-rat IgG AF488	Life	1:250
Mouse (IgG1) anti-CD69	BioLegend	1:50	Goat anti-mouse IgG1 AF568	Life	1:250
			Goat anti-mouse IgG1 AF647	Life	1:400
Mouse (IgG2a) anti-CD103	Thermo Fisher	1:400	Goat anti-mouse IgG2a AF647	Life	1:400
Rat (IgG2a) anti-Foxp3 biotin	Ebioscience	1:100	Streptavidin Cy3	Invitrogen	1:400
Mouse (IgG1) anti-PD1	Abcam	1:400	Goat anti-mouse IgG1 AF568	Life	1:400
VZV-INJECTED SKIN					
Mouse (IgG1) anti-CD4 biotin	Immunotech	1:50	Streptavidin Cy3	Invitrogen	1:400
Mouse (IgG1) anti-CD8 biotin	BD	1:50	Streptavidin Cy3	Invitrogen	1:400
Mouse (IgG1) anti-Ki67 FITC	BD	1:50	No secondary		
Mouse (IgG1) anti-CD4	Santa Cruz	1:200	Goat anti-mouse IgG1 AF488	Life	1:200
Rat (IgG2a) anti-Foxp3 biotin	Ebioscience	1:100	Streptavidin Cy3	Invitrogen	1:400

Table 2-2 Antibodies used for immunofluorescence microscopy

AbD Serotec (Kidlington, UK)
Life Technologies (Paisley, UK)
Santa Cruz Biotechnology (Dallas, USA)
Biolegend (San Diego, USA)
Ebioscience (Hatfield, UK)
Invitrogen (Paisley, UK)
BD (San Jose, USA)
Immunotech (Marseille, France)

2.6.1.2 Antibody titration

Primary antibodies were titrated before use, using frozen sections of redundant skin from plastic surgery procedures. The initial concentrations used for titration were 1:50, 1:100 and 1:200, depending on the manufacturer's recommendations. A control, using PBS instead of the primary antibody, was included. Slides were viewed using an Axioskop 2 Plus microscope (Carl Zeiss Ltd.).

2.6.1.3 Image acquisition & analysis

Normal skin

Sections were marked and slides were loaded into a Zeiss Axio Scan.Z1 slide scanner (Carl Zeiss Ltd., Cambridge, UK). Settings were checked and adjusted for focusing and exposure times for each fluorescent channel, and then the entire stained sections were scanned at x200 magnification. Slide scans were analysed using Zen lite software (Carl Zeiss Ltd.).

The cells of interest in this study were the T cells in the upper and mid-dermis, as described in previous publications by this group. T cells were identified using surface stains for CD4 or CD8, in combination with the nuclear stain DAPI. Counting of cells was performed manually using the marker function within Zen lite; a T cell was only counted if an immunofluorescent ring (representing the cell surface) was seen to envelop a DAPI-stained central nucleus. Once the total number of CD4 or CD8 cells was counted per section, additional fluorescent stains (for example for the surface marker CD69) were visualised on Zen lite; the number of previously counted T cells expressing this additional marker was then counted. This enabled a percentage of T cells expressing a certain marker to be calculated. Two sections per slide were analysed, and the average was taken to generate a data value.

VZV-injected skin

Slides were imaged using an Axioskop 2 Plus microscope (Carl Zeiss Ltd.). To assess the degree of cellular infiltration, the 5 largest perivascular infiltrates in the upper dermis were visually selected. These were photographed at x40 magnification using Axiovision software (Carl Zeiss Ltd.). Images were viewed in ImageJ (National Institute of Health, Bethesda, USA), and the total number of T cells (either CD4 or CD8) was counted per perivascular infiltrate (for method of identifying T cells, see above). T cells staining positive for a particular marker (eg. Foxp3 or Ki67) were then counted, to generate a percentage of total T cells

expressing this marker. The mean value for the 5 perivascular infiltrates was then generated. Two sections per slide were analysed, and the average was taken to generate a data value. This method of cell counting for antigen-challenged skin has previously been published by our group[149].

2.6.1.4 Autofluorescence

Immunofluorescence microscopy of skin sections was complicated by the fact that in many of the skin samples, the dermal fibres (collagen and elastin) fluoresced bright green[254]. This was particularly noticeable in the old compared to the young, presumably due to the alteration/degradation in the properties of skin collagen/elastin during the ageing process. Fibre fluorescence was much brighter in the fully stained sections compared to the controls, suggesting that the fibres were taking up primary antibody in a non-specific manner. Autofluorescence was also visible in the blood vessels of the skin, perhaps due to their collagen/elastin content. To negate autofluorescence, concentrations of secondary antibodies were kept as low as possible, and two washing steps of 10 minutes each (after secondary antibody staining) were used as part of the staining protocol.

2.6.2 In vitro functional assays

PBMCs or skin-derived cells were suspended in 1ml complete medium, and placed in 5ml polypropylene round-bottom sterile culture tubes (Greiner Bio-One). 10^6 PBMCs were used per sample; skin cell numbers ranged from 10^5 – 10^6. Cells were incubated at 37°C with humidified 5% CO_2. At the end of the incubation period, sample tubes were centrifuged at 700*g* for 5 minutes, supernatant tipped off, and tube tops were blotted on paper towel. The cell pellets were vortexed, prior to commencing antibody staining.

2.6.2.1 PMA/ionomycin stimulation

Cells were stimulated with 25ng/ml PMA and 500ng/ml ionomycin for 1 hour, followed by addition of 5 µg/ml brefeldin A (Sigma-Aldrich) and incubation for a further 5 hours. Unstimulated cells were used as the negative control. This was followed by intracellular cytokine staining for IL-2, IFN-γ, TNF-α and IL-22.

2.6.2.2 VZV stimulation

Cells were stimulated with 40 µl/ml VZV lysate (Virusys, Taneytown, USA) for 2 hours before addition of 5 µg/ml brefeldin A (Sigma-Aldrich). Unstimulated cells were used as the negative control, and cells stimulated with 1µl/ml staphylococcal enterotoxin B (SEB) were used as the positive control. Ventilation caps were left loose, and samples were incubated for 15 hours, before intracellular cytokine staining for IL-2, IFN-γ and TNF-α.

2.6.3 Flow cytometry

Multicolour flow cytometry was used for the investigation of single cells for multiple surface phenotypic markers and intracellular cytokines.

2.6.3.1 Cell surface & intracellular staining

Staining was performed in 100µl volume. A master mix of surface antibodies was made up (see Table 2-3), including 1µl Live/Dead UV stain (Invitrogen) per sample. The appropriate antibody mix volume was added to each sample tube, before vortexing and incubation in the dark for 30 minutes on ice.

Surface Antibody	Fluorochrome	Source	Volume (µl)
CD3	PE-CF594	BD	0.3
CD4	A700	BD	2
CD8	PerCP	BD	15
CD27	APC-H7	BD	5
CD45RA	BV605	BioLegend	2
CLA	FITC	BD	10

Table 2-3 Surface antibodies for flow cytometry

Following the surface staining stage, 100µl Fix and Perm Cell Permeabilization Medium A (Invitrogen) was added for 15 minutes at room temperature. Approximately 1ml PBSA (PBS with 0.2% BSA and 0.1% azide) was added to each tube before centrifuging at 700*g* for 5 minutes. The supernatant was tipped off and tube tops were dried by briefly placing on paper towel. 100 µl Fix and Perm Cell Permeabilization Medium B (Invitrogen) was next added to each tube along with the intracellular antibodies from a master mix (Table 2-4). Samples were vortexed and incubated for 30 minutes at room temperature.

Intracellular Antibody	Fluorochrome	Source	Volume (µl)
IFN-γ	V450	BD	0.8
TNF-α	APC	BD	5
IL-2 (VZV stim experiment)	PE	BD	10
IL-2 (PMA experiment)	FITC	BD	10
IL-22	PE	Biolegend	5

Table 2-4 Intracellular antibodies for flow cytometry

After the staining stages, PBSA was added to each tube before centrifuging at 700*g* for 5 minutes, tipping off the supernatant, and adding 100µl 2% paraformaldehyde to fix the cells. PBMC samples were transferred to standard flow cytometry tubes; skin cell samples were transferred to Falcon flow cytometry tubes with a 35µm cell strainer cap (BD) to filter out debris.

Single colour compensation controls were prepared using CompBeads Set (BD). The following were added sequentially to a flow cytometry tube: 1µl of antibody; 20µl of anti-mouse Ig, κ particles; 20µl negative control particles; 300µl 2% paraformaldehyde. This protocol was suitable for all antibodies used except CLA FITC, as this is a rat antibody. Therefore, a BD CD4 FITC antibody was substituted for the FITC compensation control.

An unstained control comprising 10^6 fixed PBMCs was included. A live-dead control was prepared by heating 2.5 x 10^5 PBMCs in 50µl medium on a hot plate at 70°C for 4 minutes, allowing to cool, and adding to 2.5 x 10^5 live PBMCs in 50µl medium. This control was stained with 1µl Live/Dead UV stain for 30 minutes and then fixed. Fluorescence minus one (FMO) controls, containing every fluorochrome in the panel except for the one being controlled for, were prepared and acquired.

2.6.3.2 Flow cytometers

An LSR II flow cytometer (BD, San Jose, USA), equipped with blue (488nm), red (640nm), yellow/green (561nm), violet (405nm) and ultraviolet (355nm) lasers, was used for flow cytometry experiments. Compensation control tubes were acquired and the flow cytometer used an automated compensation process to account for spectral overlap between fluorochromes. Next, the sample tubes were acquired, with up to 10 fluorochromes detected simultaneously. Data was processed by FACSDiva software (BD).

2.6.3.3 Flow cytometry data analysis

Data files were analysed using FlowJo version 10 software (FlowJo LLC, Ashland, USA). Initial gating strategy selected lymphocytes (based on forward and side scatter properties), live cells (that were negative for the live/dead UV stain), and singlet cells (based on side scatter pulse height and width). FMO controls were used to assist with gating strategy.

2.7 Statistical analysis

Graphpad Prism version 5 (Graphpad Software, Inc., La Jolla, USA) was used for statistical analysis. Tests were deemed to be statistically significant where the p value was less than 0.05. The Shapiro-Wilk test was applied to data sets to test whether these were normally distributed. Where data was non-parametric, the Mann-Whitney U test was used to compare whether the medians of 2 populations (i.e. young and old) were statistically different. The non-parametric Wilcoxon signed-rank test was used to test whether paired samples were different. Parametric data were compared with the unpaired t test for unpaired groups, or paired t test for paired groups. Linear regression was used to assess whether 2 variables were correlated.

3 AGE-RELATED CHANGES IN T CELLS WITHIN THE SKIN

3.1 Chapter Introduction

Antigen-specific immunity declines with age, and a key contributing factor is the constellation of age-related changes occurring within the circulating memory T cell compartment[41]. Skin immunity also declines with ageing, and the reasons for this have not yet been determined. T_{RM} are recognised to play a key role in the immune protection of the skin against pathogens[255], but to date they have not been characterised in the context of ageing; it is unclear whether skin T_{RM} in old individuals are susceptible to changes associated with immunosenescence. Here, $CD4^+$ and $CD8^+$ skin T_{RM} were identified and characterised in young and old individuals, with regards to numbers, phenotype, state of differentiation, and effector function. As clinical VZV reactivation is more frequent in the old[191], and this is associated with impaired DTH responses to VZV skin test antigen[143], it was also of interest to investigate VZV-specific T cells in the skin for potential age-related impairments.

3.2 Aims & Objectives

The aim of this chapter was to characterise skin resident memory T cells in young and old individuals, and to identify potential defects in the latter age group.

3.3 Results

3.3.1 Skin T_{RM} numbers

3.3.1.1 The number of skin $CD4^+$ T_{RM} is equivalent between young and old

Frozen sections of normal skin from young and old individuals were stained with anti-CD4 antibody and analysed by immunofluorescence microscopy. Cells in the upper dermis were counted, where the majority of skin T_{RM} are located. The epidermis and the lower dermis were not incorporated into cell counts as a result of intense background staining and adnexal structure staining in these areas respectively; in any case, very few T cells were observed in these areas.

A representative immunofluorescence micrograph of a skin section stained for CD4 is shown in Figure 3-1A, with a close-up of a perivascular cluster of $CD4^+$ T cells in the upper dermis in Figure 3-1B. The majority of $CD4^+$ T cells were observed to be in perivascular clusters, with additional occasional single cells also present in the dermis, consistent with previous findings[256]. The median $CD4^+$ T cell number per section was 65 for young and 67 for old (range 16-128; Figure 3-1C), with no significant difference between the age groups.

To establish the proportion of $CD4^+$ T cells that were resident memory cells (T_{RM}), as opposed to recirculating, skin sections were co-stained for the tissue residency marker CD69 (Figure 3-1A&B). The majority of $CD4^+$ T cells in the dermis were found to be $CD69^+$ (median 77.5% for young and 78.9% for old; Figure 3-1D) and there was no significant difference between age groups. Together, these data indicate that the numbers of skin resident $CD4^+$ T cells remain stable from early adulthood into old age.

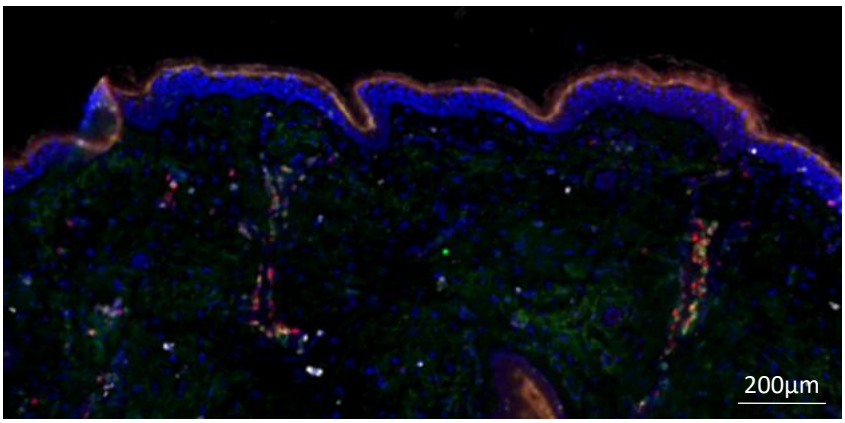
As CD69 is not solely a T cell tissue residency marker but also a T cell activation marker, the intensity and pattern of CD69 expression following T cell activation was examined by staining skin sections that had been obtained by biopsy at 7 days after cutaneous antigen challenge (Figure 3-1E). CD69 expression was seen to be intensely expressed in large numbers of perivascular T cells infiltrating into the skin. These observations highlight the usefulness of CD69 as a residency marker in unchallenged skin, and as an activation marker in challenged skin.

CD103 is a tissue residency marker, predominantly of $CD8^+$ T cells, whose expression varies depending on the tissue site, with highest expression seen in the gut[257]. Expression of CD103 in mouse skin is significantly higher than in

human skin[79,87]. $CD103^+$ T_{RM} were shown to have more potent effector abilities than $CD103^-$ T_{RM} [87], and it was of interest to determine whether the proportion of human skin T_{RM} expressing this marker changes with age.

Anti-CD103 co-staining was performed and the majority of $CD4^+CD69^+$ T_{RM} were observed to be $CD103^-$ (Figure 3-1A, B, F). The median proportion of $CD4^+CD69^+$ T_{RM} expressing CD103 was 22.2% in the young and 25.7% in the old, with no significant difference between the 2 age groups.

A



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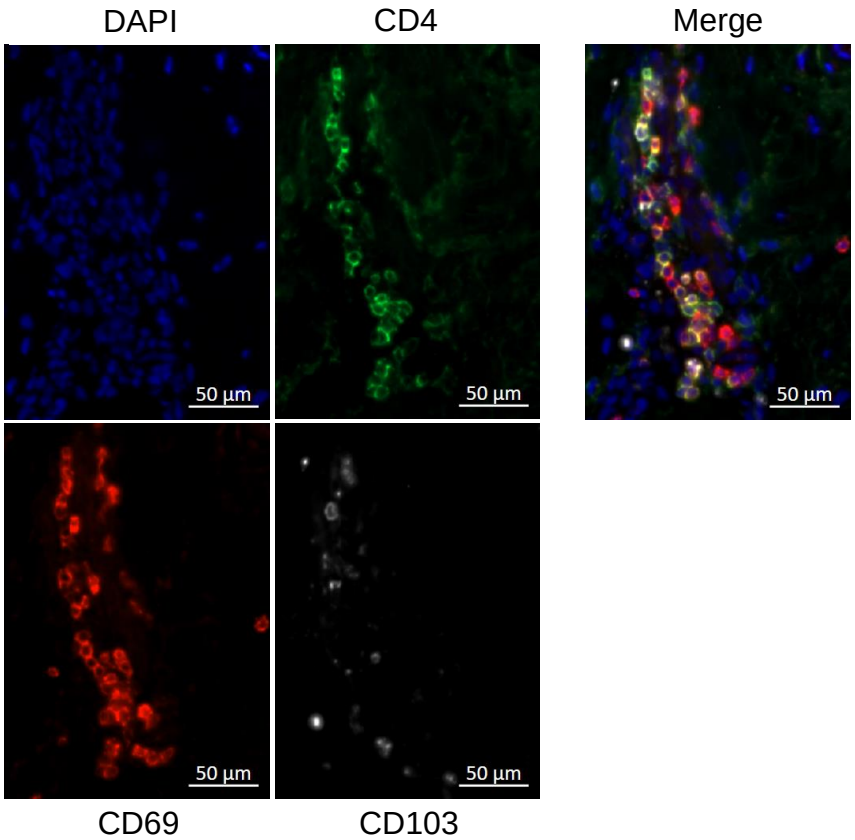

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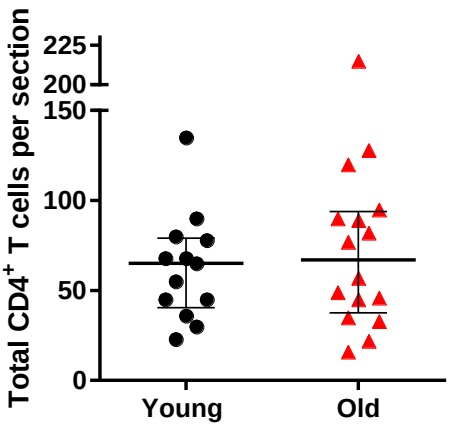

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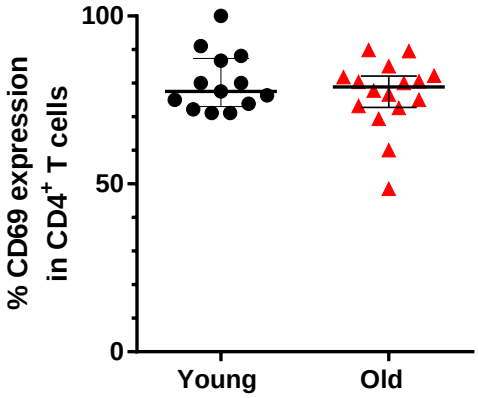

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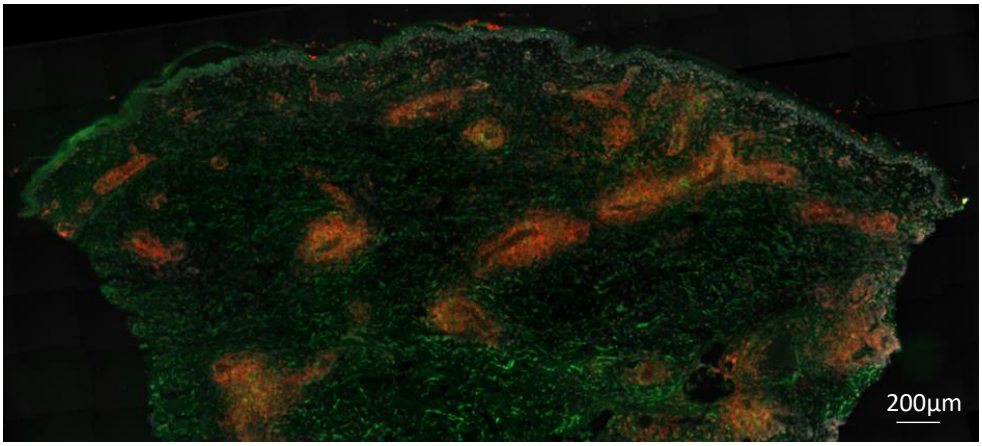

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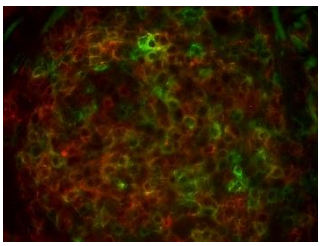

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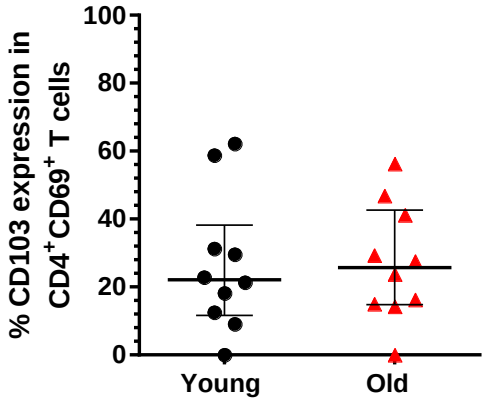


Figure 3-1 Effect of age on $CD4^+$ T_{RM} numbers

A: Immunofluorescence micrograph (x200 original magnification) of a skin section stained for DAPI (blue), CD4 (green), CD69 (red), CD103 (white).
B: Close up of a perivascular cluster of $CD4^+$ T cells in the upper dermis, the majority of which are $CD69^+$. In the merged image, $CD4^+CD69^+$ T_{RM} are seen as orange/yellow. Few of these cells are $CD103^+$.
C: Graph showing the number of dermal $CD4^+$ T cells per section in young (n=13) and old individuals (n=16).
D: Graph showing the percentage of $CD4^+$ T cells in the dermis expressing CD69 (n=13 young, 16 old).
E: Top – immunofluorescence micrograph (x200 original magnification) of a section of antigen-challenged skin stained for CD4 (green) and CD69 (red), with large numbers of activated perivascular T cells visible. Bottom – close-up view (x400 original magnification) of a perivascular cellular infiltrate, with activated $CD4^+CD69^+$ T cells seen as orange.
F: Graph showing the percentage of $CD4^+CD69^+$ T cells in the dermis expressing CD103 (n=10 young, 10 old).
Medians and interquartile ranges are displayed, and populations were compared using the Mann-Whitney test.

3.3.1.2 The number of skin $CD8^+$ T_{RM} is equivalent between young and old

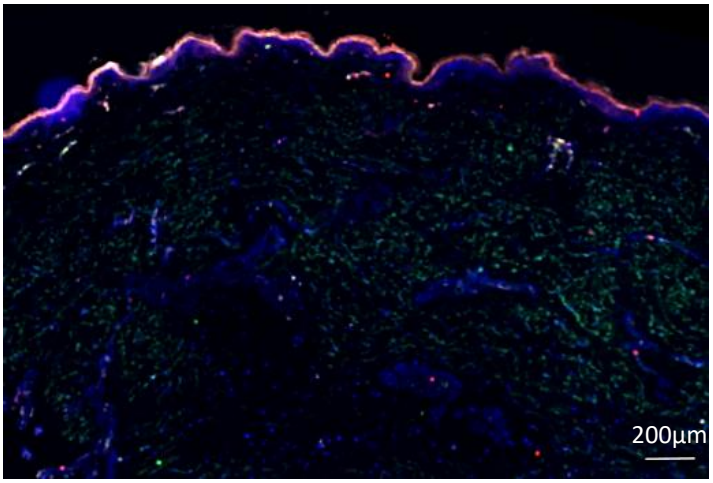
Frozen skin sections were stained with anti-CD8 antibody and analysed by immunofluorescence microscopy. $CD8^+$ T cells in the upper dermis were counted as described in Section 3.3.1.1 for $CD4^+$ T cells. A representative image of a skin section stained for CD8 is shown in Figure 3-2A, with a close-up of $CD8^+$ T cells within a perivascular cell cluster in the upper dermis in Figure 3-2B. In general, $CD8^+$ T cells were observed as a minority component of perivascular clusters or as isolated cells in the upper dermis.

$CD8^+$ T cells in the skin were observed to be more sparsely distributed throughout the skin than $CD4^+$ T cells. The median number of $CD8^+$ T cells per section was 26 for both young and old (Figure 3-2C), with no significant difference between the groups.

The median percentage of $CD8^+$ T cells in the dermis expressing CD69 was 83.6% in the young and 85.1% in the old, with no significant difference between the groups (Figure 3-2D). Together, these data indicate that the numbers of skin $CD8^+$ T_{RM} remain stable and unaltered by the ageing process.

Co-staining with CD103 demonstrated the median percentage of $CD8^+CD69^+$ T_{RM} expressing this marker was 23.3% in the young and 12.1% in the old (Figure 3-2E). There was a trend to a difference ($p=0.21$) between the two age groups.

A



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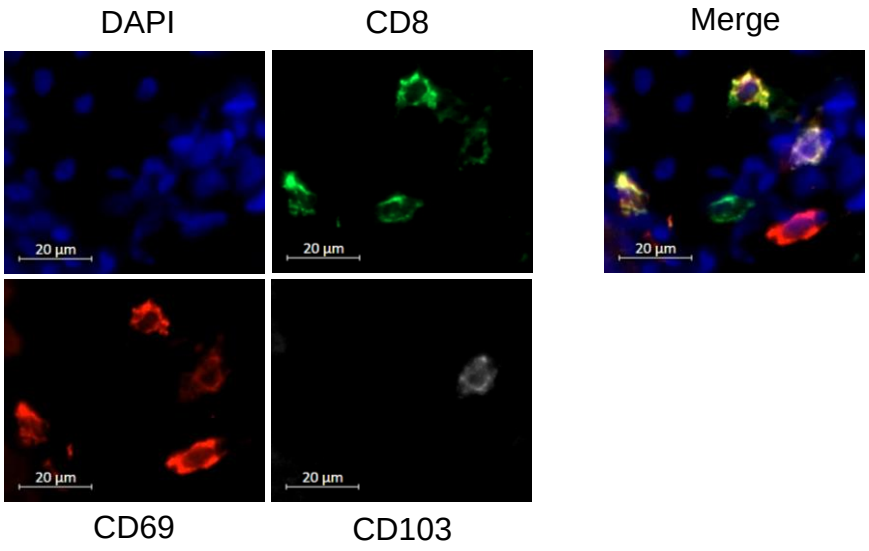


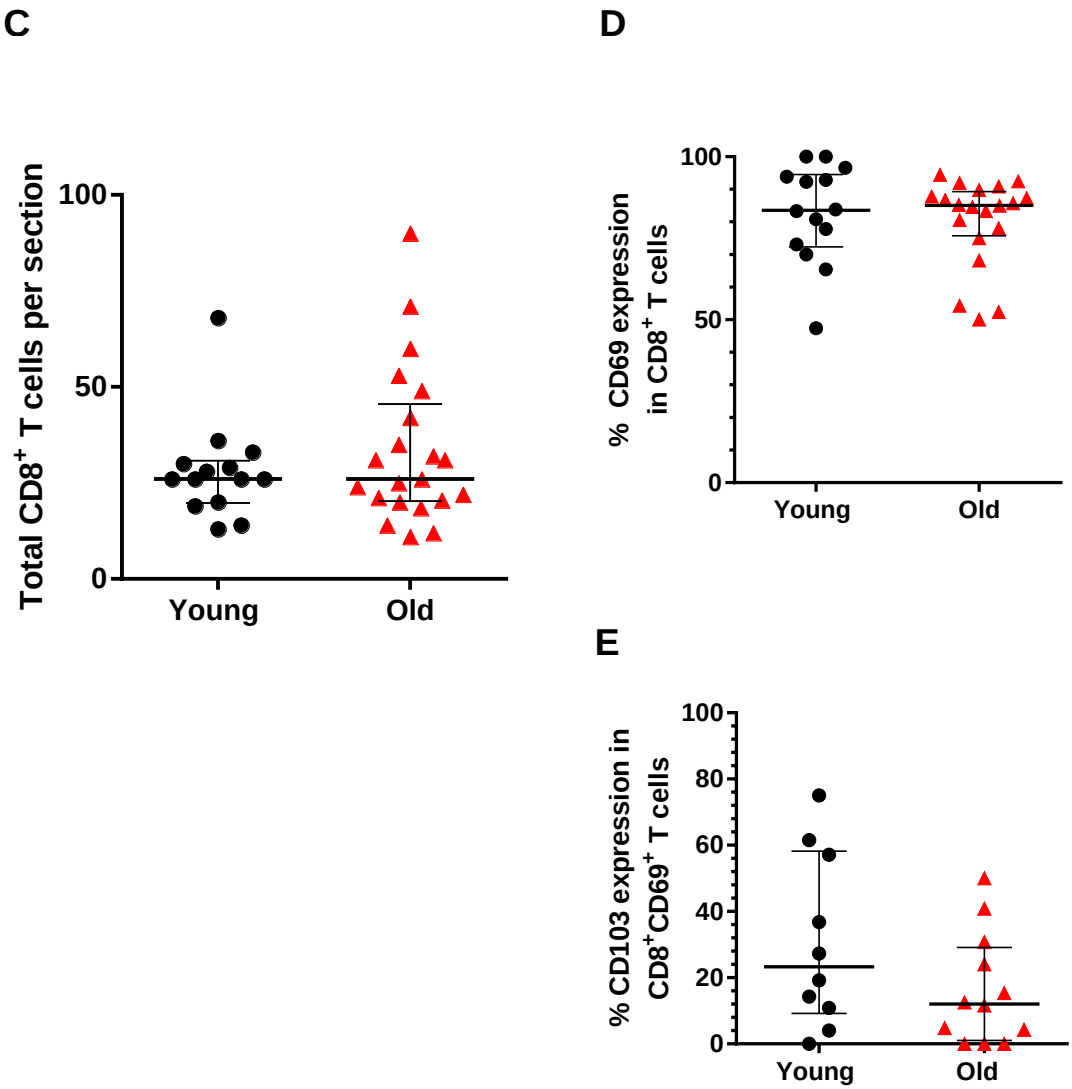


Figure 3-2 Effect of age on CD8⁺ T_{RM} numbers

A: Representative immunofluorescence micrograph (x200 original magnification) of a skin section stained for DAPI (blue), CD8 (green), CD69 (red) and CD103 (white).
B: Close-up of a perivascular cluster of CD8⁺ T cells in the upper dermis, the majority of which are positive for CD69 (red) and a minority of which are positive for CD103.
C: Graph showing the number of dermal CD8⁺ T cells per section in young (n=14) and old (n=21) individuals.
D: Graph showing the percentage of dermal CD8⁺ T cells expressing CD69 in young (n=14) and old (n=21) individuals.
E: Graph showing the percentage of CD8⁺CD69⁺ T cells in the dermis expressing CD103 (n=10 young, 12 old).
Medians and interquartile ranges are displayed, and the groups were compared using the Mann-Whitney test.

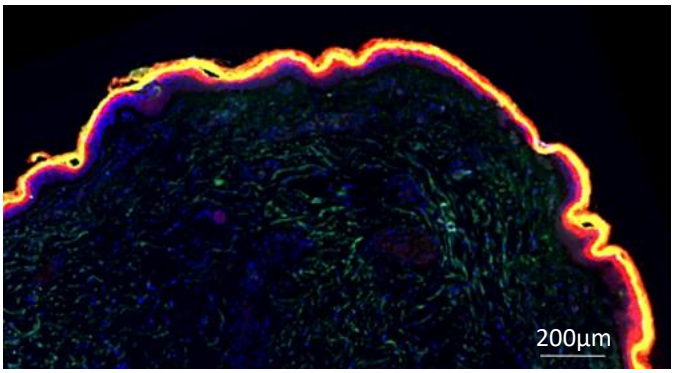
3.3.1.3 Old skin has a higher proportion of Tregs compared to young

A previous study by our group showed an increased percentage of Tregs in old skin compared to young[135]. To confirm and extend these findings, frozen skin sections were stained for CD4 and Foxp3 and analysed by immunofluorescence microscopy. The number of $CD4^+$ $Foxp3^+$ T cells in the dermis was expressed as a percentage of the total number of $CD4^+$ T cells.

A representative image of a skin section stained for CD4 and Foxp3 is shown in Figure 3-3A, with a close-up image of a perivascular cell cluster containing a number of $CD4^+Foxp3^+$ T cells shown in Figure 3-3B. The median proportion of Tregs was significantly higher in the old (9.0%) than the young (3.3%), shown in Figure 3-3C ($p=0.001$).

The proportion of Tregs that are resident in the skin is unknown, and this was investigated by co-staining with CD69. Figure 3-3D shows the median percentage of Tregs expressing CD69 in the young was 74.0%, significantly higher than the 50.0% observed in the old ($p=0.029$). Interestingly, in the old the percentage of Tregs expressing CD69 was lower than the percentage of total $CD4^+$ and $CD8^+$ T cells expressing CD69, regardless of age (see Sections 3.3.1.1 and 3.3.1.2).

A



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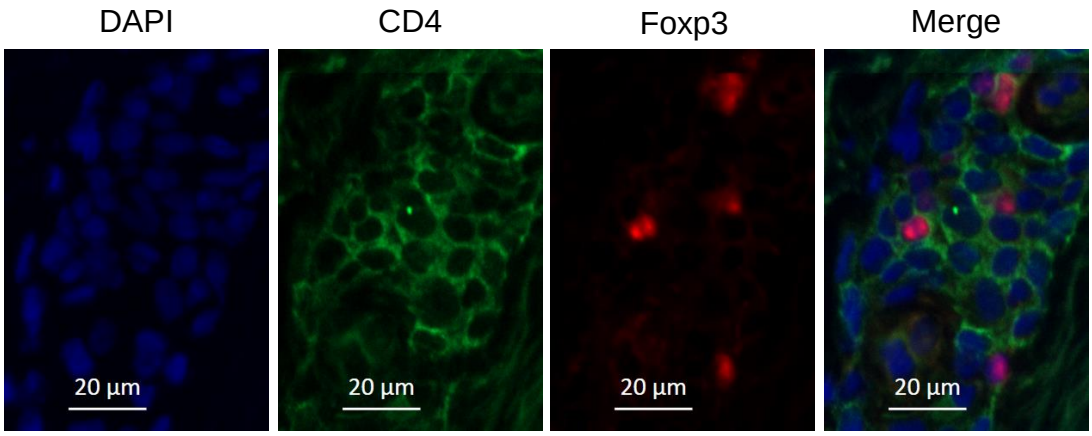


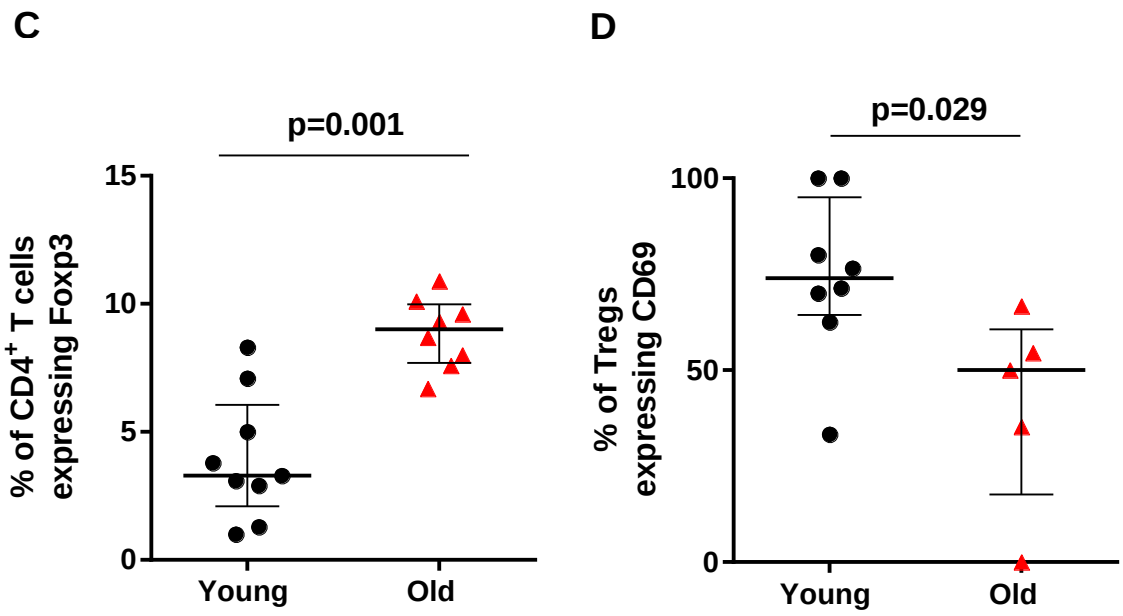


Figure 3-3 Effect of age on Tregs in the skin

A: Representative immunofluorescence micrograph (x200 original magnification) of a skin section stained for DAPI (blue), CD4 (green) and Foxp3 (red).
B: Close-up view of a perivascular cluster of $CD4^+$ $Foxp3^-$ T cells and $CD4^+$ $Foxp3^+$ Tregs (green surface with red nuclear stain) in the dermis.
C: Graph showing the percentage of $CD4^+$ T cells in the dermis expressing Foxp3, in young (n=9) and old (n=8) individuals.
D: Graph showing the percentage of Tregs in the dermis expressing CD69 in young (n=8) and old (n=5) individuals.
Medians and interquartile ranges are displayed. Both groups were compared using the Mann-Whitney test and the significance level is displayed.

3.3.2 T cell differentiation profiles

3.3.2.1 There is no increase in the proportion of terminally differentiated $CD27^-CD45RA^+$ $CD4^+$ T cells in old skin

The differentiation state, defined by the surface expression of CD27 and CD45RA, of $CD4^+$ T cells isolated from collagenase-digested skin was investigated in young and old using flow cytometry. $CD4^+$ T cells from peripheral blood were also analysed for comparison. A representative flow cytometry gating strategy is shown in Figure 3-4A. Representative CD27/CD45RA plots for $CD4^+$ T cells derived from young and old skin and blood are shown in Figure 3-4B.

Cumulative data for blood (Figure 3-4C, left graph) showed that the majority of $CD4^+$ T cells in blood in both young and old were of the naïve ($CD27^+$ $CD45RA^+$) and central memory ($CD27^+$ $CD45RA^-$) subsets, as previously described[258]. There were significant differences in the proportion of $CD4^+$ T cells represented by each of the 4 differentiation subsets between young and old, with old individuals having a smaller proportion of naïve $CD4^+$ T cells ($p<0.0001$) and greater proportions in the central memory ($p=0.0007$), effector memory ($p=0.0006$) and T_{EMRA} subsets ($p=0.0063$), consistent with published studies[258,259].

Cumulative equivalent data for skin (Figure 3-4C, right graph) demonstrated that the vast majority of $CD4^+$ T cells were $CD45RA^-$, corresponding to the central and effector memory subsets according to the standard classification used for T cells in circulation. Old individuals had a significantly smaller proportion of $CD4^+$ T cells in the $CD27^+CD45RA^-$ central memory subset ($p=0.0067$) and a significantly larger proportion in the $CD27^-CD45RA^-$ effector memory subset ($p=0.0143$) compared to the young. Importantly, there was no significant difference in proportions in the terminally differentiated $CD27^-CD45RA^+$ T_{EMRA} subset between young (median 1.7%) and old (median 1.4%).

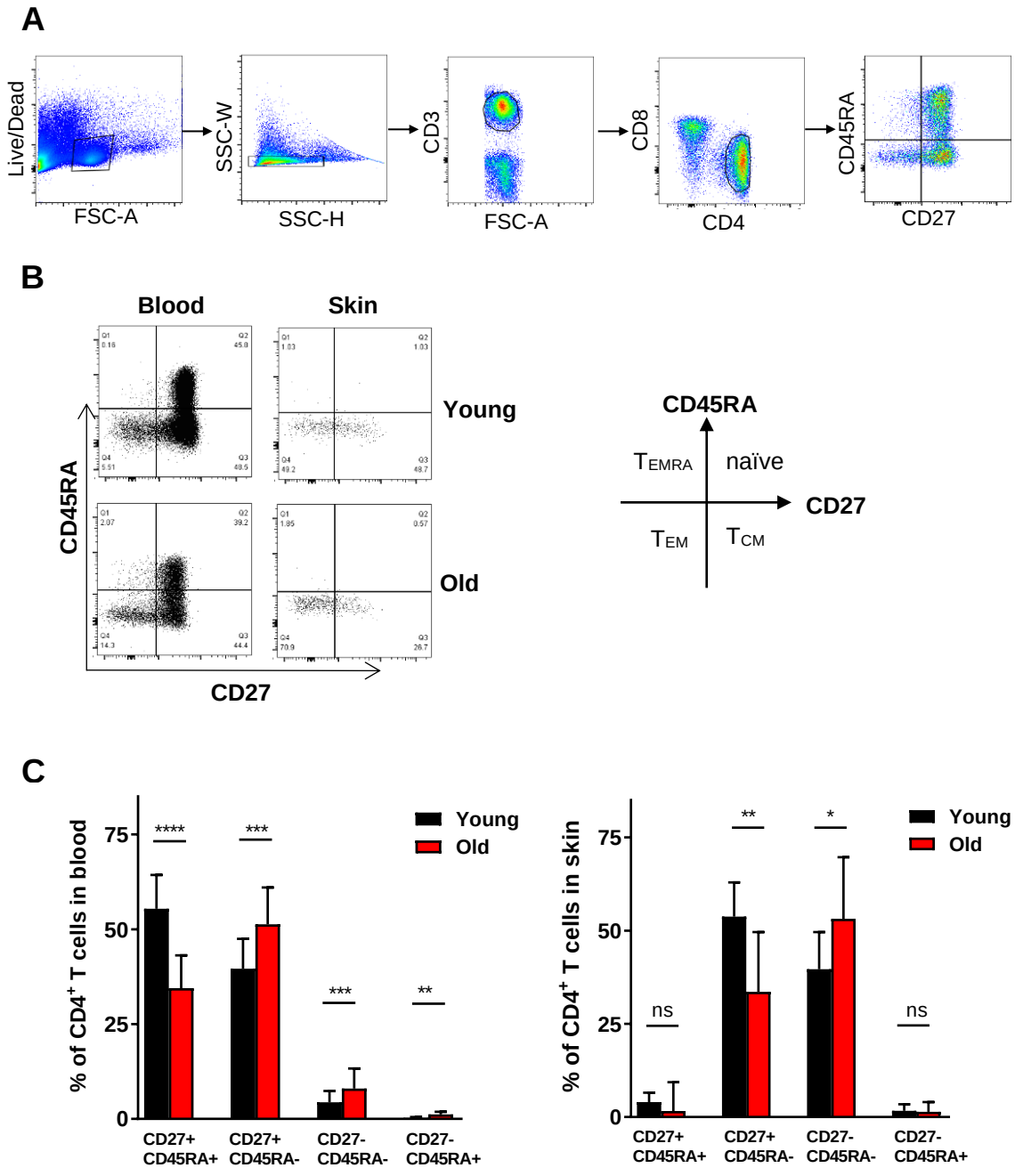


Figure 3-4 Effect of age on the differentiation status of CD4⁺ T cells in the skin

A: Representative flow cytometry dot plots of PBMCs showing the sequential gating strategy to identify CD4⁺ T cells, and division into the 4 differentiation subsets defined by CD27 and CD45RA expression.
B: Representative dot plots of the CD27/CD45RA expression profile of blood- and skin-derived CD4⁺ T cells from a young and old donor.
C: Bar graphs showing cumulative data for the proportion of CD4⁺ T cells represented by each of the 4 subsets defined by CD27 and CD45RA expression, in young and old individuals (n=23 each). Blood data is shown in the left graph and skin data in the right. Medians and interquartile ranges are shown. Subsets were compared using the Mann-Whitney test and significance levels are displayed.

3.3.2.2 There is no increase in the proportion of terminally differentiated $CD27^{-}CD45RA^{+}$ $CD8^{+}$ T cells in old skin

The differentiation state of $CD8^{+}$ T cells from collagenase-digested skin and blood samples was assessed by flow cytometry. Representative CD27/CD45RA plots for $CD8^{+}$ T cells derived from young and old blood and skin are shown in Figure 3-5A.

Cumulative data for blood (Figure 3-5B, left graph) showed that in young individuals, the vast majority of $CD8^{+}$ T cells were naïve $CD27^{+}$ $CD45RA^{+}$ cells, whereas in the old there was a more even distribution between the 4 subsets defined by CD27/CD45RA expression. There was a significant loss of naïve $CD8^{+}$ T cells in the old compared to the young ($p<0.0001$) and a gain in the effector memory ($p=0.0009$) and T_{EMRA} subsets ($p<0.0001$), consistent with published studies[258].

Cumulative data for skin (Figure 3-5B, right graph) demonstrated that the majority of $CD8^{+}$ T cells were $CD45RA^{-}$. Old individuals had a significantly smaller proportion of $CD8^{+}$ T cells in the $CD27^{+}$ $CD45RA^{+}$ naïve subset ($p=0.0002$) and a significantly larger proportion in the $CD27^{-}CD45RA^{-}$ effector memory subset ($p=0.0003$) compared to the young. Importantly, there was no significant difference in proportion in the terminally differentiated T_{EMRA} subset between young (median 7.7%) and old (median 4.5%).

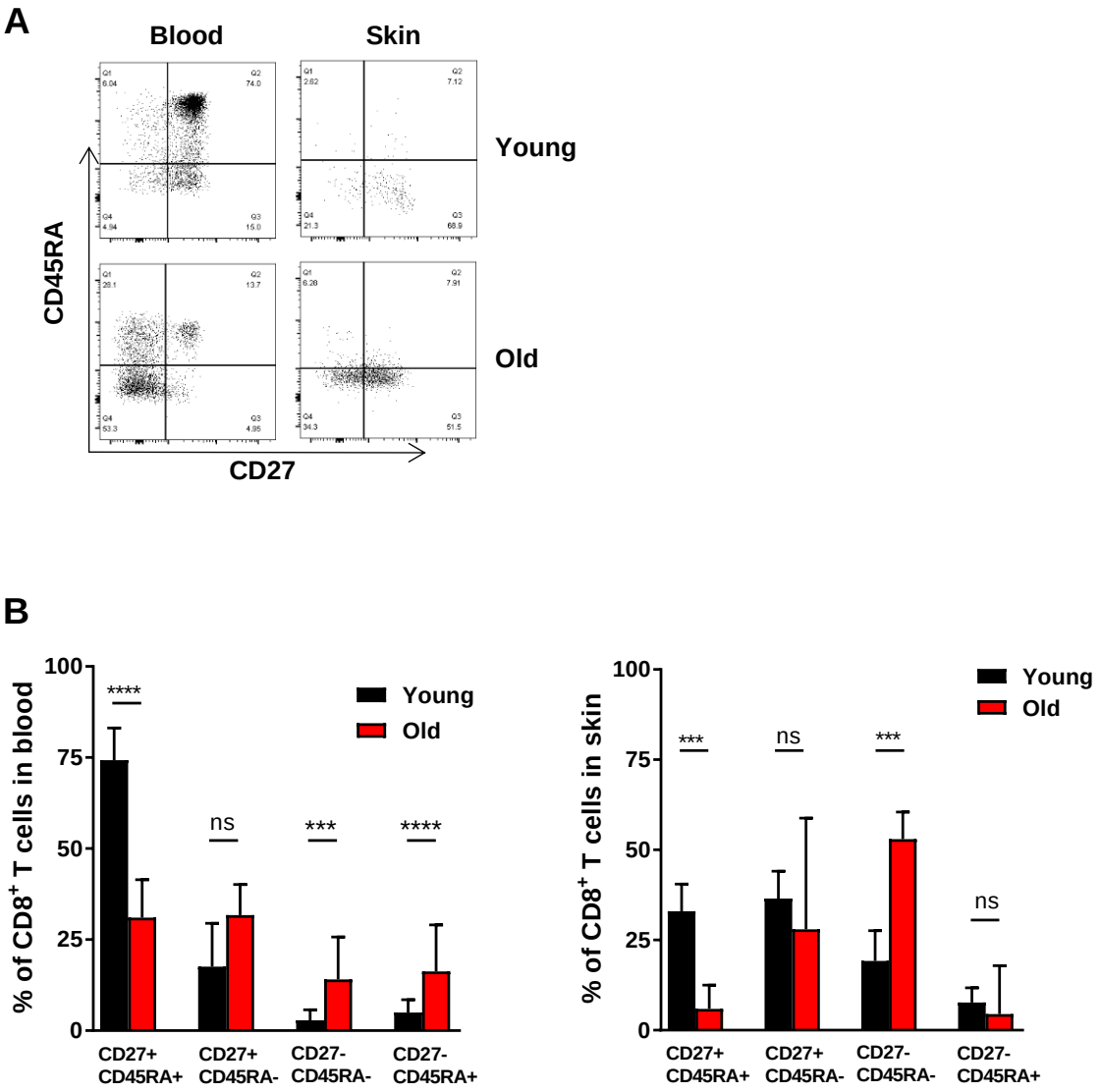


Figure 3-5 Effect of age on the differentiation status of CD8⁺ T cells in the skin

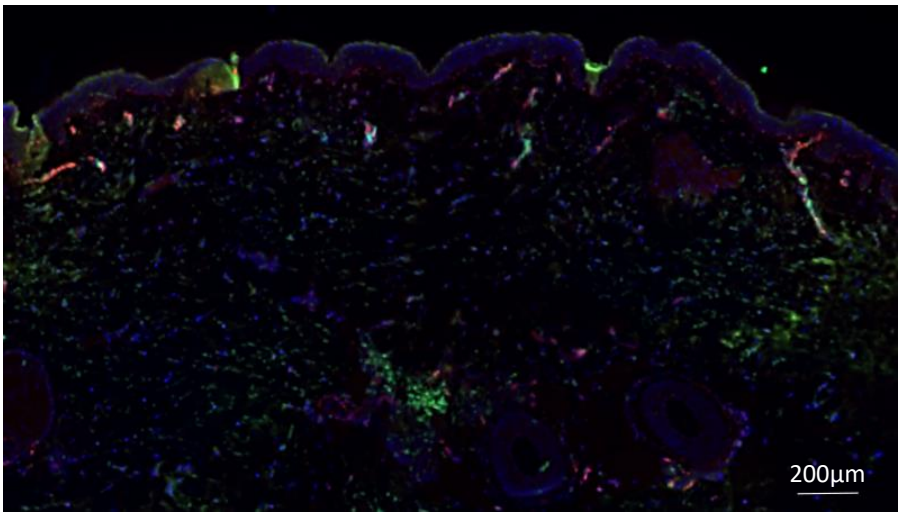
A: Representative dot plots of the CD27/CD45RA expression profile of blood- and skin-derived CD8⁺ T cells from a young and old donor.
B: Bar graphs showing cumulative data for the proportion of CD8⁺ T cells represented by each of the 4 subsets defined by CD27 and CD45RA expression, in young (n=16) and old (n=10) individuals. Blood data is shown in the left graph and skin data in the right. Medians and interquartile ranges are shown. Subsets were compared using the Mann-Whitney test and significance levels are displayed.

3.3.3 Programmed cell death 1 (PD-1)

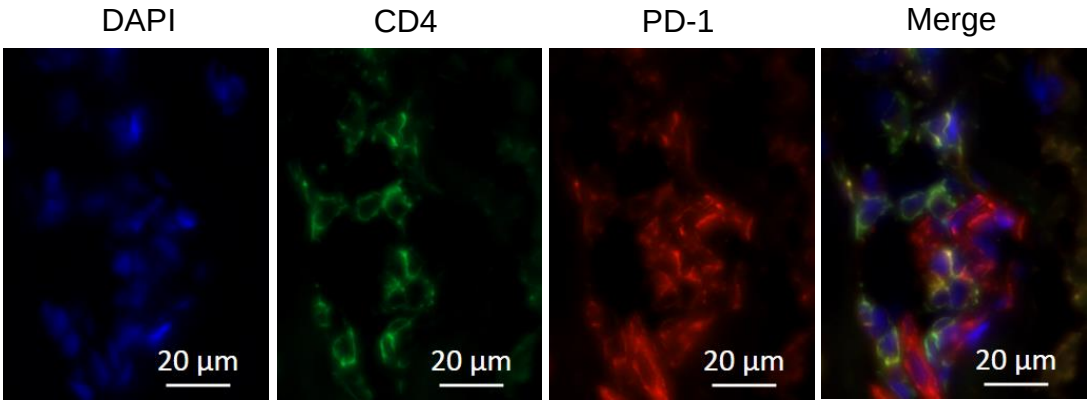
3.3.3.1 PD-1 expression on $CD4^+$ T cells in the skin increases with age

Frozen skin sections from healthy young and old donors were co-stained for CD4 and PD-1 and analysed by immunofluorescence microscopy. A representative immunofluorescence micrograph of a skin section stained for CD4 and PD-1 is shown in Figure 3-6A, with a close-up image of a cluster of dermal $CD4^+$ T cells in Figure 3-6B. The median percentage of $CD4^+$ T cells expressing PD-1 was significantly higher in the old (57.1%) than the young (27.2%), as shown in Figure 3-6C ($p=0.03$).

A



B



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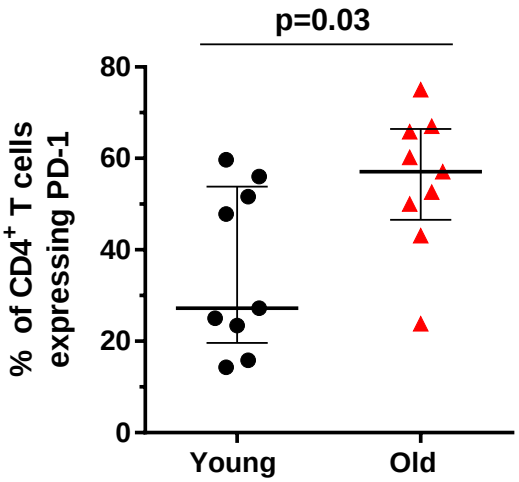


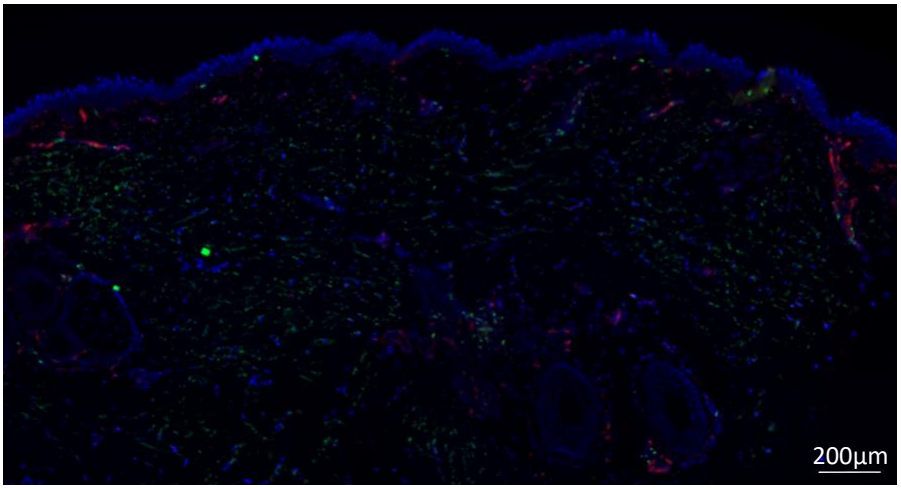
Figure 3-6 The effect of age on PD-1 expression in skin CD4⁺ T cells

A: Representative immunofluorescence micrograph (x200 original magnification) of a skin section stained for DAPI (blue), CD4 (green) and PD-1 (red).
B: Close-up image of a perivascular cluster of T cells; CD4⁺ PD-1⁺ T cells are seen as orange/yellow in the merged image.
C: Graph showing the percentage of dermal CD4⁺ T cells expressing PD-1 in young (n=9) and old (n=9). Medians and interquartile ranges are shown. Young and old were compared using the Mann-Whitney test, and the significance level is shown.

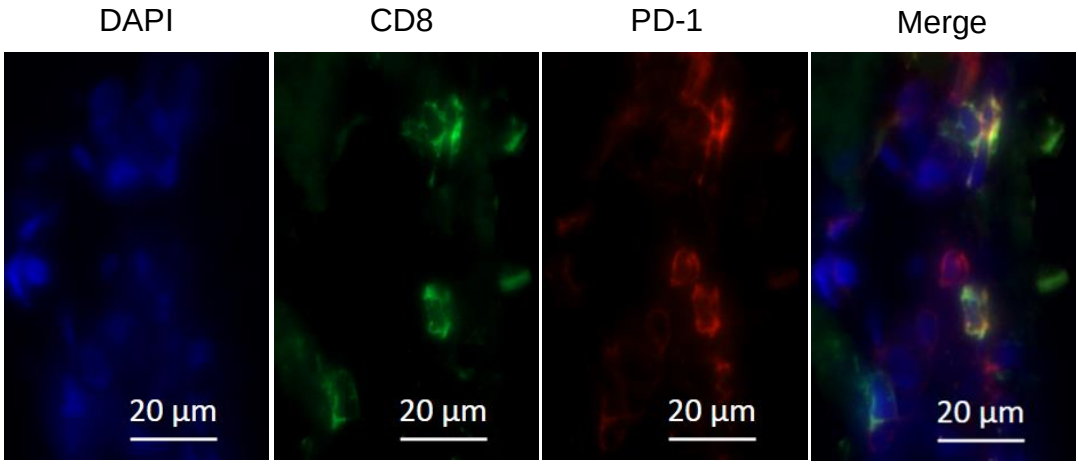
3.3.3.2 PD-1 expression on $CD8^+$ T cells in the skin increases with age

Frozen skin sections from healthy young and old donors were co-stained for CD8 and PD-1 and analysed by immunofluorescence microscopy. A representative immunofluorescence micrograph of a skin section stained for CD8 and PD-1 is shown in Figure 3-7A, with a close-up image of a cluster of dermal $CD8^+$ T cells expressing PD-1 in Figure 3-7B. The median percentage of $CD8^+$ T cells expressing PD-1 was significantly higher in the old (70.7%) compared to the young (23.6%) as shown in Figure 3-7C ($p=0.001$).

A



B



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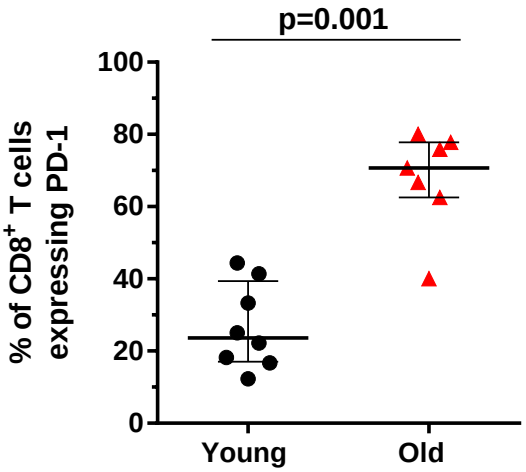


Figure 3-7 The effect of age on PD-1 expression in skin $CD8^+$ T cells

A: Representative immunofluorescence micrograph (x200 magnification) of a skin section stained for CD8 (green) and PD-1 (red).
B: Close-up image of dermal T cells, with $CD8^+$ $PD\text{-}1^+$ T cells seen as orange/yellow in the merged image.
C: Graph showing the percentage of dermal $CD8^+$ T cells expressing PD-1 in young (n=8) and old (n=7). Medians and interquartile ranges are shown. Young and old were compared using the Mann-Whitney test, and the significance level is shown.

3.3.4 T cell effector function

3.3.4.1 Skin-derived $CD4^+$ T cells from old individuals are functionally competent in vitro

To assess whether $CD4^+$ T cells isolated from old skin were functionally competent in vitro, leukocytes derived from collagenase-digested skin were stimulated with PMA/ionomycin for 6 hours, and intracellular cytokine production by these cells was measured by flow cytometry, specifically IFN-γ, TNF-α, IL-2 and IL-22. Unstimulated cells were used as negative controls.

Representative dot plots showing the proportion of skin-derived $CD4^+$ T cells producing IFN-γ and TNF-α in a young and an old individual are shown in Figure 3-8A. Cumulative data are shown in Figure 3-8B. Although there was no significant difference in the percentage of cytokine-producing $CD4^+$ T cells between young and old for any of the 4 cytokines measured, there was a trend towards an increase for IFN-γ, TNF-α and IL-2 in the old. IL-22 production was minimal.

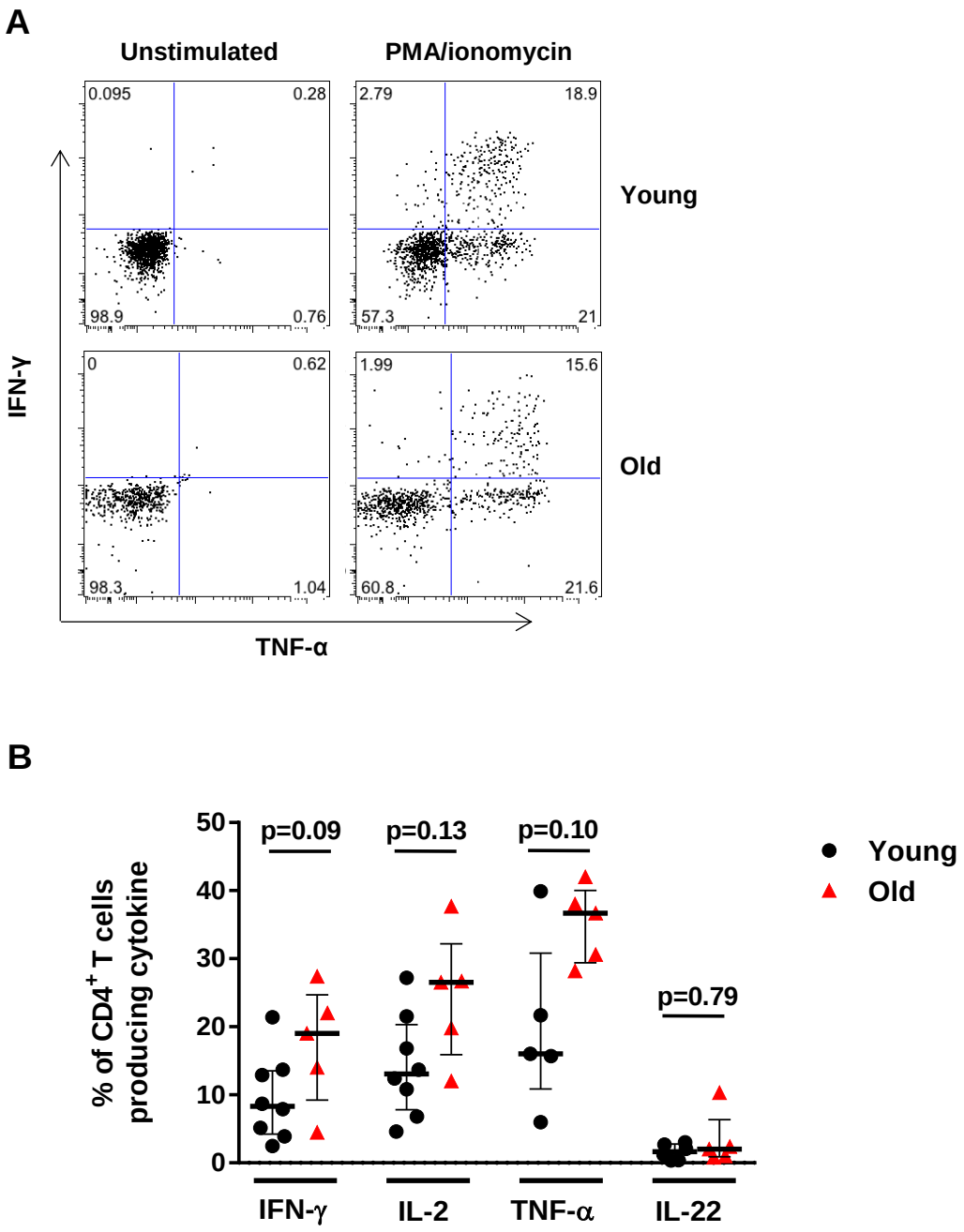


Figure 3-8 Effect of age on cytokine production by skin CD4⁺ T cells

A: Representative dot plots showing the proportion of PMA/ionomycin-stimulated and unstimulated skin-derived CD4⁺ T cells producing IFN-γ and TNF-α in a young and old donor.
B: Graph showing cumulative data for the percentages of CD4⁺ T cells producing cytokines in the skin of young (n=8) and old (n=5) individuals after stimulation. Medians and interquartile ranges are shown. Young and old were compared using the Mann-Whitney test; significance levels are shown.

3.3.4.2 Skin-derived $CD8^+$ T cells from old individuals are functionally competent in vitro

Activated $CD8^+$ T cells produce a variety of effector molecules in response to recognised pathogens, including the pro-inflammatory cytokines IFN-γ and TNF-α [260]. To investigate whether there was any age-related loss of cytokine-producing ability by skin $CD8^+$ T cells, skin-derived leukocytes from young and old were stimulated with PMA/ionomycin for 6 hours, and intracellular cytokine production (IFN-γ, TNF-α, IL-2 and IL-22) by $CD8^+$ T cells was measured. Unstimulated cells were used as negative controls.

Representative dot plots showing the proportion of stimulated skin-derived $CD8^+$ T cells producing IFN-γ and TNF-α in young and old individuals are shown in Figure 3-9A. Cumulative data (Figure 3-9B) showed a significant increase in the proportion of IL-2-producing $CD8^+$ T cells in the old compared to the young ($p=0.045$). There was no significant difference in the proportion of $CD8^+$ T cells producing IFN-γ, TNF-α or IL-22 between young and old, although there was a trend to an increase in the old for each of these 3 cytokines. As with skin-derived $CD4^+$ T cells, only a minority of $CD8^+$ T cells produced IL-22.

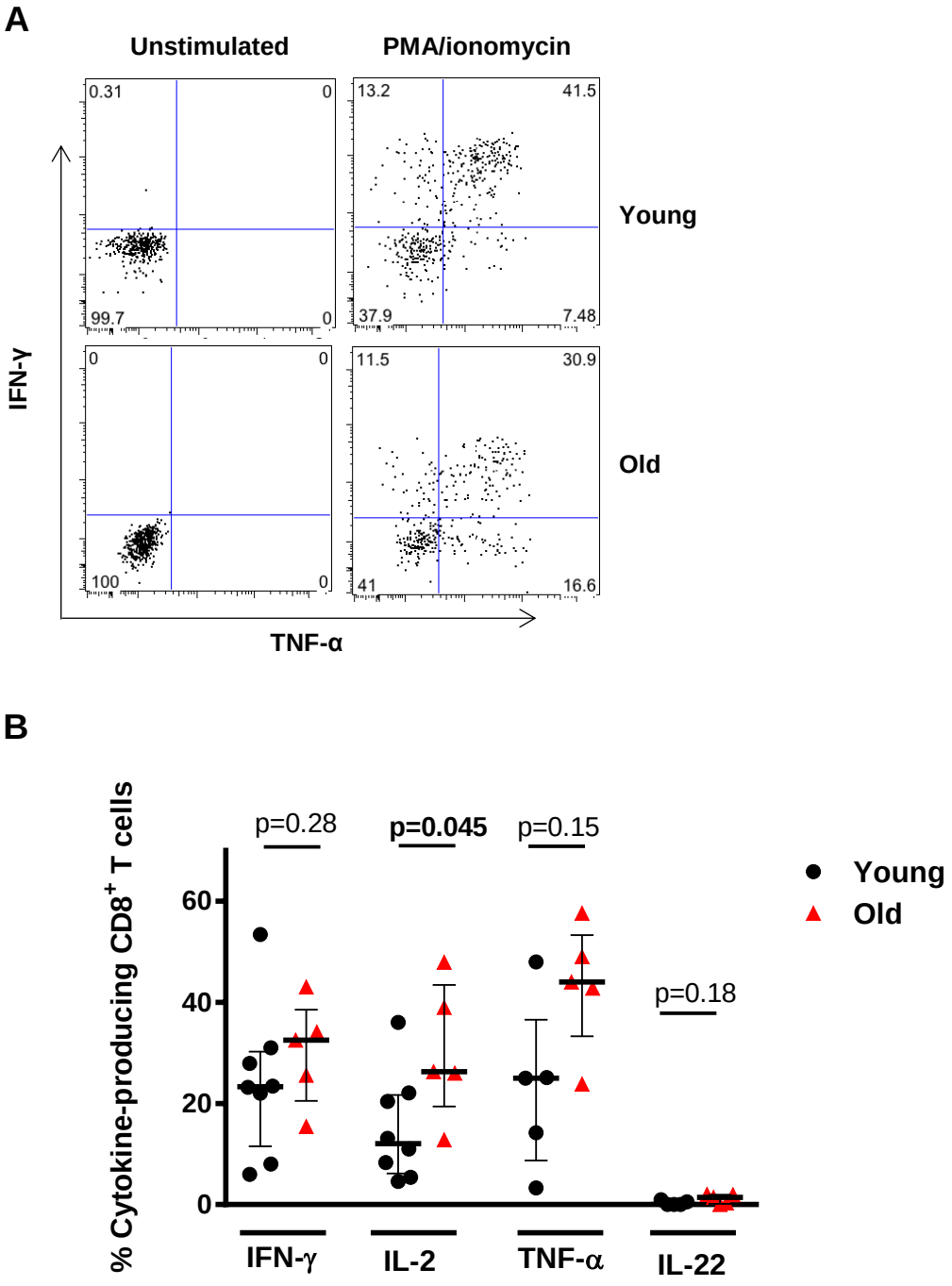


Figure 3-9 Effect of age on cytokine production by skin CD8⁺ T cells

A: Representative dot plots showing the proportion of PMA/ionomycin-stimulated and unstimulated skin CD8⁺ T cells producing TNF-α and IFN-γ in a young and an old individual.
B: Graph showing cumulative data for the percentages of CD8⁺ T cells producing cytokines in the skin of young (n=8) and old (n=5) individuals after stimulation. Medians and interquartile ranges are shown. Young and old were compared using the Mann-Whitney test; significance levels are shown.

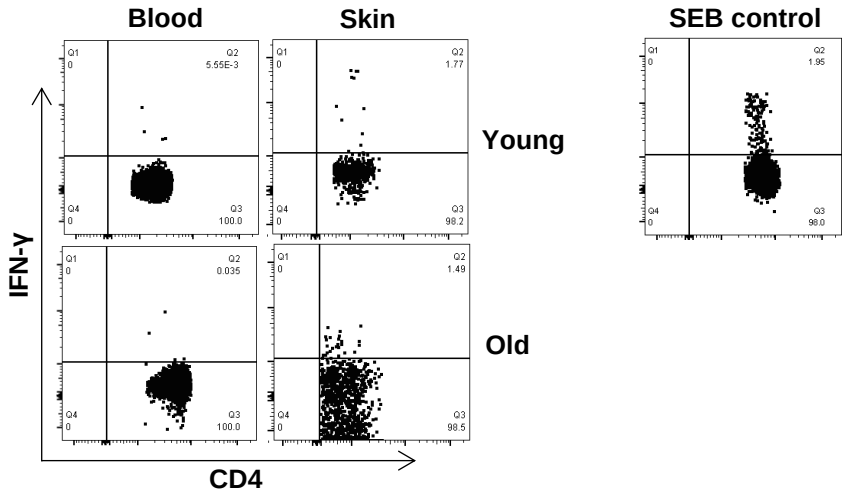
3.3.5 VZV-specific $CD4^+$ T cells

3.3.5.1 Skin-derived VZV-specific $CD4^+$ T cells do not decline with increased age

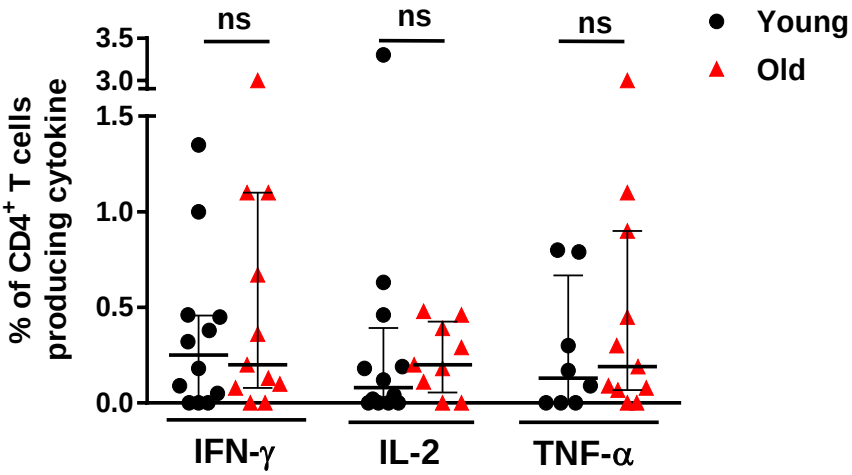
To identify VZV-specific $CD4^+$ T cells, leukocytes from blood and skin biopsies from young and old individuals were stimulated overnight with VZV lysate, in the presence of brefeldin A. The following day, intracellular cytokine production (IFN-γ, TNF-α, IL-2) was measured by flow cytometry.

Representative dot plots of the percentage of IFN-γ-producing (i.e. VZV-specific) $CD4^+$ T cells present in the blood and skin are shown in Figure 3-10A. Cumulative data demonstrated no significant difference in the percentage of cytokine-producing $CD4^+$ T cells between young and old, whether for IFN-γ, IL-2 or TNF-α (Figure 3-10B). Individuals in whom IFN-γ- or TNF-α-producing $CD4^+$ T cells could be detected in the skin were pooled together (young and old) for an analysis of paired blood and skin samples (Figure 3-10C). This revealed that as a group, the proportion of VZV-specific $CD4^+$ T cells was significantly greater in the skin than the blood ($p<0.0001$).

A


Blood
Skin
SEB control
Young
Old
IFN-γ
CD4
Q1
Q2
Q3
Q4
5.55E-3
1.77
1.85
100.0
98.2
98.0
0.035
1.49
100.0
98.5

B


ns
ns
ns
Young
Old
% of CD4+ T cells producing cytokine
3.5
3.0
1.5
1.0
0.5
0.0
IFN-γ
IL-2
TNF-α

C

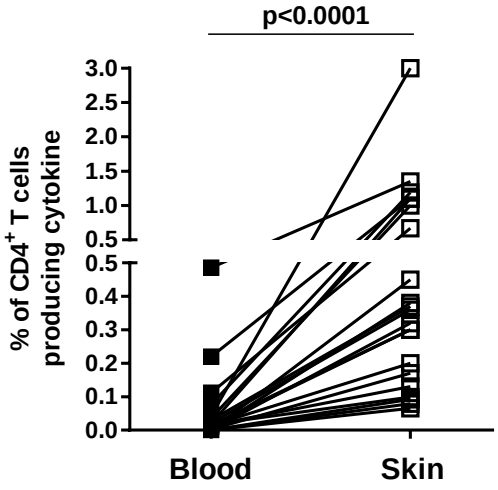


Figure 3-10 Effect of age on the frequency of VZV-specific CD4⁺ T cells in the skin

A: Representative dot plots showing the percentage of IFN-γ-producing CD4⁺ T cells in the blood and skin in a young and old donor. SEB-stimulated PBMC control is shown on the right.
B: Graph showing cumulative data for the percentages of cytokine-producing CD4⁺ T cells in the skin of young and old individuals (n=11 young, 10 old). Medians and interquartile ranges are shown. Young and old were compared using the Mann-Whitney test.
C: Graph showing the percentage of cytokine-producing CD4⁺ T cells in blood and skin samples for pooled young and old individuals (n=21). The difference between blood and skin percentages was analysed by the Wilcoxon matched-pairs signed rank test and the significance level is shown.

3.3.5.2 There is an increase in the proportion of terminally differentiated CD27$^-$CD45RA$^+$ VZV-specific CD4$^+$ T cells in old skin

The differentiation state of VZV-specific CD4$^+$ T cells in the skin was investigated by performing CD27 and CD45RA surface staining, in conjunction with VZV-specific CD4$^+$ T cell detection as described in 3.3.5.1. Cumulative data of the percentages of circulating and skin-derived VZV-specific CD4$^+$ T cells within each of the four CD27/CD45RA differentiation subsets are shown (Figure 3-11). The majority of circulating and skin-derived VZV-specific CD4$^+$ T cells were CD45RA negative, consistent with a memory phenotype.

In the circulating compartment, there were no differences between young and old in the proportions of VZV-specific CD4$^+$ T cells within each subset of differentiation (Figure 3-11, left graph). In the skin compartment, the percentage of VZV-specific CD4$^+$ T_{EMRA} cells was greater in the old compared to the young (p=0.029), while there were no differences within the other subsets (Figure 3-11, right graph).

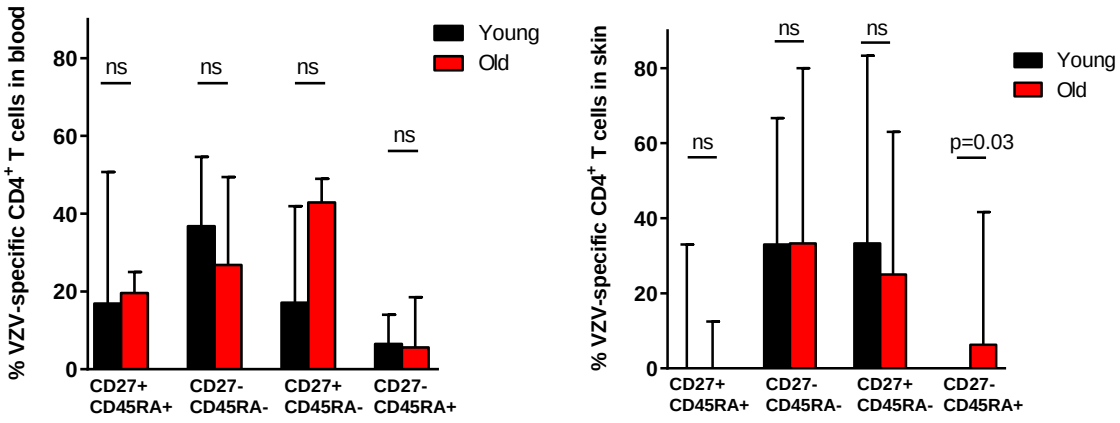


Figure 3-11 Effect of age on the differentiation state of VZV-specific CD4$^+$ T cells in the skin and blood

Bar graphs showing the percentage of VZV-specific CD4$^+$ T cells within each subset of differentiation, defined by CD27/CD45RA expression. Cumulative data is shown for blood (left) and skin (right). Medians and interquartile ranges are shown. Young and old were compared by Mann-Whitney test (n=15 young, 9 old) and significance levels are shown.

3.4 Discussion

The results showed that the total numbers of $CD4^+$ and $CD8^+$ T cells in the dermis (including both resident and recirculating T cells) were maintained in the older age group. Thus, impaired cutaneous immune responses in the old cannot be explained by a fall in the absolute $CD4^+$ and $CD8^+$ T cell numbers in the skin. In comparison, Martinet et al found that in ageing mice, there was no change in $CD4^+$ T cell numbers in lung or liver, and there was an increase in these cells in gut mucosa-associated lymphoid tissue (GALT), accompanied by a decrease in SLOs, compared to young mice[261]. These age-related changes were speculated to be due to redistribution of $CD4^+$ T cells from the SLOs to the periphery as there was no increased proliferation seen in the GALT-based $CD4^+$ T cell population[261]. In the same study, analysis of ageing mice showed that numbers of $CD8^+$ T cells were maintained in the liver, SLOs and Peyer's patches, and increased in the lungs as well as small and large bowel lamina propria[261]. T cells in the skin were not considered, but the study demonstrated that other peripheral tissues (albeit in mice) also preserve their numbers of $CD4^+$ and $CD8^+$ T cells during the ageing process.

Various studies have shown that in the circulation, total T cells, total $CD4^+$ T cells and total $CD8^+$ T cells all decline with increasing age[258,262,263]. This decline is primarily due to a fall in the number of naïve T cells in circulation[258,263] as a consequence of thymic involution. The decline in naïve T cell numbers is only partially offset by an increase in numbers of circulating memory $CD4^+$ T cells[263] and memory $CD8^+$ T cells[258]. Skin T_{RM} numbers are negligible at birth[87] and accumulate during life after every cutaneous pathogen exposure[98,105]. The age-related decline in circulating naïve T cell numbers might have meant that old individuals were less able to generate adequate numbers of skin-homing memory T cells following primary immune responses to newly encountered pathogens in the skin. However, by the age of 65, it is expected that most commonly occurring skin pathogens would have already been encountered earlier in life, such that a plateau in numbers of memory T cells found in the skin may be reached earlier on in adulthood. Therefore, the age-related decline in circulating naïve T cells does not appear to have an impact on the total numbers of T cells in the skin.

The majority of T cells found in the skin are permanently resident ($CD69^+$ T_{RM}) and a minority are recirculating ($CD69^-$ T_{CM} and T_{MM})[87]. The results in this work

demonstrated that there was no difference in the proportion of $CD4^+$ T cells in the dermis expressing CD69 between young (77.5%) and old (78.9%). This is consistent with a study by Watanabe et al, who found that 77.6% of $CD4^+$ T cells in human dermis (5 donors, age not mentioned) expressed CD69 [87]. Similarly, there was no significant difference in the percentage of dermal $CD8^+$ T cells expressing CD69 between the young (83.6%) and the old (85.1%). The equivalent figure from the aforementioned study was 86.7% [87]. Therefore, for both $CD4^+$ and $CD8^+$ T cells in the skin, the proportion expressing CD69 did not decline with increasing age.

A subset of $CD69^+$ T_{RM} cells also express CD103. This work showed that the proportion of $CD4^+$ $CD69^+$ cells expressing CD103 was 22.2% in the young and 25.7% in the old, with no difference between the groups. For $CD8^+$ $CD69^+$ cells the proportion was 23.3% in the young and 12.1% in the old. There was a trend to a decrease in the old group but this did not reach statistical significance. The equivalent proportions from the study by Watanabe et al[87] using flow cytometric analysis were 37.9% (extrapolated) for $CD4^+$ T cells and 38.5% (extrapolated) for $CD8^+$ T cells[87]. One possible reason for the lower values in this work compared with the published study is that flow cytometry may be more effective at identifying dimly positive cells.

While skin T_{RM} are generally defined in the scientific literature by their expression of CD69 and CD103 [87,264,265], expression of the latter marker is significantly higher in mouse than in human skin[79,87,113]. CD103 expression is therefore not a defining characteristic of skin T_{RM} in humans. In this work, there is only one data set (CD69 expression on each of the $CD4^+$ and $CD8^+$ T cell populations; Figure 3-1C and Figure 3-2C) referring to a human skin T_{RM} phenotype. As only one marker has been used to detect the skin resident T cell population in this work, caution is needed in making definitive conclusions about the identity and function of this specific cell type. Other works have suggested the absence of the surface markers CD62L and CCR7 also denote a skin T_{RM} phenotype[85,97], as well as expression of the transcription factors Hobit and Blimp1 [84], and the use of these additional markers should be considered in future work.

The skin data presented here on VZV-specific T cells, T cell differentiation profiles (based on CD27 and CD45RA expression), and T cell cytokine-producing ability all relied on enzymatic extraction of leukocytes from skin biopsies, using

collagenase type IV. During our lab's development of the protocol for overnight collagenase digestion of skin, lymphocytes extracted in this way demonstrated a reduction in the intensity of expression of certain surface markers, such as CD4. To verify whether this observation was specific to lymphocytes isolated from skin, PBMCs were incubated in collagenase type IV overnight – and also manifested reduced surface marker expression. Mulder et al found that this effect was due to protease contamination of commercially available collagenase[266], and demonstrated that addition of serum abolished the effect. We found that increasing the serum concentration in the digestion medium from 10% to 20% prevented the loss of surface marker expression, and so 20% FBS was used in all experiments using the collagenase digestion technique.

The frequency of VZV-specific T cells in the skin has not been investigated previously in the context of ageing. This work showed that the proportion of VZV-specific $CD4^+$ T cells in the skin did not decline with age, when identified by overnight VZV stimulation in vitro and measurement of intracellular cytokine production. These findings were confirmed by other group members, who identified VZV-specific T cells by binding to an HLA-DRB1*1501-restricted IE63 tetramer[267]. Thus, an age-related change in the frequency of VZV-specific T cells in the skin is not responsible for the increased susceptibility of old individuals to cutaneous reactivation of VZV. An additional finding was that the frequency of VZV-specific $CD4^+$ T cells in the skin was significantly greater than in the blood, for both the young and the old. This may reflect the fact that the range of antigens encountered by skin T_{RM} is likely to be less diverse than those encountered by T cells in circulation, so cutaneous VZV-specific T cells represent a greater overall proportion of the skin T_{RM} population. Additionally, VZV-specific T cells may be more highly concentrated in the skin so as to be strategically positioned to counteract any potential VZV invasion of the skin from local sensory nerves.

Terminally differentiated memory T cells, particularly $CD8^+$ T cells, accumulate in the circulation of old humans[50]. This work confirmed the proportions of circulating $CD4^+$ T_{EMRA} and $CD8^+$ T_{EMRA} cells were significantly increased in the old compared to the young. However, there was no corresponding increase in the proportion of $CD4^+$ T_{EMRA} or $CD8^+$ T_{EMRA} cells in the skin. These findings suggest that impaired cutaneous immune responses in the old are not due to a functional impairment arising from accumulation of terminally differentiated memory T cells

in the skin. The disparate findings for T cells in the blood and skin suggest that the T cell population in the skin is relatively protected from the circulatory environment that predisposes T cells to terminal differentiation in the old. Repeated cycles of proliferation drive the increased differentiation of circulating T cells in the old[28]. It is possible that low homeostatic proliferation by skin T_{RM} accounts for their relatively less differentiated state.

We have commented on the differentiation state of skin-derived VZV-specific T cells in Section 3.3.5.2, by analysing CD27 and CD45RA expression on gated $CD4^+$ IFN-γ^+ T cells[267]. A caveat to the findings presented here is that the number of VZV-specific T cells identified in the skin for each donor was small (generally represented by only a few dots in the $CD4^+$ IFN-γ^+ quadrant). We are therefore aware that strong conclusions should not be drawn when the cell yield of the population of interest is so low in number.

In this work, a greater percentage of $CD4^+$ and $CD8^+$ T cells in old skin expressed PD-1 compared to young skin. Due to limitations in staining for multiple antigens together using immunofluorescence microscopy, it was not possible to analyse PD-1 expression by memory subset here. PD-1 expression increases with chronic antigen exposure[28]. T_{RM} in old skin can be presumed to have encountered more antigen throughout their lifespan than those in young skin, so this may explain the increased PD-1 expression in the old. Increased T cell PD-1 expression is likely to contribute to impaired cutaneous immune responses in ageing skin. Melanoma-infiltrating $CD8^+$ T cells demonstrate increased PD-1 expression; evidence for the inhibitory role of PD-1 in the skin comes from the use of the anti-PD-1 immunotherapy drugs nivolumab and pembrolizumab as treatment options for patients with metastatic melanoma[268]. These drugs powerfully stimulate the T cell anti-tumour response and lead to tumour regression and prolonged survival.

In this work, skin-derived $CD4^+$ and $CD8^+$ T cells were just as able to produce cytokines in vitro in the old compared to the young. Therefore, despite increased PD-1 expression, T cells did not appear to be functionally exhausted in the old. It has been mentioned that a plateau in the number of skin T_{RM} may be reached in early adulthood. In an aged individual, the pool of skin T_{RM} specific to a given pathogen would likely have first been generated from a relatively ‘young’ naïve T cell at the time of initial pathogen exposure. The pool of T_{RM} cells is likely to be

long-lived, with maintenance of its numbers from low-level cell division in steady state, as well as rapid pool expansion during episodes of subsequent exposure to the same pathogen[24]. A study in mice has shown that memory T cells generated from aged naïve T cells demonstrated impaired responses to their cognate antigen, with less proliferation, reduced levels of cytokines, and reduced cognate helper function, compared with memory T cells generated from young naïve cells[45]. This suggests that it is the age of the naïve T cell that first gives rise to memory T cells, as opposed to the age of the memory T cell, that determines the strength of the immune response to re-encountered antigen. If this concept can be transferred to humans, then the robust ability of stimulated skin T_{RM} to produce cytokines in the old may be due to the fact that these memory T cells were first generated not from aged naïve T cells but from naïve T cells during earlier stages of life.

The percentage of $CD4^+$ $Foxp3^+$ Tregs in relation to total $CD4^+$ T cells in old skin was more than double that compared to young skin, which is consistent with previous work[135]. This age-related increase is mirrored by studies on human blood that have shown the proportion of Tregs in relation to total $CD4^+$ T cells increases with age[170,171]. Increased skin Tregs in the old may contribute to impaired skin immunity in this age group. Reasons for the increased frequency include a compensatory response to chronic inflammation[64] or to chronic lifelong antigen exposure[124].

4 THE EFFECT OF SHINGLES VACCINATION ON T CELL POPULATIONS AND VZV-SPECIFIC IMMUNE RESPONSES IN THE SKIN

4.1 Chapter Introduction

The mechanisms by which the live attenuated shingles vaccine Zostavax® prevents shingles are not fully understood. The vaccine is observed to boost circulating VZV-specific $CD4^+$ T cell responses and VZV-specific antibody[239], but its effects on immune cell populations within the skin are unknown. As the skin is the first site to which virus particles migrate from the affected sensory nerve during VZV reactivation, we would assume skin T_{RM} play a role in immune protection against shingles infection. Therefore, the effects of Zostavax on skin T_{RM} were investigated here.

It was shown in Chapter 3 that there was no reduction in numbers or function (in vitro) of total or VZV-specific T_{RM} in old compared to young skin. However, ageing skin was shown to be a more inhibitory environment than young skin, with higher Treg frequency and PD-1 expression on T cells in the old. A potential mechanism of action of Zostavax® might therefore be a remodelling or reversal of the inhibitory influences that exist in ageing skin.

Individuals over the age of 70 years (the age group for which the vaccine is recommended) were recruited to our shingles vaccine study. T_{RM} populations in normal skin were enumerated before and after vaccination. The extent to which vaccination altered the level of immune inhibition in the skin was quantified by analysis of T cell PD-1 expression and Treg frequency in the skin before and after vaccination.

The effect of vaccination on an in vivo cutaneous VZV-specific immune response was studied by inducing a DTH response to VZV skin test antigen before and after vaccination; the DTH site was sampled at various time-points to investigate the kinetics of the cellular response. It was hypothesised that Zostavax® confers enhanced protection against shingles by boosting VZV-specific T cell responses in the skin, thereby overcoming the greater inhibitory environment in ageing skin.

4.2 Aims & Objectives

The aim of this section was to identify whether the shingles vaccine induces in ageing skin:

1. A change in the numbers of T_{RM} present
2. A less inhibitory skin microenvironment
3. An enhancement of in vivo VZV-specific immune responses (and to investigate the cellular kinetics involved)

4.3 Results

4.3.1 Shingles vaccination does not alter the number of $CD4^+$ T_{RM} in normal skin

Frozen sections of normal skin from vaccinated individuals were stained with anti-CD4 antibody and analysed by immunofluorescence microscopy. As previously, cell counts were performed for cells in the dermis, and the data are shown in Figure 4-1A. The median number of $CD4^+$ T cells per section was not significantly different between vaccinated (59) and unvaccinated individuals (75).

The proportion of $CD4^+$ T cells resident in the skin was analysed by co-staining with CD69 (Figure 4-1B). 80.2% of $CD4^+$ T cells in the dermis were $CD69^+$ in vaccinated individuals, with no significant difference when compared with the unvaccinated old (median 80.4%). These data suggest that the shingles vaccine does not lead to the generation of additional skin $CD4^+$ T_{RM} distant to the vaccination site.

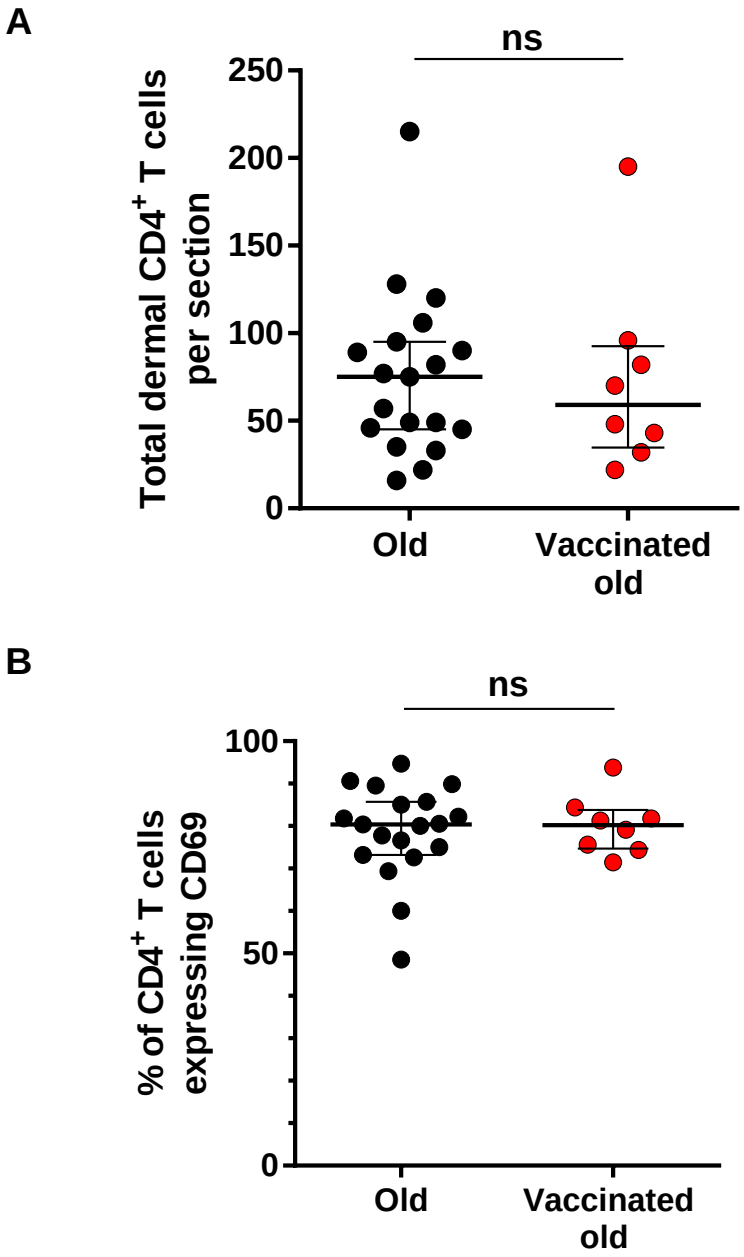


Figure 4-1 Effect of vaccination on numbers of skin CD4⁺ T_{RM}

A: Graph showing the number of dermal CD4⁺ T cells per section in vaccinated individuals (n=8). Data for unvaccinated old are shown for comparison.
B: Graph showing the percentage of CD4⁺ T cells in the dermis expressing CD69 (vaccinated individuals n=8).
Medians and interquartile ranges are displayed, and the populations were compared using the Mann-Whitney test.

4.3.2 Vaccination boosts the frequency of VZV-specific $CD4^+$ T cells in circulation but not skin

The effect of vaccination on the frequency of VZV-specific $CD4^+$ T cells in the skin and blood was studied. Leukocytes were isolated from paired skin biopsies and blood samples, before and after vaccination. As previously, VZV-specific $CD4^+$ T cells were identified by IFN-γ production following in vitro overnight stimulation with VZV lysate.

As shown in Figure 4-2, there was a significant increase in the proportion of VZV-specific $CD4^+$ T cells in the blood after vaccination (median 0.046%) compared to before (median 0.032%; p=0.027). Surprisingly, there was no associated increase in the proportion of VZV-specific $CD4^+$ T cells in skin (median 0.065% both before and after vaccination). These results indicate that the vaccine induces a boost in circulating VZV-specific T cell immunity, but not necessarily in the skin.

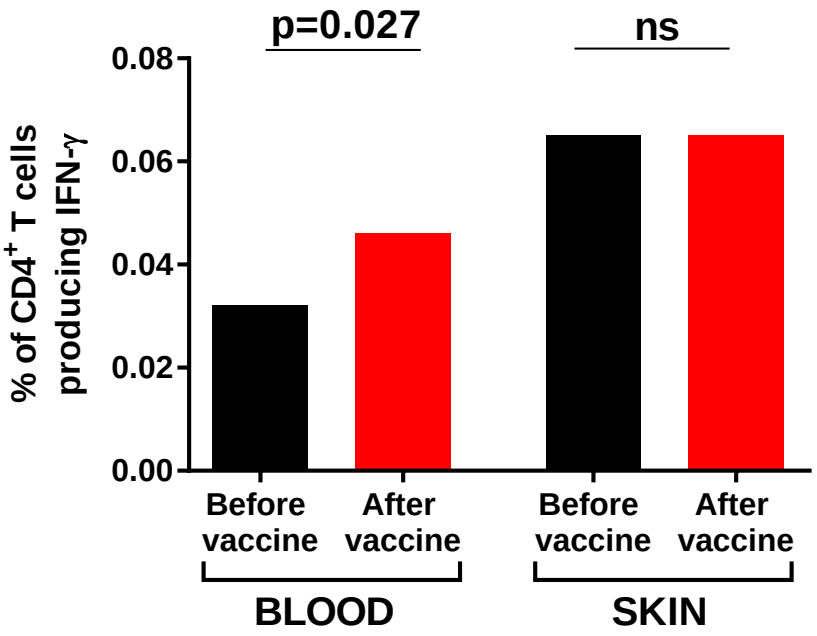


Figure 4-2 Effect of vaccination on the frequency of VZV-specific CD4⁺ T cells in the blood and skin

Graph showing cumulative data for the percentages of IFN-γ-producing CD4⁺ T cells in the blood and skin, before and after vaccination (blood n=10, skin n=6). Medians and significance levels are shown. Data were compared using the Wilcoxon matched-pairs signed rank test.

4.3.3 Shingles vaccination does not alter the number of skin $CD8^+$ T_{RM} in normal skin

Immunofluorescence microscopy experiments demonstrated no significant difference in the median number of $CD8^+$ T cells per section between vaccinated (21.3) and unvaccinated old individuals (26.5), as shown in Figure 4-3A. There was also no significant difference in CD69 expression on $CD8^+$ T cells between the vaccinated (median 84.3%) and the unvaccinated (median 85.2%) – shown in Figure 4-3B. Thus, as was the case with $CD4^+$ T cells, the shingles vaccine did not appear to generate increased $CD8^+$ T_{RM} distant to the site of vaccine administration.

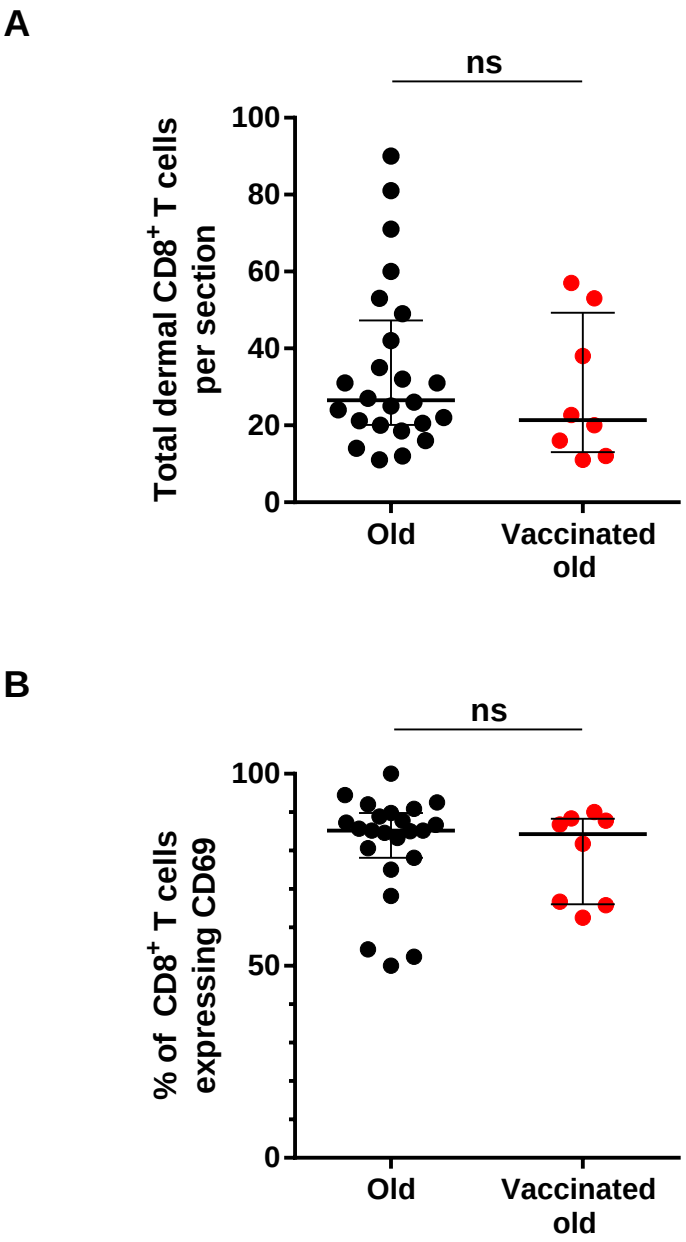


Figure 4-3 Effect of vaccination on numbers of skin CD8⁺ T_{RM}

A: Graph showing the number of dermal CD8⁺ T cells per section in vaccinated individuals (n=8). Data for unvaccinated old are shown for comparison.
B: Graph showing the percentage of CD8⁺ T cells in the dermis expressing CD69 (vaccinated individuals n=8).
Medians and interquartile ranges are displayed, and the populations were compared using the Mann-Whitney test.

4.3.4 Shingles vaccination does not alter the proportion of Tregs in normal skin

The effect of the vaccine on the proportion of $CD4^+$ T cells in the skin displaying a regulatory phenotype was analysed by immunofluorescence microscopy. As shown in Figure 4-4A, the median percentage of Tregs in vaccinated individuals (12%) was not significantly different to the percentage seen in the unvaccinated (8.7%). Figure 4-4B demonstrates the median percentage of Tregs expressing CD69 in the vaccinated (54.3%) was not significantly different compared to the unvaccinated (48.8%). Therefore, the Treg frequency in the skin appeared unaltered after vaccination.

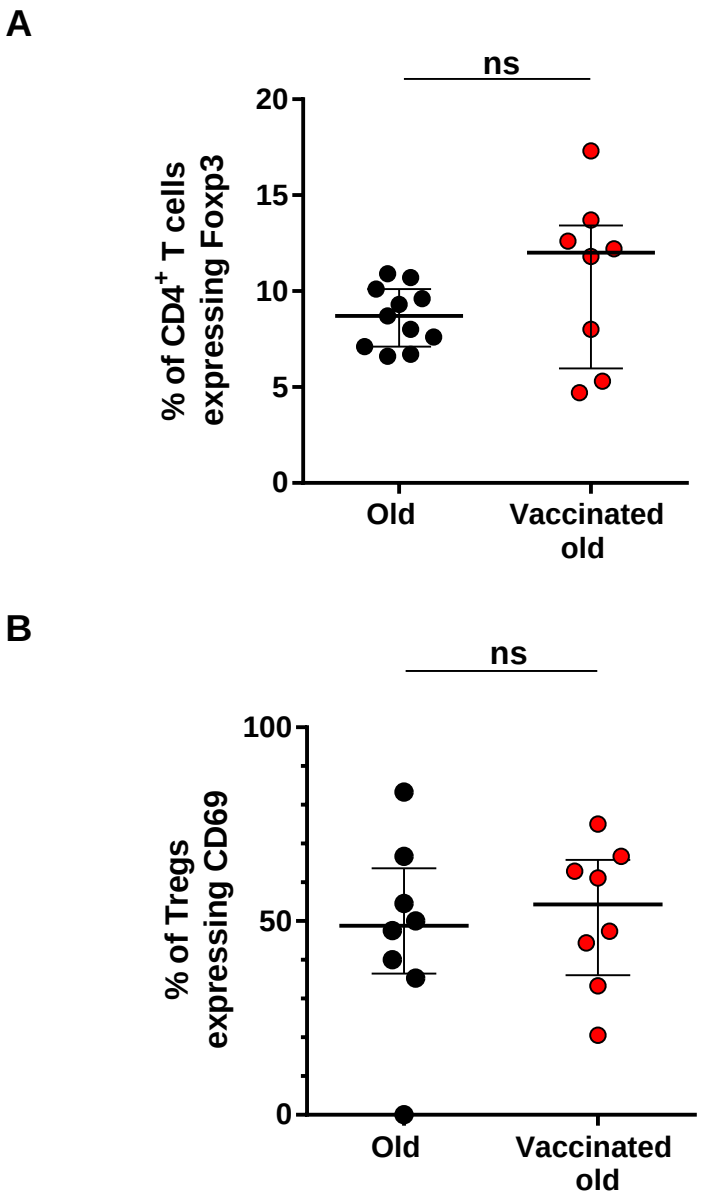


Figure 4-4 Effect of vaccination on the proportion of Tregs in the skin

A: Graph showing the percentage of $CD4^+$ T cells in the dermis expressing Foxp3 in vaccinated old individuals (n=8). Data for unvaccinated old are shown for comparison.
B: Graph showing the percentage of $CD4^+Foxp3^+$ Tregs in the dermis expressing CD69 in vaccinated old individuals (n=8). Data for unvaccinated old are shown for comparison.
Medians and interquartile ranges are displayed. Populations were compared using the Mann-Whitney test and significance levels are shown.

4.3.5 Shingles vaccination increases PD-1 expression on skin $CD4^+$ T cells

In Section 3.3.3, PD-1 expression was shown to be increased in $CD4^+$ and $CD8^+$ T cells in ageing skin. As vaccination boosts immunity, it was of interest to investigate whether PD-1 expression in the skin was reduced following vaccine administration.

Interestingly, the median percentage of $CD4^+$ T cells expressing PD-1 was higher in the vaccinated individuals (75.2%) compared to the unvaccinated (58.7%) as seen in Figure 4-5A ($p=0.031$). In contrast, the median percentage of $CD8^+$ T cells expressing PD-1 was not significantly different between the vaccinated (63.9%) and unvaccinated (68.7%) individuals.

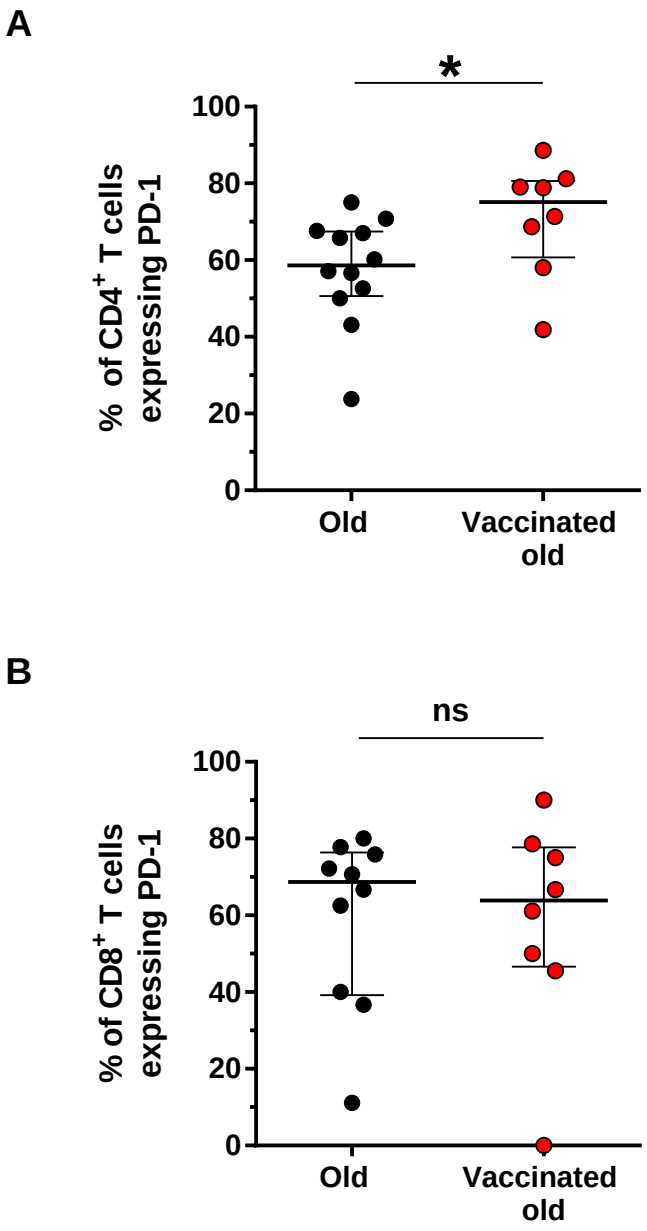


Figure 4-5 Effect of vaccination on PD-1 expression by skin $CD4^+$ and $CD8^+$ T cells

Graph showing the percentage of $CD4^+$ T cells **(A)** and $CD8^+$ T cells **(B)** in the dermis expressing PD-1 in vaccinated old individuals (n=8). Data for unvaccinated old individuals are shown for comparison. Medians and interquartile ranges are displayed. Groups were compared using the Mann-Whitney test and significance levels are shown.

4.3.6 The shingles vaccine boosts the clinical response to VZV antigen challenge

Ageing volunteers were challenged with the VZV skin test antigen before and after vaccination, and the clinical response at Day 3 was measured. Participant and clinical score data are listed in Table 4-1. Out of a total of 42 volunteers who consented to the study, 4 dropped out (3 for medical reasons, 1 declined further participation). A further 8 volunteers were excluded from the study due to a highly robust clinical score ≥ 5. Of the remaining 30 volunteers, 5 had a starting score of 4, reflecting a moderately robust response; none of these 5 experienced a boost in clinical response after vaccination. The age range was 70-93; median age 75.5; 10 male and 20 female.

Donor	Age	Gender	Clinical Score	
			Before vaccine	After Vaccine
1	82	M	0	3
2	80	F	0	3
3	82	M	0	Dropped out (medical reasons)
4	70	F	0	0
5	74	F	1	4
6	84	M	4	4
7	81	F	1	3
8	82	F	1	Dropped out
9	77	M	1	1
10	74	F	3	4
11	79	F	1	7
12	82	F	0	0
13	72	M	6	Excluded (high score)
14	80	F	1	1
15	71	F	3	6
16	71	F	0	3
17	70	M	6	Excluded (high score)
18	72	F	1	6
19	70	F	1	4
20	75	M	0	0
21	73	F	0	Dropped out (medical reasons)
22	84	M	0	Dropped out (medical reasons)
23	76	F	0	0
24	75	F	4	4
25	83	M	0	3
26	93	M	0	3
27	85	M	1	3
28	71	F	5	Excluded (high score)
29	70	F	4	3
30	70	F	6	Excluded (high score)
31	72	M	4	4
32	75	F	3	4

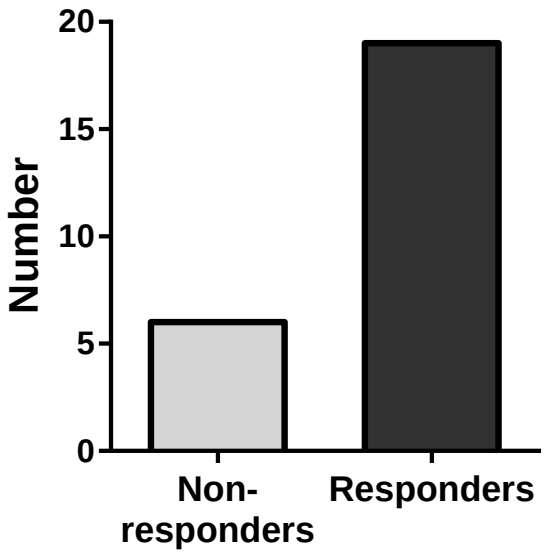
33	76	F	3	4
34	71	F	1	2
35	73	F	3	5
36	73	M	5	Excluded (high score)
37	78	M	0	4
38	77	F	6	Excluded (high score)
39	74	F	9	Excluded (high score)
40	74	F	4	4
41	76	F	5	Excluded (high score)
42	74	M	1	4

Table 4-1 Table showing participant age, gender, and clinical score before and after vaccination

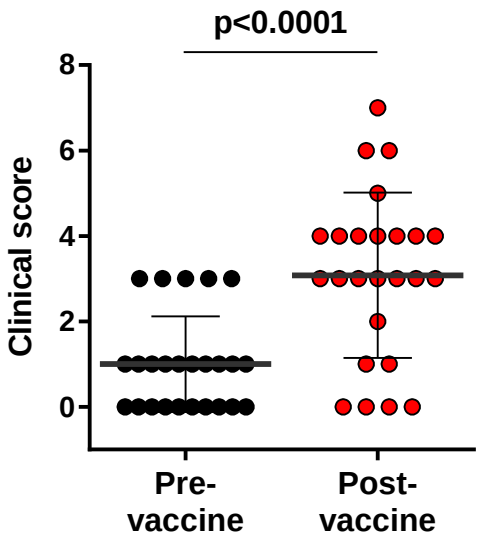
Before and after analysis was performed for the remaining 25 volunteers who had a starting clinical score ≤3. As shown in Figure 4-6A, 19 out of 25 (76%) had an enhanced clinical response after vaccination, with the others showing no improvement. There was a significant increase in clinical score after vaccination (mean score 3.1) compared to before (mean score 1) – as shown in Figure 4-6B & C ($p<0.0001$).

To demonstrate whether repeated VZV skin testing per se enhances the clinical score, we re-challenged 14 volunteers with the VZV skin test without any attendant intervention, and found no significant enhancement on the second test (Figure 4-6D; $p>0.99$).

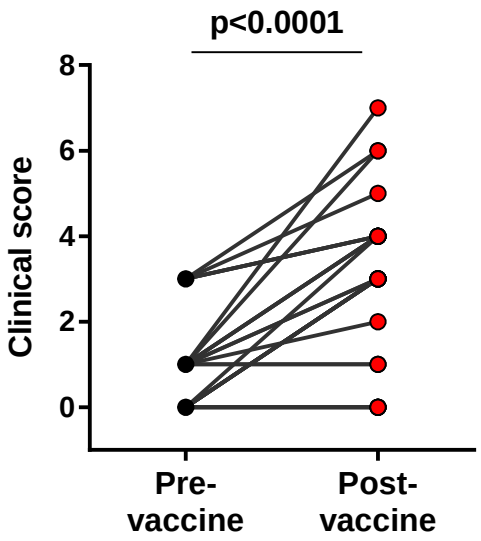
A


20
15
10
5
0
Number
Non-
responders
Responders

B


p<0.0001
8
6
4
2
0
Clinical score
Pre-
vaccine
Post-
vaccine

C


p<0.0001
8
6
4
2
0
Clinical score
Pre-
vaccine
Post-
vaccine

D

Donor	Clinical score 1	Clinical score 2
1	1	0
2	2	3
3	0	3
4	4	4
5	0	0
6	4	4
7	1	1
8	0	0
9	1	1
10	0	0
11	0	0
12	4	4
13	4	3
14	4	4

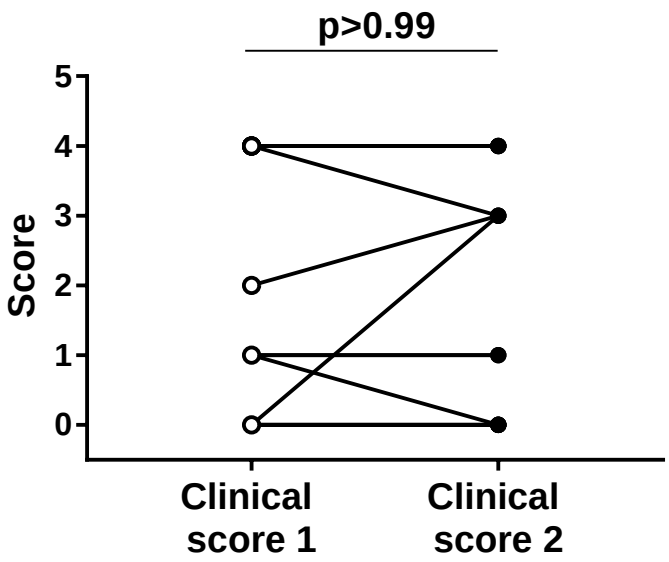


Figure 4-6 Effect of vaccination on clinical score following VZV skin challenge

A: Graph showing the number of individuals whose clinical score improved (responders) and did not improve (non-responders) after vaccination (n=25).
B&C: Graphs showing clinical scores of volunteers before and after vaccination (n=25). Means with standard deviations are shown in **B**. Values were compared using the paired t test and the significance level is shown.
D: Clinical scores of 14 volunteers who underwent VZV skin test re-challenge. Data were analysed using the Wilcoxon matched-pairs signed ranked test and the significance level is shown.

4.3.7 The kinetics of the DTH response to VZV skin challenge

The numbers of $CD4^+$ and $CD8^+$ T cells, as well as Tregs, within perivascular infiltrates at the site of VZV antigen challenge were quantified by immunofluorescence microscopy in old individuals before and after vaccination. The 5 largest perivascular infiltrates in the upper dermis were used to generate a mean value per infiltrate. By sampling the test site at various time points in different individuals, a time course was plotted to trace the kinetics of the T cell response. These data were compared with young donor data that had already previously been acquired by our research group[142]. Young data are shown in the graphs for comparison.

4.3.7.1 Vaccination induced an enhanced $CD4^+$ T cell skin response to VZV challenge

Representative immunofluorescence micrographs showing perivascular $CD4^+$ T cells in an old donor, before and after vaccination, are displayed in Figure 4-7A. The mean numbers of $CD4^+$ T cells per perivascular infiltrate at the various time-points are shown in Figure 4-7B. Over the 7-day time-course, the young mounted a robust cellular response, with large numbers of $CD4^+$ T cells accumulating within perivascular infiltrates by Day 7 ($p=0.0016$). In contrast, the unvaccinated old generate a weak response, with significantly fewer $CD4^+$ T cells than the young at Days 3 ($p=0.014$) and Day 7 ($p=0.0025$). Vaccinated individuals had enhanced cellular infiltration, with significantly more $CD4^+$ T cells at Days 3 ($p=0.019$) and Day 7 ($p=0.030$) than the unvaccinated.

Cellular proliferation was analysed by staining with Ki67 (Figure 4-7A). The percentage of $CD4^+$ T cells expressing Ki67 within perivascular infiltrates was calculated and the time-course is shown in Figure 4-7C. T cell proliferation was not detectable in unchallenged skin. Over the 7-day time-course, $CD4^+$ T cell proliferation increased significantly in the young ($p=0.036$). Contrastingly, proliferation remained low in the unvaccinated old, with a significant difference from the young at Days 3 ($p=0.0020$) and 7 ($p=0.030$). In the vaccinated group there was a trend to increased proliferation compared to the unvaccinated group at Day 7 ($p=0.082$).

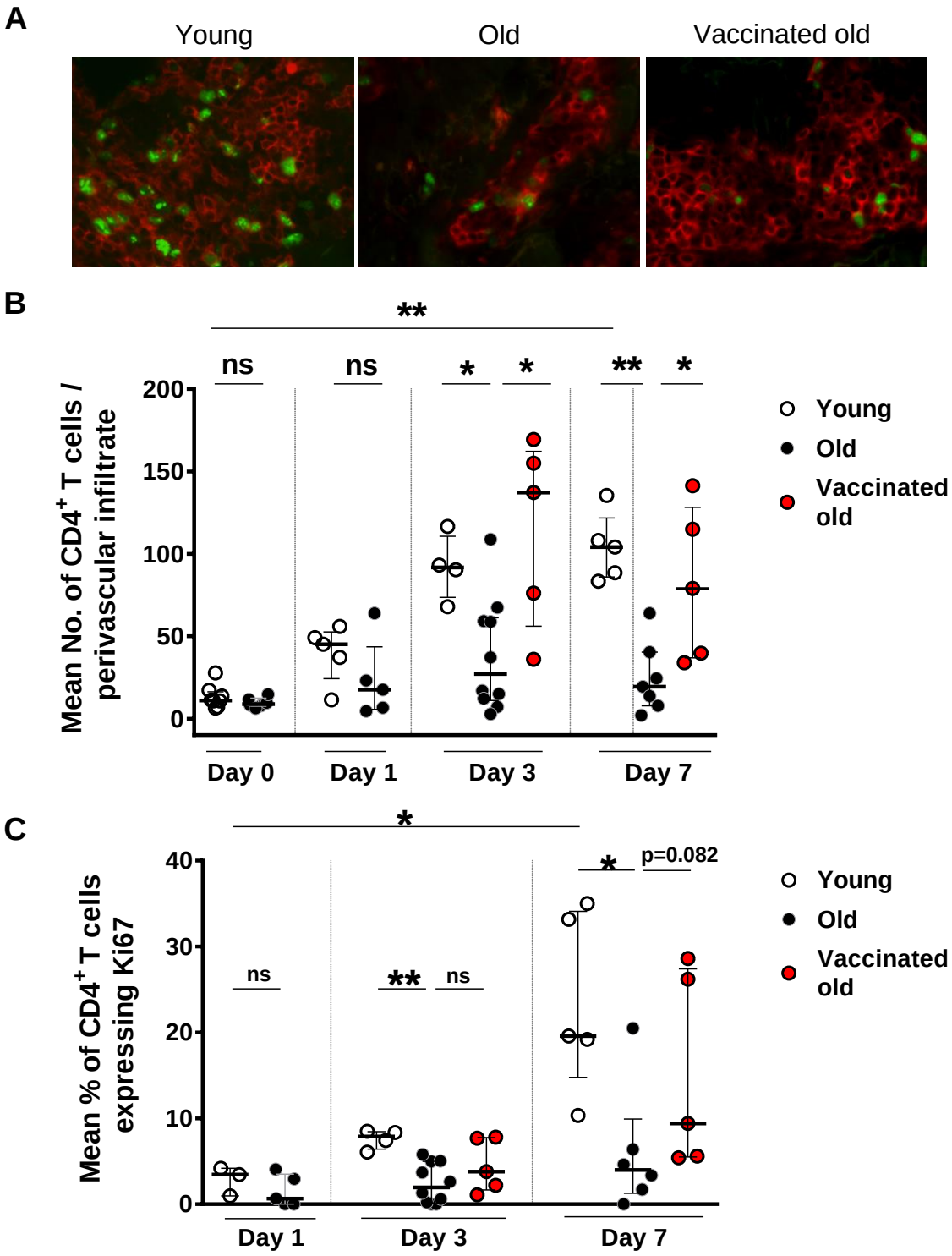


Figure 4-7 Time-course of infiltrating CD4⁺ T cells at the site of VZV challenge, and the effect of vaccination

A: Representative immunofluorescence micrographs (x400 original magnification) of perivascular CD4⁺ T cells (red) co-stained with Ki67 (green), in an old donor before vaccination (middle) and after (right image). An image from a young donor (left) is shown for comparison.
B: Graph showing the mean number of perivascular CD4⁺ T cells at various time-points in young, unvaccinated and vaccinated old donors. Different donors were used for each time-point.
C: Graph showing the percentage of proliferating CD4⁺ T cells during the 7-day time-course in the different volunteer groups.
Medians and interquartile ranges are shown. Groups were compared using the Mann-Whitney test and significance levels are shown.

4.3.7.2 Vaccination induced an enhanced CD8$^+$ T cell skin response to VZV challenge

Representative immunofluorescence micrographs showing perivascular CD8$^+$ T cells in an old donor, before and after vaccination, are shown in Figure 4-8A. The mean numbers of CD8$^+$ T cells per perivascular infiltrate at the different time-points are shown in Figure 4-8B. In the young, a robust CD8$^+$ T cell response was seen over the 7-day time-course ($p=0.0043$). In contrast, the unvaccinated old generated a poor response, with significantly fewer CD8$^+$ T cells than the young at Days 3 ($p=0.0080$) and Day 7 ($p=0.0043$). Vaccinated individuals had significantly more CD8$^+$ T cells at Days 3 ($p=0.0080$) and Day 7 ($p=0.030$) than the unvaccinated.

The percentage of CD8$^+$ T cells expressing Ki67 within perivascular infiltrates (Figure 4-8A) was calculated and the time-course is shown in Figure 4-8C. CD8$^+$ T cell proliferation increased significantly in the young over the 7-day time-course ($p=0.0079$). Proliferation remained low at all time-points in the unvaccinated old, with a significant difference from the young at Days 3 ($p=0.0014$) and 7 ($p=0.025$). Among old individuals, proliferation was significantly increased in the vaccinated compared to the unvaccinated at Day 3 ($p=0.0030$) and Day 7 ($p=0.0163$).

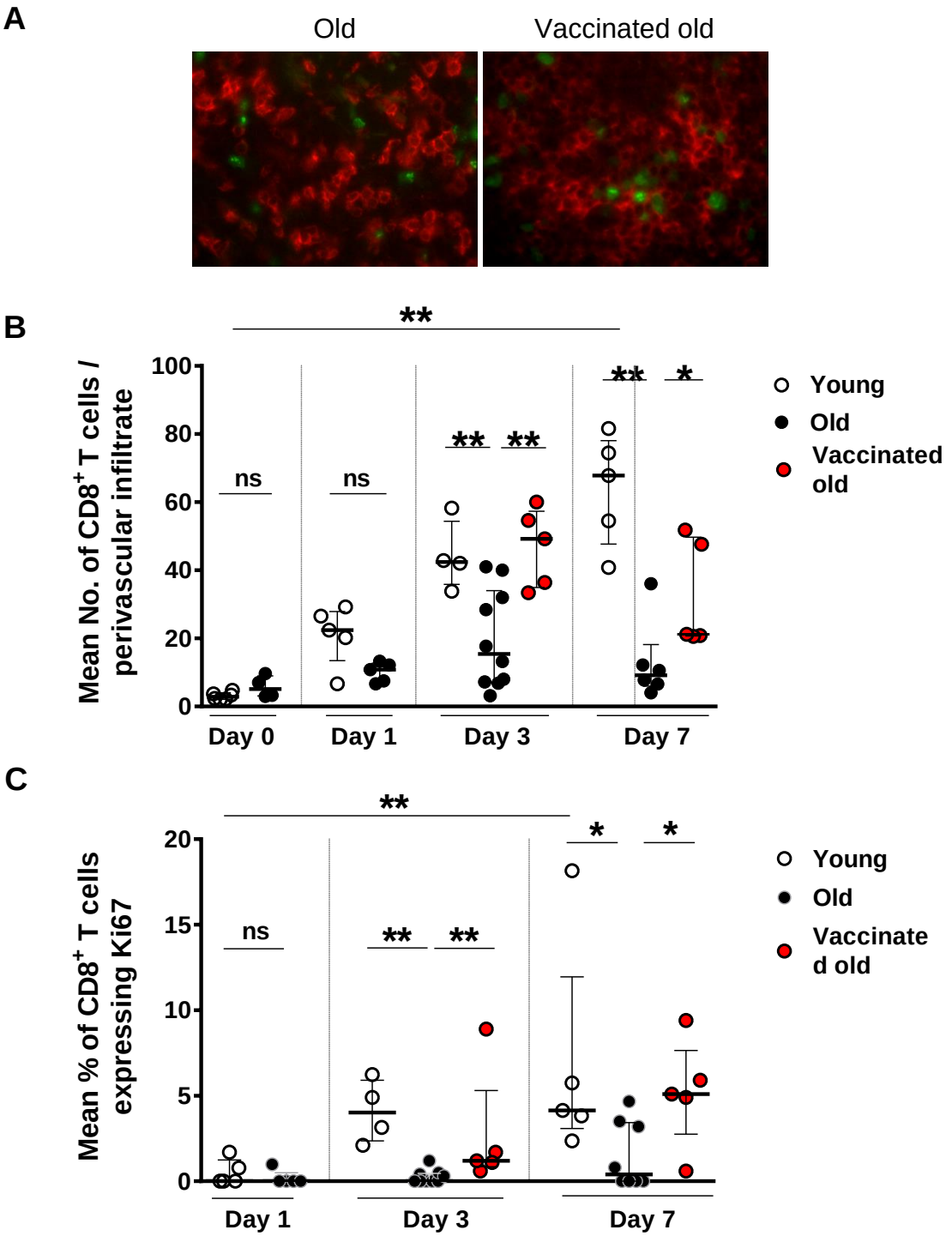


Figure 4-8 Time-course of infiltrating CD8⁺ T cells at the site of VZV challenge, and the effect of vaccination

A: Representative immunofluorescence micrographs (x400 original magnification) of perivascular CD8⁺ T cells (red) co-stained with Ki67 (green), in an old donor before vaccination (left) and after (right).
B: Graph showing the mean number of perivascular CD8⁺ T cells at various time-points in young, unvaccinated and vaccinated old donors. Different donors were used for each time-point.
C: Graph showing the percentage of proliferating CD8⁺ T cells during the 7-day time-course in the different groups.
Medians and interquartile ranges are shown. Groups were compared using the Mann-Whitney test and significance levels are shown.

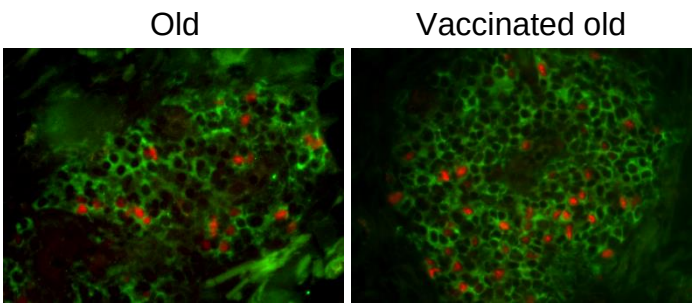
4.3.7.3 Vaccination led to a reduced proportion of perivascular Tregs at the site of VZV challenge

Representative immunofluorescence micrographs showing perivascular $CD4^+$ T cells co-stained with Foxp3 in an old donor, before and after vaccination, are shown in Figure 4-9A. The mean number of Tregs per perivascular infiltrate at the various time-points is shown in Figure 4-9B. Over the 7-day time-course, the number of Tregs per perivascular infiltrate increased progressively in the young ($p=0.0016$) but not in the old. By Day 7 there were significantly more Tregs in the young than the old ($p=0.030$). When the vaccinated old were compared with the unvaccinated old, there was no significant difference in Treg numbers at either Day 3 or Day 7.

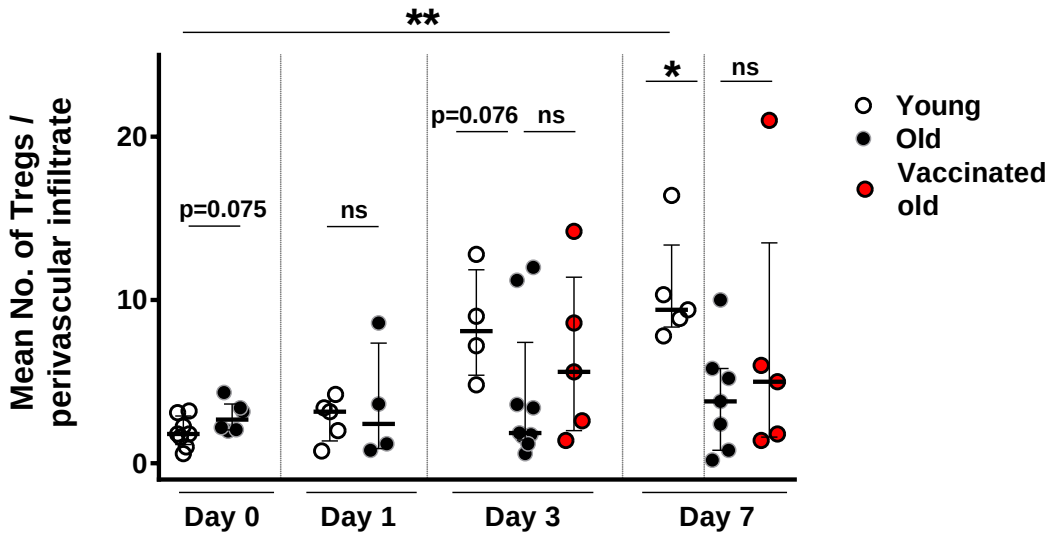
The mean percentage of perivascular $CD4^+$ T cells displaying a regulatory phenotype was calculated (Figure 4-9C). Old individuals had a greater percentage of Tregs than the young in perivascular clusters in unchallenged skin (median 30.1% in the old and 11.9% in the young; $p=0.0062$). After antigen challenge, the percentage values fell in both young and old, but the old maintained a higher percentage than the young at all time-points (Day 1 $p=0.032$; Day 3 $p=0.11$; Day 7 $p=0.030$). Interestingly, vaccinated individuals had a lower percentage of Tregs than the unvaccinated at both Days 3 ($p=0.0070$) and Day 7 ($p=0.019$).

Treg proliferation was analysed by co-staining with Ki67 (Figure 4-9D). There was a trend to an increase in Treg proliferation in the young at Day 7 compared to Day 1 ($p=0.071$), but no change in the old. At Day 1 there was no significant difference in Treg proliferation between the young and the old, but at Days 3 and 7, there was a trend to an increase in the young (Day 3 $p=0.11$; Day 7 $p=0.15$). In the vaccinated individuals there was a trend to an increase in Treg proliferation at both Day 3 and Day 7 compared to the unvaccinated (Day 3 $p= 0.095$; Day 7 $p=0.11$).

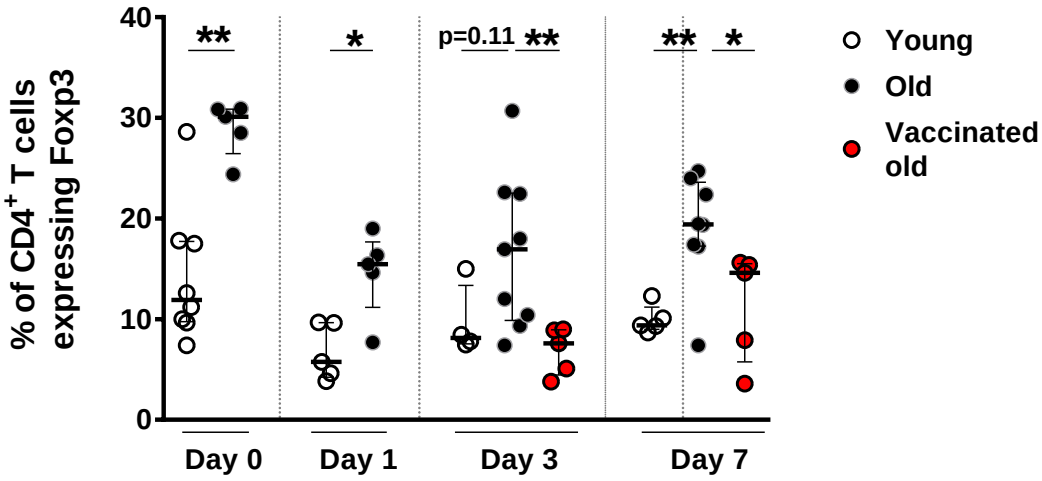
A


Old
Vaccinated old

B


**
*
ns
p=0.076
ns
p=0.075
ns
Mean No. of Tregs / perivascular infiltrate
20
10
0
Day 0
Day 1
Day 3
Day 7
Young
Old
Vaccinated old

C


**
*
p=0.11
**
**
*
% of CD4+ T cells expressing Foxp3
40
30
20
10
0
Day 0
Day 1
Day 3
Day 7
Young
Old
Vaccinated old

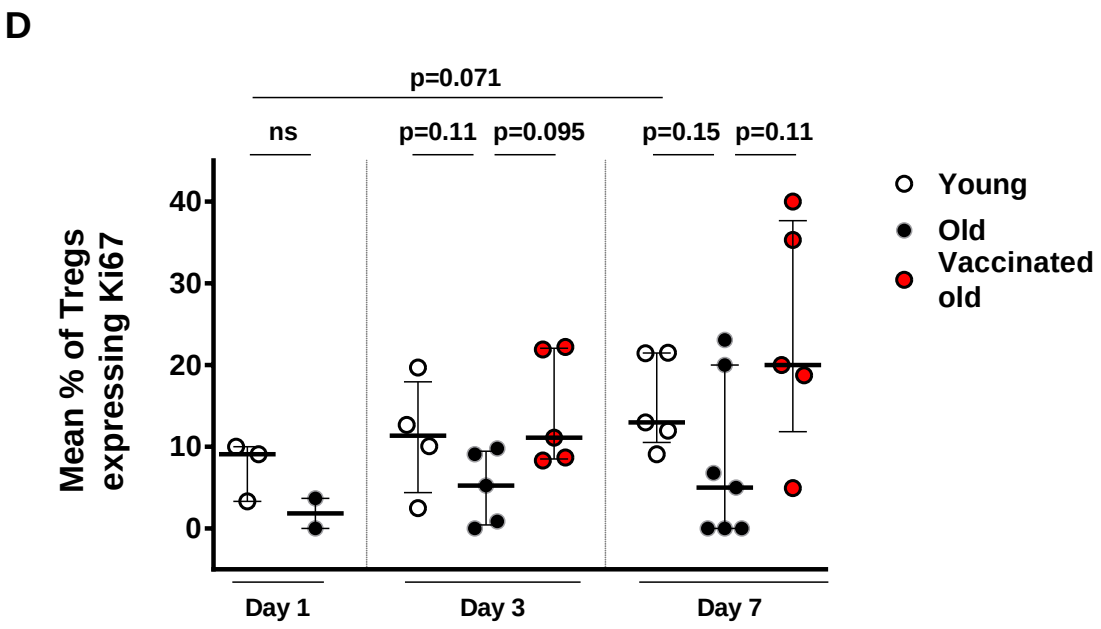


Figure 4-9 Time-course of infiltrating regulatory T cells at the site of VZV challenge, and the effect of vaccination

A: Representative immunofluorescence micrographs (x400 original magnification) of perivascular $CD4^+$ T cells (green) co-stained with Foxp3 (red), in an old donor before vaccination (left) and after (right image). Cells with a red nuclear stain surrounded by a green surface membrane stain are Tregs.
B: Graph showing the mean number of Tregs within perivascular infiltrates at various time-points in young, unvaccinated and vaccinated old donors.
C: Graph showing the percentage of perivascular $CD4^+$ T cells displaying a regulatory phenotype at the various time-points.
D: Graph showing the percentage of Tregs that were proliferating during the 7-day time-course. The median and interquartile range for each group is displayed. Significance levels are shown, and groups were compared using the Mann-Whitney test.

4.4 Discussion

4.4.1 Vaccination and skin T_{RM} numbers

The shingles vaccine did not lead to an increase in the number of skin $CD4^+$ or $CD8^+$ T_{RM} at the site of sampling (forearm). There was also no increase in the frequency of VZV-specific $CD4^+$ T cells in the skin, although a boost in the frequency of these cells was seen in the peripheral blood. Therefore, the mechanism of enhanced protection against shingles is not via an increase in the number of skin T_{RM}. This is perhaps surprising, as the skin is the first tissue to which the virus migrates following reactivation in the dorsal root ganglion, and is the site where shingles infection becomes clinically apparent.

As with many vaccines, the mechanisms by which the body processes the contents of Zostavax® and how this initiates an immune response are not fully understood. Following injection of a vaccine, particles less than 200nm can directly access lymphatic capillaries, with the optimal size for efficient passage being ~40nm, while particles larger than 200nm must be transported into the lymphatic system by DCs[269]. VZV virions are 150-200nm in diameter[270] and therefore likely to be able to access the lymphatic system directly. Additionally, after vaccination with a live vaccine, live virions rapidly disseminate via the vascular route, as occurs in natural infection, and activate DCs at multiple sites[225].

Live viral vaccines are considered to be effective in activating DCs via their PRRs[225]. These DCs migrate to the corresponding draining LNs and initiate multiple foci of T and B cell activation[225]. This is one explanation for the greater immunogenicity of live versus non-live vaccines[225]. Furthermore, this method of lymphocyte activation means that the subcutaneous and intramuscular routes of vaccine administration are similarly immunogenic for live vaccines[271].

Following the activation and proliferation of antigen-specific T cells in the lymph node, a proportion persists as long-lived memory T cells in the circulation[25], where they are detectable by peripheral blood sampling. Thus, the detection of an increased frequency of VZV-specific $CD4^+$ T cells in the blood following vaccination in this study is not surprising. Similar findings were demonstrated in an immunology substudy accompanying the original clinical trial of Zostavax®, in which vaccination of older individuals led to an increase in T cell-mediated immunity (CMI) in peripheral blood at all time points between 6 weeks and 3

years, as measured by $CD4^+$ responder cell frequency (RCF) and IFN-γ ELISPOT assays[239].

There may be several explanations for the lack of an increase in the number of T_{RM} or frequency of VZV-specific T cells in the skin following vaccination. As live vaccine virus particles are able to migrate from the vaccine site to the draining LN without relying on uptake by DCs, imprinting of virus-specific lymphocytes in the LN with information relating to the site of inoculation may not occur, meaning that these lymphocytes may not acquire homing markers to that site[100,272]. Conversely, when DCs encounter antigen in the skin and present this to lymphocytes in the draining LN, skin-related signals are imprinted onto these lymphocytes, inducing their expression of skin homing receptors and facilitating their recruitment to the skin[272].

Zostavax® is injected into the subcutaneous layer in the shoulder/upper arm region. The subcutaneous layer is made up of adipose tissue, predominantly consisting of adipocytes and fibroblasts. Even if the majority of Zostavax® virus particles end up migrating through the vascular network to reach the LNs, it is still possible that some particles are taken up by DCs at the site of vaccination, processed and transported via lymphatic capillaries to the regional lymph node. The lymphatic system is intimately related to adipose tissue, and dermal lymphatic capillaries are located in close proximity to subcutaneous fat[273]. However, in contrast to the skin, the subcutaneous layer is relatively devoid of resident immune cells[274]. Despite this paucity of immune cell numbers, a variety of immune cell types have been identified in healthy, non-obese adipose tissue. The most numerous are Tregs, iNKT cells, macrophages and eosinophils, while B cells, Th1 $CD4^+$ T cells, $CD8^+$ T cells and mast cells are more sparsely present[275]. DCs in adipose tissue in healthy humans have not been well-characterised but are considered to be scant in number, particularly in comparison to the neighbouring tissues of skin and muscle[225], which represent alternative options for vaccination sites. DCs within adipose tissue have been better characterised in obese individuals, in whom they are proposed to have a pro-inflammatory role[276]. In summary, DC activation by Zostavax® vaccine antigen within the adipose tissue is unlikely to play a major role in the induction of immune memory and formation of VZV-specific T_{RM}.

If Zostavax® does lead to the generation of VZV-specific effector T cells in the draining LN that home to the site of injection, then this may well lead to the formation of adipose tissue T_{RM}[275], but not skin T_{RM}. To demonstrate whether adipose tissue T_{RM} are generated by subcutaneous vaccination, samples of subcutaneous adipose tissue would need to be analysed, which was beyond the scope of the project detailed here. In any case, even if there was an expansion of VZV-specific T cells resident within adipose tissue, these would be unlikely to play an important contributory role in the defence against VZV reactivation, as virus particles are shed from the sensory nerve into the dermis and not into the subcutis.

Leishmania major-specific $CD4^+$ T_{RM} were shown to be generated throughout the skin surface of mice following localised infection with *L. major*. These cells persisted at least 1 year and were protective against further infection[107]. Another study involving mice showed that acute epicutaneous infection (via skin scarification) with vaccinia virus led to the generation of antigen-specific $CD8^+$ T_{RM} at the site of infection, and these cells were long-lived and highly protective against vaccinia re-infection[105]. Importantly, antigen-specific $CD8^+$ T_{RM} were also generated throughout the entire skin surface, albeit at lower numbers than at the original site of infection. These cells were also long-lived, protective against vaccinia infection, and further increased in number with every subsequent episode of vaccinia infection[105]. Local inflammation and antigen recognition encourage lodgement and retention of T_{RM} [277], which may explain the greater numbers of T_{RM} at the original site of infection.

The subcutaneous adipose tissue layer is directly underneath and in contact with the dermis. It is thus conceivable that vaccine contents injected into the subcutaneous layer may come into contact with the overlying dermis and the dermal dendritic cells (DDCs) within. It is therefore possible that Zostavax® antigens are taken up by DDCs, which present antigen to and activate VZV-specific T cells in the skin or in the draining LN, ultimately leading to an expanded population of VZV-specific T cells in the skin. If this is indeed the case, then the technology used in this study for identifying VZV-specific T cells in the skin is not sensitive enough to detect an increase following vaccination.

The number of live T cells identified by flow cytometry following overnight collagenase digestion of 2 x 5mm punch biopsies, a subsequent 15-hour in vitro

stimulation with VZV lysate in the presence of brefeldin, and then a surface and intracellular flow cytometry staining protocol – is low, perhaps a few thousand cells on average. T cells from the skin are prone to death in culture in the absence of homeostatic cytokines, and the above experimental protocol from the time of biopsy to the cell fixing stage is prolonged (may be up to 48 hours depending on time of biopsy) and testing. If the frequency of VZV-specific $CD4^+$ T cells is, for example, 0.2% for a given donor, and the total number of skin-derived $CD4^+$ T cells obtained from 2 biopsies is, say 1000 (both of which are plausible figures), then the number of VZV-specific $CD4^+$ T cells identified on the flow cytometer will be 2. Such a low absolute number of antigen-specific cells calls into question the reliability of this technique. For example, if 1 more or 1 fewer VZV-specific T cell is detected, this would increase or decrease the calculated frequency by 50%, giving rise to a wide confidence interval.

Furthermore, it must be noted that in the study volunteers, the site of vaccination (upper arm/shoulder) was different to the site sampled by skin biopsy (forearm). As the highest concentration of skin T_{RM} occurs at the site of original infection, perhaps if the skin overlying the vaccination site was sampled, an increase in VZV-specific $CD4^+$ T cells may have been seen.

Additionally, in this study the time interval between vaccination and skin biopsy sampling was 2-8 months. A uniform time point of 2 months was aimed for but for a variety of reasons, including volunteer availability, this could not be adhered to. Once antigen-specific T_{RM} in the skin have been generated after skin infection, they remain long-term but their frequency may decline gradually over time in the absence of further antigenic stimulation[105]. Peak Zostavax®-induced VZV-specific T cell responses in the circulation were observed at 6 weeks, and the sharpest decline was seen between 6 weeks and 1 year[239]. Therefore, it is possible that if all skin samples in our study were consistently taken at an early time-point, such as 6 or 8 weeks after vaccination, then a boost in VZV-specific T cells in the skin may have been seen.

4.4.2 Alternative routes of vaccination

For reasons explained above, the intradermal route of vaccination may be more effective at generating skin T_{RM} than the hypodermic route. The skin has a dense, well-characterised network of DCs[278], meaning that antigen uptake, initiation of

inflammation, and antigen presentation to T cells are more likely to lead to the efficient generation of antigen-specific T_{RM}.

Several studies have compared the immunogenicity of reduced dose intradermal vaccines versus full dose hypodermic vaccines, with the former superior or equivalent to the latter. Rabies vaccine, which contains killed rabies virus, is given intradermally at one tenth of the dose as intramuscularly and generates similar antibody responses[279], demonstrating the highly immunogenic nature of vaccination into the dermis. Consequently, reduced-dose intradermal rabies vaccine has been adopted by a number of developing countries to reduce the economic burden of mass vaccination with a vaccine that is expensive to produce[279]. Kenney el al. showed that when influenza vaccine, containing purified surface antigen from the influenza virus, was administered intradermally at one fifth of the standard dose, it generated equivalent or superior antibody responses as when administered at full dose via the intramuscular route[280]. Hepatitis B vaccine (containing hepatitis B surface antigen), when administered intradermally at one sixth the dose of the intramuscular route, led to similar seropositivity rates over 2 years (protective levels), although antibody titres were higher in the intramuscular group[281].

The mechanism of immune memory generation for these non-live vaccines is distinct from live vaccination, however. Activation of DCs at the site of vaccination is less critical for live viral vaccines than it is for non-live vaccines, as free virus particles can make their own way to LNs via the lymphovascular system[225]. Beals et al. compared the immune responses generated by subcutaneous and intradermal routes of administration of Zostavax®; the intradermal route led to a more pronounced increase in VZV IgG at 6 weeks (3.25-fold increase) compared to the subcutaneous route (1.74-fold increase)[282]. Importantly, fold-change in VZV IgG at 6 weeks post-vaccination has been shown to be an excellent correlate of protection against shingles[245]. At 18 months the fold increase in antibody titre remained higher in intradermally vaccinated compared to subcutaneously vaccinated participants[282]. However, there was no difference in the cell-mediated immune response, as measured by IFN-γ ELISPOT, between both routes of administration. There is no licence in place for Zostavax® to be administered to individuals via the intradermal route, and this is unlikely to change given the increased pain associated with this route.

Another route of vaccination that is yet more immunogenic than intradermal is the epicutaneous route, using skin scarification. Variola major infection (smallpox) killed 300-500 million people in the 20th century, yet was eradicated by mass vaccination with the related live vaccinia virus via skin scarification.

Studies in mice have demonstrated the usefulness of vaccination by scarification to generate antigen-specific immunity in the skin. Scarification of mice with vaccinia virus led to upregulation of skin-homing molecules on $CD8^+$ T cells in the draining lymph nodes, with subsequent migration of a proportion of these cells back to the skin[283]. Liu et al showed that mice vaccinated with live vaccinia virus via skin scarification generated more IFN-γ-producing $CD8^+$ T cells, a superior recall IFN-γ response and superior humoral responses, compared to the intradermal, subcutaneous or intramuscular routes[284]. The skin scarification route led to prominently higher viral gene expression at the inoculation site than the other vaccination routes[284], suggesting enhanced viral activation and presumably also heightened stimulation of the immune system via this route. Mice previously immunised by skin scarification were shown to be better protected against secondary vaccinia skin challenge than mice which had been immunised via the intradermal, subcutaneous, intramuscular or intraperitoneal routes; this enhanced protection was shown to be due to a boost in T cell memory[283,284]. Furthermore, it was particularly the skin T_{RM} generated by the original skin scarification procedure that mediated the enhanced protection[284]. Importantly, increased numbers of $CD3^+$ T cells were observed in the skin of mice previously vaccinated by scarification but not via any of the other routes[284]. This demonstrates that scarification is a more effective method of generating skin T_{RM} than other routes of vaccination.

In humans, skin scarification with vaccinia virus protects against natural smallpox infection, which is acquired via the respiratory route. Interestingly, mice vaccinated by scarification were completely protected against intranasal vaccinia infection, in contrast to the mice vaccinated by all the other routes which developed clinical signs of infection[284]. This suggests that scarification is far superior to all other routes of vaccination at generating immunity at distant peripheral tissue sites. A combination of T cells resident within the upper respiratory mucosa and T_{CM} in the lymph nodes draining respiratory mucosa was needed to provide full protection against intranasal vaccinia infection[284].

Thus, in general it appears that skin scarification is superior to intradermal vaccination, which itself is superior to the hypodermic route. It is recognised that a successful vaccine must be capable of inducing a robust innate immune response, which then translates into vigorous stimulation of the adaptive immune system and generation of long-lived and potent immune memory. The differences in efficacy between the various routes of vaccination are likely to be due to the varying ability to initiate activation of the innate immune system. Hypodermic vaccination bypasses the dense network of APCs in the skin and targets anatomical areas that are relatively devoid of APCs[274], a clear disadvantage for the induction of immune memory. The hypodermic route is effective at generating antibody responses[285,286], useful for preventing systemic infection, but where the aim is prevention of infection at an epithelial site such as the skin, then the generation of T_{RM} at that site may be more desirable[286] – an aim that is more likely to be achieved by introducing the vaccine directly into the skin.

The usefulness of administering a vaccine at the same anatomical site as the entry point for the pathogen is demonstrated by the efficacy of the oral polio vaccine, oral rotavirus vaccine and intranasal influenza vaccine. Mucosal vaccination has been shown to mediate humoral and cell-mediated immune protection both at mucosal surfaces and systemically[287]. This route of vaccination also strongly induces long-term T and B cell memory[287].

In summary, future strategy for vaccination against pathogens that invade the body through the skin should take into consideration the option for administering vaccines via skin scarification or intradermally. Such skin-tropic pathogens include Staphylococcus aureus, Streptococcus pyogenes, herpes simplex, human papillomavirus, Candida albicans and dermatophyte fungi. The problem with the skin scarification route is that, in the context of smallpox vaccination, it led to morbidity and mortality in people with compromised immune systems or atopic dermatitis[288]. Intradermal vaccination is more painful than the subcutaneous or the intramuscular routes, and the injected volume is restricted to a maximum of 200µl, whereas large volumes can be injected via the hypodermic route[274].

Using skin scarification to vaccinate against pathogens that do not typically invade the body via the skin (such as smallpox) should also be considered as a future strategy, given that this method of immunisation appears effective in

generating cell-mediated immunity at other epithelial barriers (eg. lung) as well as in circulation.

4.4.3 Vaccination and PD-1 expression in the skin

PD-1 expression was increased on dermal $CD4^+$ but not $CD8^+$ T cells in the vaccinated compared to the unvaccinated group. There may be several explanations for this finding. Firstly, it is possible that the increased PD-1 expression was transient and due to $CD4^+$ T cell activation following viral antigen encounter. This could be investigated further with serial biopsies at various time points, and a reduction in PD-1 expression back to baseline over time would support this possibility.

Another explanation is that the vaccine induced a relative state of T cell exhaustion, due to its high antigenic load. Previous work showed that T cells in the skin experienced accelerated telomere erosion as early as 19 days after induction of a cutaneous secondary immune response to PPD[169]. It is conceivable therefore that skin resident T cells have a heightened propensity to exhaustion and senescence following secondary immune responses.

4.4.4 Improved clinical response to VZV skin challenge following vaccination

Vaccination of old volunteers with Zostavax® led to a significant improvement in the clinical score (a visible marker of inflammation) at the site of VZV antigen challenge. The mechanism for this restoration of inflammation is unclear. In this chapter it was shown that skin T_{RM} numbers remained the same following vaccination, so a boost in T_{RM} numbers prior to DTH induction cannot account for the enhanced inflammation. Khan et al showed antigen-specific $CD8^+$ T_{RM} in mouse skin were potent stimulators of localised inflammatory responses after cognate antigen challenge[112]. Therefore, it is possible that after vaccination, VZV-specific T_{RM} have a lower threshold for activation, and undergo more rapid proliferation and produce more pro-inflammatory cytokines, thereby driving the inflammatory process early on during the response. Another possibility is that the increased frequency of VZV-specific T cells in circulation after vaccination leads to enhanced recruitment of these cells to the skin after VZV challenge. With both these possibilities, greater amounts of T-cell-derived IFN-γ would lead to

enhanced activation of skin resident macrophages and increased macrophage-derived TNF-α production, perpetuating the inflammatory response.

Endothelial expression of E-selectin and VCAM-1 was equivalent between young and old at 6 hours after induction of the DTH response to VZV antigen, and was significantly elevated compared to unchallenged skin in both age groups[143]. This suggests that endothelial cells in the old are functional at the early stages of the DTH response. However, from 24 hours onwards, there was a significant increase in endothelial E-selectin expression in the young compared to the old, implicating active inhibition in the later stages of the immune response or a failure of immune response amplification in the old, or both[143]. It would be interesting to measure endothelial expression of E-selectin from 6 hours onwards in the vaccinated old, to investigate whether vaccination restores the expression of this molecule to levels seen in the young. If confirmed, this would highlight endothelial expression of cell adhesion molecules as a critical mechanism for the generation of robust antigen-specific immune responses, and would be a candidate for therapeutic manipulation to boost immunity in the old.

76% of volunteers in our study had an improved clinical score after vaccination. Several other studies have investigated the effect of VZV vaccination on the clinical response to VZV skin test antigen in older adults and the results are summarised in Table 4-2.

Study	Participant age	Time after vaccine	Skin test response measure	Response rate
Takahashi et al. 2003[289]	70-79	4-6 weeks	Negative/weak positive to positive	76.2% (16/21)
Takahashi et al. 2001[290]	>50	5-7 weeks	Negative/weak positive to positive	76.9% (10/13)
Takahashi et al. 1992[291]	60-82	2 weeks	Negative to positive	56.3% (9/16)

Table 4-2 Previous studies examining the effect of VZV vaccination on clinical response to VZV skin test

These studies first confirmed the loss of clinical response to VZV skin test antigen in older adults, and then demonstrated the boost provided by VZV vaccination in

a proportion of volunteers. The response rates from the two more recent studies[289,290] are similar to the percentage of responders in our study. These two studies used a higher vaccine dose of 3.0 x 10^4 PFU per vaccine, compared with 1.94 x10^4 PFU in our study. The vaccine dose in the 1992 study was not stated but if a lower dose was used then this may explain the lower response rate in that study.

In our study, volunteers with a pre-vaccination score of 4 or above, indicating at least moderately robust VZV-specific immunity in the skin, were excluded from further participation. The reason for this is that the aim of our work is to understand how the immune system declines in old age, and to investigate strategies to boost failing immunity in this age group. The scope for enhancing immunity in an ageing individual with good baseline immune function is therefore limited. A similar strategy for excluding ageing volunteers with good baseline immunity (as indicated by the robustness of the VZV clinical score) before investigating the immune effects of the VZV vaccine was also adopted in two of the studies listed in the table above[289,291]. A caveat in our work is that the volunteers who did complete the study may not be wholly representative of the ageing population, as undoubtedly the ageing population contains a minority of individuals who demonstrate vigorous immune responses akin to those in early adulthood.

The clinical response to VZV skin test antigen has been shown to be an excellent correlate of clinical protection against shingles[147]. Additionally, the clinical response is inversely correlated with the severity of shingles skin lesions and associated pain[292]. Oxman et al showed that in the over 70s Zostavax® reduced shingles incidence by only 37.6%, and burden of illness (taking into account duration and severity of pain) by 55.4%[238]. This suggests that an improvement in cell-mediated VZV-specific immunity in the skin following vaccination does not necessarily equate to protection against VZV reactivation.

Interestingly, our figure of 76% is not dissimilar to the percentage reduction in the incidence of post-herpetic neuralgia (PHN) in the >70-year-old population, found to be 66.8% in the study by Oxman et al[238]. Imoto et al demonstrated that a lack of clinical response to the VZV skin test is an important predictive factor for PHN[148]. It is possible that the boost in clinical response to VZV skin test after vaccination may be correlated with a reduction in incidence of PHN. It is believed

that PHN results from viral and inflammatory damage to sensory nerves, so it is possible that enhanced cell-mediated immunity to VZV after vaccination protects the nerves from viral attack.

The possibility that the first VZV skin test leads to a boosting of the response induced by the second skin test was considered. Ideally this study would have involved a control group that did not receive a vaccine, but there were an inadequate number of volunteers to achieve this. In parallel studies we showed that re-challenge of old volunteers with VZV skin test antigen did not significantly boost their original clinical scores.

To consider the immunogenicity of the VZV skin test in comparison to live attenuated VZV vaccine, Takahashi et al measured the glycoprotein content of both preparations[289]. Their standard 100µl dose of VZV skin test contained 0.37µg of glycoproteins, whereas the VZV vaccine (3.0×10^4 PFU per dose) contained 9.18µg of glycoproteins per dose. We used the same skin test but at a dose of 20µl, and Zostavax® consists of 1.94×10^4 PFU per dose and may be a different preparation to the vaccine used in the referenced study from 2003. Nevertheless, adjusting their calculated glycoprotein content to match the doses used in our study, the VZV skin test contains 0.07µg per dose, and Zostavax may contain approximately 5.94µg per dose – meaning that the vaccine contains approximately 80 times more glycoprotein than the skin test at the doses used in our study. This would suggest that the immunogenicity of the skin test is negligible in comparison to the vaccine. Furthermore, skin test antigen is soluble, a less immunogenic form than particulate antigen as contained in live VZV vaccine[289,293].

Several studies have investigated whether repeated injection of the VZV skin test leads to an enhancement of the VZV-specific cell-mediated and antibody immune response[146,289,291,294–298]. Guinea pigs immunised with live VZV vaccine developed VZV-specific humoral and skin test responses[291,294]. Heat-inactivated vaccine induced only a humoral response but no skin response[291]. Unimmunised guinea pigs were injected with 1ml of VZV skin test antigen subcutaneously 2-3 times, and this led to a VZV-specific antibody response but no visible skin reaction[291,294]. Injection of 0.1ml VZV skin test via the intradermal route 5 times did not lead to an antibody or visible skin response[291,294]. These results

demonstrated an absence of boosting with repeated intradermal skin test injections.

In a study of 60 humans Berger et al found the VZV skin test was unable to induce an antibody response in seronegative individuals or an antibody booster response in seropositive individuals[298]. Furthermore, volunteers who had a negative VZV-specific lymphocyte transformation test (a measure of CMI) remained negative 2 weeks after administration of the VZV skin test[298].

In a study by Somekh et al, none out of 4 VZV seronegative participants seroconverted 1 month after having a negative VZV skin test[146]. An antibody booster effect was observed in 6 out of 12 (50%) seropositive and skin test-positive participants[146]. LaRussa et al performed VZV serology 2 to 3 weeks after initial screening with the VZV skin test, VZV serology and lymphocyte proliferation assay (LPA)[296]. They found that out of 7 individuals with no evidence of past VZV infection, none of them seroconverted after receiving the VZV skin test[296]. In contrast, 6 out of 17 (35%) individuals with evidence of immunity to VZV demonstrated an antibody booster effect after the VZV skin test[296].

In another study of 4 VZV-immune volunteers, the VZV skin test antigen boosted the level of VZV-specific antibody after 2 weeks, with minimal further increase after vaccination with Oka VZV vaccine[295]. In this study, the VZV skin test was injected 5 times over 7 months, but the VZV vaccine was administered after the first skin test, complicating the interpretation of the effect of the skin test only. In any case, the clinical response fluctuated between the 4 tests administered during the 6-month post-vaccine period in an apparently sporadic manner. In total there were 7 sequential decreases and 5 sequential increases among the 4 volunteers. No consistent boosting effect was seen.

There is one study by Duchateau et al, involving 30 individuals over the age of 70, which concluded that repeated VZV skin tests led to a boosting of the skin immune response after 4 weeks[297].

These studies investigating the immunogenicity of the VZV skin test are limited in number, making interpretation of the results difficult. Additionally, they predominantly focus on the humoral response to the VZV skin test while our study focused on the skin test response. Bearing in mind the limitations regarding the available published data, it appears that in individuals with no history of VZV infection, the skin test does not induce detectable VZV-specific humoral or cell-

mediated responses. In VZV-immune volunteers however, the VZV skin test appears to boost the humoral response, but the effect on further skin test responses remains unclear[289]. With the exception of the one study by Duchateau et al[297], it is of the opinion of the authors of the studies investigating this question that the VZV skin test has low immunogenic potential and unlikely to lead to a boosting of the cell-mediated response[291,294–296]. All but one of these studies are authored by researchers who were involved in the original development of the VZV skin test in Japan in the late 1970s and early 1980s[291,294,295].

The effect of vaccination on DTH responses to other pathogens has been investigated in ageing individuals previously. Tetanus toxoid has been shown to be an effective recall antigen for evaluating CMI in the skin, with response rates in young healthy individuals ranging from 79% to 90% [299–301]. However, in an older population (mean age 54) the response rate was only 45.5% [302], reflecting the diminishment of CMI with increased age. French et al identified 27 individuals with an approximate mean age of 60 years who failed to respond to intradermal challenge with a 1:10 dilution of tetanus toxoid[302]. These volunteers were randomised to either receive 0.5ml tetanus toxoid vaccine (n=14) or no intervention (n=13). 10 weeks later the volunteers were retested with intradermal tetanus toxoid, and surprisingly none of the vaccinated group, or unvaccinated group, responded.

A reason for the variance of this study's findings with our work may be the different formulations of the vaccines used. Tetanus toxoid contains an inactive form of *Clostridium tetani*-derived toxin and its primary aim is to stimulate an antigen-specific antibody response, whereas Zostavax® contains live attenuated virus designed to boost CMI. It therefore appears that the latter vaccine is more potent at enhancing a DTH response than the tetanus vaccine. The difference in response between both vaccines highlights the problems regarding the efficacy of vaccination in the older population.

4.4.5 Enhanced cellular responses to VZV skin challenge after vaccination

There was a boost in the $CD4^+$ and $CD8^+$ T cell responses (greater numbers and proliferation) to VZV skin challenge after vaccination. The enhanced responses were detectable at both time-points measured in the vaccinated – Days 3 and 7. As discussed in the previous section, the clinical response to VZV antigen

challenge, measured at Day 3, was also boosted in the vaccinated group. Previous work by our group has shown that the Day 3 clinical score is positively correlated with the mean number of $CD4^+$ T cells per perivascular infiltrate[142]. Young individuals have more robust clinical and cellular responses to VZV challenge than the old[135,252], so vaccination of the old appeared to partially restore impaired VZV-specific cutaneous immune responses to the more robust responses associated with the young.

Previous work using skin suction blisters in robust responders to VZV skin challenge showed that approximately 5% of infiltrating $CD4^+$ T cells at the site of VZV challenge at Day 7 were antigen-specific, measured both by IFN-γ production after in vitro VZV-restimulation and also by tetramer staining[142]. In a study of skin immune responses to the Mantoux test, the proportion of IFN-γ-producing antigen-specific $CD4^+$ T cells accumulating at the challenge site was significantly greater in the young than the old[135]. Presumably therefore, the proportion of VZV-specific $CD4^+$ T cells accumulating at the site of VZV challenge would be higher in the vaccinated than the unvaccinated old, and further experiments should be performed to verify this is the case.

In Section 4.3.2 it was shown that the frequency of VZV-specific $CD4^+$ T cells after vaccination remained the same in unchallenged skin but was boosted in the blood. Therefore, if indeed a greater proportion of VZV-specific $CD4^+$ T cells at the site of VZV challenge were confirmed in the vaccinated, the origin of these cells should be considered. After pathogen re-encounter, tissue resident DCs can directly present antigen to antigen-specific T_{RM} in the skin, leading to proliferation and effector function by these latter cells in situ[111,303]. Therefore, it is conceivable that after vaccination, activation and proliferation of VZV-specific $CD4^+$ skin T_{RM} occurs more rapidly than before vaccination.

Another explanation, consistent with the data generated in this project, is that the higher frequency of VZV-specific $CD4^+$ T cells in the circulation after vaccination leads to enhanced recruitment of these cells into the skin. As the predominant memory phenotype of circulating VZV-specific $CD4^+$ T cells is the T_{CM} ($CD27^+$ $CD45RA^-$) subset[267], these cells have access to the LNs, where they could encounter cognate antigen transported from the skin, become activated, and generate effector T cells that home to the skin to participate in the immune response. A greater initial frequency of VZV-specific $CD4^+$ T cells in the

circulation would presumably lead to a larger pool of expanded effector T cells after antigen challenge, which would then home to the challenged skin site.

It has also been shown that circulating memory T cells can be recalled to infected tissue in a lymph-node-independent manner[119,304]. After recall antigen challenge, successful initiation of inflammation and activation of the local endothelium leads to significant recruitment of T cells from the circulation to the skin[111]. This predominantly occurs in a non-specific manner, but some antigen-specific T cells are also recruited in this way without encountering antigen in the lymph nodes[111,304]. Furthermore, these antigen-specific T cells proliferate in infected tissue preferentially to non-specific T cells, leading to a pool of expanded specific T cells at the site of infection[142,304].

The association of reduced endothelial expression of E-selectin with diminished T cell infiltration in old humans during a DTH response to VZV antigen suggests that lymphocyte recruitment from the circulation is essential for amplification of the cutaneous immune response[143]. Studies in rodents have shown that inhibition of leukocyte trafficking to the skin by separate blockade of E-selectin[305], E-selectin ligands[306], LFA-1 and VLA-4[307], and CCR4[308] respectively led to impaired T cell accumulation in cutaneous hypersensitivity models. This provides further evidence for the importance of lymphocyte recruitment from the circulation to participate in the DTH response. Therefore, the association between the Zostavax®-induced boost in frequency of circulating VZV-specific $CD4^+$ T cells and boost in clinical response to VZV antigen appears to be a highly important one.

Proposed mechanism of action for Zostavax®

Combining the findings of Zostavax®-induced enhanced clinical and cellular responses after VZV antigen challenge in the old, a proposed mechanism of action for the vaccine is proposed. In the old, there was an excessive and dysregulated non-specific inflammatory response at 6 hours in VZV-challenged (and saline-challenged) skin, and this was associated with reduced clinical DTH responses to VZV[143]. At 24 hours and beyond, there was impaired endothelial activation and DC accumulation[143], suggesting that early (6h) excessive inflammation prevents subsequent leukocyte recruitment via the endothelium. To correct for these defects in the old, it is now proposed that the vaccine leads to an early enhancement of the cytokine response from skin resident VZV-specific

T cells. IFN-γ secreted by these T cells activates local macrophages, which produce TNF-α in a regulated manner, in turn activating local endothelium. Memory T cells are thus recruited from the circulation in a non-specific manner, but some of these will include VZV-specific T cells, which are more numerous in circulation due to the boosting effect of the vaccine. VZV-specific T cells infiltrating the site of antigen challenge proliferate and produce cytokines, further amplifying the response. Within days, VZV-specific T cells are also recruited from the draining lymph node and generate larger clones of effector T cells due to increased baseline numbers following vaccination. These effector T cells home to the site of antigen challenge and magnify the immune response yet further.

The role of Tregs in the DTH response after vaccination

Interestingly, the percentage of Tregs in the vaccinated was significantly lower than for the unvaccinated at both Days 3 and 7. This may be explained by the significant accumulation of effector $CD4^+$ T cells and the lack of an increase in absolute Treg numbers in the vaccinated (although there was a trend to increased Treg proliferation in the vaccinated).

It may be that the absolute number of Tregs within perivascular infiltrates is a reflection of the need to terminate the immune response once foreign antigen has been eliminated. In the young, as the skin immune response is more robust with more effector T cells present, more Tregs are required to subdue the response once it has peaked. Conversely, in the old, fewer absolute numbers of Tregs are needed to terminate the more attenuated immune response. With regards to the vaccinated individuals, the median Treg number did lie between the medians for the young and for the unvaccinated, and so this hypothesis may still hold true; however, the differences observed were not large enough to be significant. Perhaps more n numbers would elucidate this further.

Tregs are thought to exert their suppressive function at least in part via cell-cell contact[13], and for this reason the proportion of Tregs out of the total $CD4^+$ T cell population should be considered when analysing the inhibitory influence of this cell type on the immune response. The lower percentage of Tregs in the vaccinated compared to the unvaccinated may be due to one of two separate mechanisms. Firstly, and more likely, the vaccine directly leads to a marked accumulation of effector T cells but not Tregs, thereby lowering the observed Treg percentage within perivascular infiltrates. Secondly, after vaccination it is possible

that via an unknown mechanism perivascular Tregs are prevented from exerting their inhibitory effects, thereby allowing larger numbers of effector T cells to accumulate, which secondarily leads to a drop in the Treg percentage.

4.4.6 The transcriptional response to vaccination

In work performed by Akbar group members, global gene expression analyses were performed to identify genes that may be associated with the improved response to VZV antigen challenge following vaccination. In each donor, 3mm skin biopsies were taken from the site of VZV antigen challenge (at 6h and 72h post-injection), both pre- and post-vaccination. The gene expression was compared to biopsies taken from normal, un-injected skin. In a previous study we showed that in normal steady state skin, there was little difference in gene expression in young or old age groups[267]. At 6 hours post-VZV antigen challenge there was a relatively minor difference in the numbers of differentially expressed genes (DEG) before and after vaccination (Figure 4-10A & B). Genes associated with migration (e.g. *SELL, CXCL10, SELE, ICAM1, IL-8*) were amongst the most upregulated genes in both groups (pre- and post-vaccine) as compared to normal skin although the expression was relatively higher post-vaccination.

At 72h after VZV antigen challenge, vaccination led to a strong transcriptional response that was considerably stronger than the response prior to vaccination (>5000 DEG post-vaccine (false discovery rate, FDR<0.05 and fold-change>2); 1711 DEG pre-vaccine, Figure 4-10A & B). The top 30 significantly DEG following vaccination are shown in Figure 4-11, indicating that the same genes were upregulated after intradermal VZV challenge but to a significantly higher extent following vaccination. Genes associated with migration (*CXCL10, CXCL9, SELL, CCL5, ITGAL*), T cell and DC activation including *ITGAX* (which encodes CD11c), *CD2, CD28*, *CD69, EOMES, ICOS* and *STAT1* were more highly expressed in the skin at 72h after VZV antigen challenge in vaccinated compared to unvaccinated individuals (Figure 4-11). This supports the histological observations showing an increase in T cell accumulation in the skin after vaccination (Figure 4-7B and Figure 4-8B).

These data also showed marked upregulation of genes involved in signaling pathways associated with innate immune responses, inflammation, immune response to viruses and inflammatory cytokine signaling (type I IFN, TNFα and IFN-γ) in vaccinated old individuals. Interestingly, many genes associated with

the activity of $CD8^+$ cytotoxic T cells including *GZMB, GNLY, GZMA* and *CD8A* were highly induced in vaccinated individuals, coinciding with the significantly increased $CD8^+$ T cell proliferation at the site of intradermal VZV challenge at 72 hours (Figure 4-8C). This indicates that Zostavax vaccination enhances the activity of VZV-specific $CD8^+$ T cells in the skin.

In a previous study, whereas circulating $CD4^+$ T cell responses were observed in response to multiple VZV antigens, only ORF-9 induced significant $CD8^+$ T cell responses[247]. Zostavax® vaccination led to an increase in the frequency of ORF-9-specific $CD8^+$ T cells producing IFN-γ by 2.8-fold and TNF-α by 4.9-fold[247]. Additionally, a 20-fold increase in the frequency of IFN-γ^+ perforin$^+$ $CD8^+$ T cells was observed following vaccination in the study[247]. VZV vaccination therefore appears to induce a robust $CD8^+$ T cell response in the circulation, and ORF-9 appears to be the key VZV antigen that mediates this response. The enhanced $CD8^+$ T cell signature in the skin after VZV challenge in vaccine recipients in our study indicates that it will be important to determine the specificity of these cells and their role in the protection against shingles.

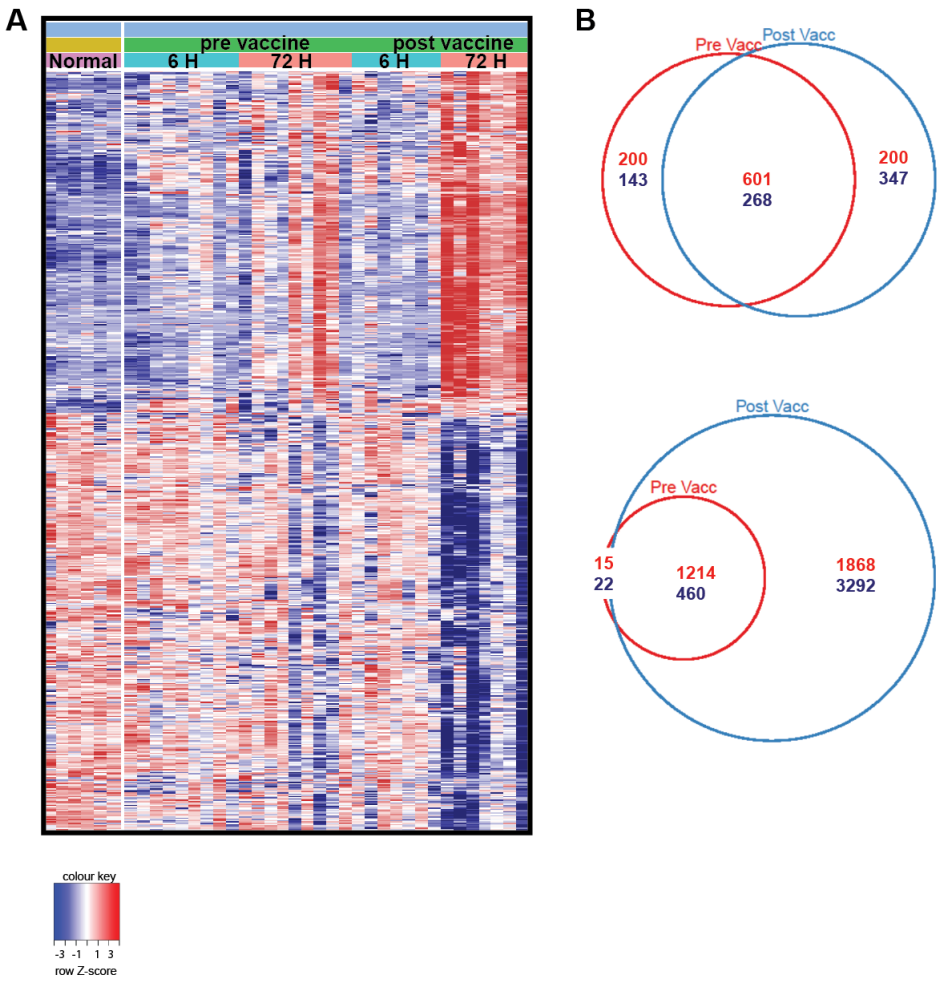


Figure 4-10 Transcriptomic analysis of VZV-challenged skin before and after vaccination

Skin biopsies were taken at the site of VZV challenge (n=9 pre-vaccine, 7 post-vaccine) and from normal skin in an additional group of volunteers (n=6). Total skin RNA was isolated, amplified and hybridized to Affymetrix Human Genome U133 plus 2.0 arrays

A: Heatmap showing the relative expression of differentially expressed genes between normal skin (left panel) and VZV-injected skin pre-vaccination (middle panel) and post-vaccination (right panel) at Fold change>2 and FDR>0.05.

B: Venn diagrams showing numbers of DEG at 6h (top) and 72h (bottom) following VZV antigen challenge, compared to normal skin. Up-regulated genes are shown in red, down-regulated genes in blue.

Akbar group data

SYMBOL	Pre-Vaccination	Post-Vaccination
CXCL10	133.14	1348.46
GZMB	59.1	639.74
CXCL9	61.22	462.7
SERPINA1	43.09	415.92
FCGR1A	42.87	319.15
FPR1	34.34	312.21
GNLY	24.14	294.75
IDO1	49.07	288.4
PLAC8	24.19	258.1
LYZ	63.89	251.81
KLRC2	12.02	235.13
SELL	29.07	228.41
FCGR1B	32.03	224.66
PRKCQ	19.76	145.44
XCL1	15.21	138.26
CCL5	17.62	135.75
GBP5	16.94	116.11
ITGAL	17.52	114.84
CD2	17.39	113.07
UBD	24.73	111.99
KLHDC7B	25.1	108.41
CTLA4	10.21	106.16
TRGV9	11.44	105.69
CLEC4E	14.62	105.62
GZMA	19.25	100.97
EOMES	10.52	100.84
CLEC12A	17.88	99.52
TARP	11.32	95.48
TRGC2	11.56	94.85
CD8A	22.12	91.79

Figure 4-11 Genes upregulated in the skin after vaccination

Table showing the top 30 genes upregulated at 72 hours post-VZV challenge pre- and post-vaccination.
Akbar group data

4.4.7 Zostavax® mechanisms and VZV-specific TRM

If VZV reactivates and is not contained within the ganglion, virus is able to migrate to the skin, where it will encounter a dense network of resident immune cells, including virus-specific T cells. In an Israeli population-based study, the incidence of shingles in 25-34 year-olds (n=291,314) was 2.35 per 1000 person-years, compared to 10.23 per 100 person-years in 65-74 year-olds (n=83,511)[309]. This suggests that, although far more common in the old, shingles is not a rare disease even in healthy young individuals. Therefore, once in the skin, reactivated VZV appears at times to be able to overcome host defence by VZV-specific T cells.

In the skin, VZV suppresses innate responses, including disruption of IFN-mediated responses in infected cells, and activation of STAT3 which upregulates survivin, a protein that is necessary to support VZV infection[177]. Thus the inhibition of innate immune responses in the skin by VZV may lead to a failure to adequately activate skin resident VZV-specific T cells, and these combined events may impede VZV-specific T cell recruitment from the circulation, thereby permitting clinical disease to develop[310]. In the old, impairment of innate responses in the skin and of VZV-specific T cell immunity in the blood is therefore likely to increase the risk of shingles.

Zostavax® may provide immune protection by:

1. Enhancing the responsiveness of skin resident VZV-specific T cells, to compensate for their impaired activation by innate cells
2. Boosting circulating VZV-specific T cells, enhancing recruitment to the skin and ganglia during VZV reactivation

As argued by Kupper, robust immune protection against secondary infection in peripheral tissue relies on the successful integration of rapid responses by T_{RM} that act against pathogens invading through epithelial surfaces, as well as a secondary wave of circulating T_{CM} that can expand massively to provide reinforcements[286]. There is now evidence that Zostavax® boosts the latter; however, the extent to which it may boost skin T_{RM} responsiveness remains uncertain and requires further investigation. Perhaps the lack of evidence for a boost in VZV-specific T_{RM} frequency in this work may partially explain why the vaccine is only 37.6% effective in the over-70 population[238]. The accumulating evidence for enhanced immune protection against repeated infection by peripheral tissue T_{RM} suggests that vaccines designed to boost this particular cell

population at the initial site of infection may well improve the likelihood of optimal immune protection[31,286,311–314].

As an example, Stary et al. found that mucosal vaccination of mice against genital *Chlamydia trachomatis* (Ct) infection led to rapid seeding of the uterine mucosa with T_{RM}, and optimal clearance of subsequent infection with Ct required both local T_{RM} as well as infection-recruited circulating memory T cells[315]. Similarly, Iijima and Iwasaki found that intravaginal immunisation of mice with HSV-2 virus protected the host against lethal genital HSV-2 challenge, and $CD4^+$ T_{RM} were shown to be responsible for this protection[316].

A potentially effective vaccination strategy against shingles might be to enhance VZV-specific T_{RM} in the ganglia as well as the skin. In support of this, Khan et al showed in the context of mouse HSV-1 infection that vaccination boosted the number of trigeminal ganglion-resident $CD8^+$ T cells (and effector memory $CD8^+$ T cells) and reduced recurrent ocular infection[317]. A key difference between latent human VZV and HSV infections, however, is that ganglion-resident antigen-specific T cells have been shown to exist for the latter but not the former. Therefore, introduction of VZV-specific T_{RM} to a niche which is unaccustomed to hosting such cells may not be possible.

4.4.8 Age-related immune defects that may impair the efficacy of Zostavax®

Zostavax® led to a broadening of the TCR repertoire in healthy adults[246,248], and recruitment of both naïve and memory VZV-specific $CD4^+$ T cells were involved in this response. In ageing individuals there is an abrupt decline in the naïve $CD4^+$ T cell population after the age of 75 [318], suggesting a reduced potential for this T cell subset to contribute to Zostavax®-induced immune responses in the old. Furthermore, in ageing individuals there is a contraction of the TCR repertoire accompanied by oligoclonal expansion of memory $CD4^+$ T cells, particularly in the context of chronic viral infection such as CMV[318–320], as well as a baseline reduction in the frequency of VZV-specific T cells[186,241,267,321], and these factors are likely to impair the capacity of memory $CD4^+$ T cells to expand following stimulation with Zostavax®. In support of this, although not conclusive evidence, there was a trend to a positive correlation between fold-change in VZV-specific T cell frequencies from Day 0 to Day 28 after vaccination and initial VZV-specific T cell frequency ($p=0.12$) [241].

Interestingly, the proliferative capacity of PBMCs in response to stimulation with a range of concentrations of VZV lysate in vitro was equivalent between young and old, except at the lowest concentration used, where proliferation was higher in the young[267]. This is perhaps a surprising finding given that ageing in general is associated with impaired T cell proliferation. Using a systems biology approach, Qi et al showed that the initial expansion of VZV-specific T cells after vaccination was relatively independent of age. By contrast, there was a greater loss of VZV-specific T cells following peak expansion in the old, and this accounted for the impaired generation of long-lived VZV-specific memory T cells in the old[241].

Gene expression arrays of whole blood before and after vaccination identified a change in the expression of a small number of monocyte-related, but very few lymphocyte-related, genes. Pathways involving TNF-α and STAT3 appeared to influence the expansion and apoptosis of short-lived effector T cells, suggesting a central role for monocyte activation in the generation of T cell responses following vaccination[241]. Overall, however, there was far less expression of innate immune system genes with Zostavax® as compared to other live vaccines[322,323].

The only inflammatory cytokine that increased in serum after vaccination was resistin, a member of the adipokine family[241]. The observed increase in resistin after vaccination was associated with reduced T cell expansion[241]. Resistin has been shown to induce other pro-inflammatory cytokines including TNF-α and IL-6[324,325], although in the context of adaptive immunity it may have an inhibitory influence by expanding Tregs through the inhibition of DCs[326].

In addition to environmental factors, investigation of $CD4^+$ T cell-specific factors demonstrated that genes regulating cell division and DNA repair mechanisms were associated with long-term survival of VZV-specific T cells following vaccination. There was a suggestion that these processes were more likely to be dysregulated in the old. Interestingly, molecules involved in apoptosis or markers of cellular senescence were not found to correlate with the attrition of effector T cells[241].

Overall, the data from the study by Qi et al highlighted the critical importance of the T cell contraction phase after peak expansion in determining long-lived VZV-specific T cell memory, and this was particularly true for older individuals. Contraction was largely regulated by cell-intrinsic gene expression patterns, with serum cytokine levels of lesser importance to this phase of the immune

response[241]. These findings suggest that therapeutic targeting of the contraction phase may be a useful strategy for improving shingles vaccine responses in the old.

Weinberg et al performed a study comparing the cellular immune responses to Zostavax® in young and old individuals[327]. Dual-function (expressing any 2 out of IL-2, IFN-γ, CD107a) VZV-specific $CD4^+$ and $CD8^+$ T cells increased at Day 7 after vaccination in the young but not the old. At baseline, VZV-specific $CD8^+$ T cells had higher expression of PD-1 and CD57 in the old compared to the young. It is possible the increased expression of these two markers was driven by chronic stimulation by VZV antigens, maybe during episodes of silent reactivation or exogenous exposure to overtly infected individuals. After vaccination there was a marginal increase in VZV-specific $CD8^+$ $CD57^+$ senescent T cells in the old. An increase in VZV-stimulated $CD4^+$ $CD69^+$ $CD57^+$ $PD\text{-}1^+$ and $CD8^+$ $CD69^+$ $CD57^+$ $PD\text{-}1^+$ T cells from baseline to post-vaccination was associated with concurrent decreased $CD4^+$ VZV-memory and $CD8^+$ effector responses, respectively, in older adults. Blocking PD-1 during ex vivo re-stimulation increased $CD4^+$ and $CD8^+$ T cell proliferation but not effector cytokine production, while TIM-3 blockade did increase IFN-γ production in $CD8^+$ T cells. This study suggested that increased senescent ($CD57^+$) and exhausted ($PD\text{-}1^+$) VZV-specific T cells may contribute to poor vaccination-induced effector responses in the old[327], and targeting exhaustion and senescence pathways may therefore be useful strategies to boost vaccination responses in this age group.

Poly-functional $CD4^+$ T cells make up the majority of VZV-specific T cells and these cells produce the largest amount of pro-inflammatory cytokines on a per-cell basis, before and after vaccination[247]. Additionally, after vaccination ORF-9-specific poly-functional $CD8^+$ T cells produced ~10-fold higher levels of pro-inflammatory cytokines compared to mono-functional $CD8^+$ T cells[247].

Poly-functional VZV-specific $CD4^+$ T cells ($IL\text{-}2^+$, $IFN\text{-}\gamma^+$, $CD107a^+$) but not $CD8^+$ T cells appeared to be increased in frequency in the old compared to the young[327]. Van Epps et al studied the poly-functionality of $CD4^+$ and $CD8^+$ T cells by differentiation subset, in response to stimulation with SEB, and found an increase in poly-functional effector memory $CD8^+$ (but not $CD4^+$) T cells in the old. This was accompanied by a decrease in poly-functional central memory $CD4^+$ and $CD8^+$ T cells in the old[328]. Other studies have shown that the poly-

functionality of memory $CD8^+$ T cells elicited by acute West Nile virus or chronic CMV or EBV infection was not impaired by old age[329,330]. Furthermore, Riddell et al demonstrated that poly-functional CMV-specific $CD4^+$ T cells are not restricted by ageing[331]. Overall, therefore, it appears that T cell poly-functionality may remain preserved in old age, and loss of VZV-specific T cell poly-functionality does not appear to be responsible for impaired VZV vaccine responses in the old.

4.4.9 Strategies to improve the efficacy of zoster vaccination in the old

Evidence gathered from the studies mentioned in the previous section allows for the identification of possible specific targets for improving VZV vaccine responses. Starting with the loss of VZV-specific naïve cells during the ageing process, this is unlikely to be a reversible process in view of the permanent thymic atrophy that occurs in adolescence. Narrowing of the TCR repertoire occurs due to the combination of loss of naïve T cells and oligoclonal expansion of dominant clones, such as CMV-specific and EBV-specific T cells. Given that CMV and EBV are ubiquitous viruses in the environment, these chronic viral infections may not be preventable and TCR repertoire narrowing may therefore be unavoidable. There remains a theoretical potential to introduce widespread CMV and EBV vaccination, but this seems unrealistic and may not necessarily avoid the problem of oligoclonal expansion of memory T cells.

It is unclear why the frequency of circulating VZV-specific T cells declines with age, but replenishing this population appears critical to the success of VZV vaccination. Currently Zostavax® is designed to be administered as a one-off dose to older adults and there are no recommendations in place for a booster programme – despite evidence that immune protection significantly declines and may not be protective by Year 8 post-vaccination[244,332,333]. Levin et al showed that >70-year-olds receiving a booster dose of Zostavax® 10 years after the first dose mounted a greater VZV-specific T cell response at 1 and 52 weeks, as compared with age-matched controls receiving their first dose only[334]. This demonstrates that a booster strategy would be a useful means of enhancing VZV vaccination efficacy.

Another strategy might be to increase the dose of the vaccine from 19,400 plaque-forming units (PFU). Doses up to 67,000 PFU have been given, and while there was no clear evidence of an immediate boost in T cell response[189], some

evidence exists for larger doses giving rise to a delayed boost in VZV-specific T cell memory[335].

The contraction following peak expansion of VZV-specific effector T cells after Zostavax® appears to be a critical factor that impairs the induction of long-term immune memory in the older population[241]. Therefore, investigation into the factors that contribute to effector T cell contraction in the old may yield potential therapeutic targets to boost vaccination efficacy.

Fang et al found that CD39, a cell membrane ATPase, was more frequently induced on activated $CD4^+$ T cells in the old than the young. $CD39^+$ $CD4^+$ effector T cells were highly differentiated, with greater expression of Tbet, IFN-γ, IL-4 and IL-17. These cells were unlikely to be Tregs as Foxp3 expression was not raised and there was no association with suppressive activity. $CD39^+$ T cells were metabolically stressed, with increased phosphorylation of AMPK, reduced cytoplasmic ATP, and dysfunctional mitochondria. Furthermore, $CD39^+$ T cells were highly prone to apoptosis, and this was shown to be a consequence of the ATPase activity of the CD39 molecule[336].

Older individuals over 50 years of age with genetically determined low CD39 expression had a trend to a greater fold increase in VZV-specific T cells 4 weeks after Zostavax® than those with a high CD39 genotype ($p=0.097$). Similar, but this time significant, findings were obtained with influenza-specific T cells after administration of 2 out of 3 strains of influenza vaccine in a group of over-60-year-olds. This suggests that CD39 impairs the long-term survival of virus-specific T cells after vaccination in ageing individuals. Interestingly, in the context of chronic viral infection, high CD39 genotype was associated with a greater proportion of T_{EMRA} cells in CMV-positive individuals over 60 years of age[336]. The findings from this study suggest that CD39 inhibitors may enhance vaccine responses to Zostavax®.

Increased PD-1 expression on circulating $CD4^+$ and $CD8^+$ T cells after Zostavax® correlated with impaired VZV-specific $CD4^+$ T cell memory and $CD8^+$ effector T cell responses, and blocking PD-1 boosted T cell proliferation[327]. In this work, increased PD-1 expression was also seen on $CD4^+$ T_{RM} in the skin after Zostavax® (Section 4.4.3), and so anti-PD1 therapy may be a potential strategy for boosting immunity with the shingles vaccination.

Another pathway that could potentially be manipulated to enhance the survival of effector T cells in the old is the type I interferon signalling pathway. This pathway has been shown to be impaired in activated naïve $CD4^+$ T cells during ageing, due to recruitment of SHP-1 to the IFN receptor signalling complex. This results in the loss of ability to produce IL-2, thereby impairing survival and the generation of antigen-specific memory $CD4^+$ T cells[226,337]. Restoration of $CD4^+$ T cell responsiveness to type I IFN in the old may therefore promote enhanced VZV-specific memory $CD4^+$ T cell formation following Zostavax® administration.

Several gene modules expressed by activated $CD4^+$ T cells on Days 8 and 14 after Zostavax® correlated with increased frequency of VZV-specific T cells on Day 28. These modules were functionally related to cell cycle control or DNA repair[241]. Modulation of cell cycle pathways or DNA repair mechanisms may therefore facilitate the survival of VZV-specific T cells after vaccination, and such strategies should be investigated further.

4.4.10 Shingrix®: A new recombinant subunit shingles vaccine

A phase 3 clinical trial investigating the efficacy of a novel recombinant subunit shingles vaccine was recently completed[338]. The vaccine consists of VZV glycoprotein E (gE) and the $AS01_B$ adjuvant system, and is known as the HZ/su vaccine or Shingrix®. It is now licensed for use in the USA. VZV gE was selected as it is the most abundant glycoprotein on infected cells, is essential for viral replication and cell-cell spread, and is a primary target for VZV-specific antibody and T cell responses[338–340]. The $AS01_B$ adjuvant system was chosen as it promotes robust humoral and $CD4^+$ T cell immune responses against recombinant proteins[338,341–343]. The vaccine is administered via the intramuscular route and 2 doses are given, 2 months apart.

This landmark clinical trial recruited 15411 participants to receive Shingrix® or placebo, and had a remarkable overall efficacy of 97.6% in the over 60s during a 3.2-year period[338]. This compared highly favourably with Zostavax®, which had a 51.3% efficacy in the over 60s over 3.1 years[240]. Critically, Shingrix® did not lose efficacy with increased participant age, with 96.6% efficacy in the 50-59 year-old age group, 97.4% in the 60-69s and 97.9% in the over 70s[338]. In comparison, the efficacy of Zostavax® declined sharply with increased age, with efficacy rates of 69.8%, 63.9% and 37.6% for the same corresponding age groups[238].

The 2-dose as opposed to 1-dose schedule was shown to be an important factor in the immunogenicity of Shingrix®, as there was a 3-fold increase in the generation of gE-specific $CD4^+$ T cells after 2 doses compared with a single dose[343]. The adjuvanted vaccine induced substantially higher immune responses compared to the unadjuvanted formulation, highlighting the importance of $AS01_B$ [343].

Immunological investigation into the effects of Shingrix® demonstrated robust $CD4^+$ but not $CD8^+$ T cell responses in the over 60s [343]. This supports previous data that $CD4^+$ T cells dominate the immune response to VZV[340,344–346]. The peak frequency of gE-specific $CD4^+$ T cells occurred at 3 months (i.e. 1 month after the second dose), and there was an approximately 50% decline from 3 to 12 months[343,347]. The drop in this period was the largest seen over the 6-year follow-up period. gE-specific $CD4^+$ T cell frequency continued to decline until 4 years after which a plateau was reached[347]. At 6 years the gE-specific $CD4^+$ T cell frequency was still 4-fold greater than pre-vaccination levels[347].

Similarly, peak anti-gE antibody levels were reached at 3 months, declining sharply by 12 months, and reaching a plateau by 24 months[347]. At month 72 the level of anti-gE antibody was 7.3 times higher than pre-vaccination levels[347]. The duration of immune responses to Shingrix® over at least several years corresponds to a clinical study demonstrating no significant loss of protection against shingles for 4 years post-vaccination[348]. A recent study that continued to follow up a subgroup of the original Shingrix® study participants demonstrated the frequency of gE-specific $CD4^+$ T cells and concentration of gE-specific antibody remained higher at 9 years compared to pre-vaccine levels regardless of age group; statistical modelling predicted immune responses would remain above baseline for up to 15 years after vaccination[349]. This compares favourably with Zostavax®, where immune responses above baseline could only be demonstrated for 8 years after vaccination[244].

Importantly, the T cell and antibody responses to Shingrix® were comparable between 60-69 year-olds and over-70s at all time points studied over 6 years[347]. These remarkable findings suggest that this vaccine in particular is able to overcome mechanisms of immunosenescence that dampen VZV-specific T cell responses in older age. The maintenance of immunogenicity and efficacy of Shingrix® in older adults confers a vital advantage over Zostavax®, as it is the

oldest people in society who are most vulnerable to shingles and its complications. A study directly comparing the immunogenicity of both vaccines is currently underway (NCT02114333) and its results are keenly awaited. A secondary advantage of Shingrix® is its suitability in immunocompromised patients in whom live vaccines are contra-indicated, such as organ transplant recipients, HIV patients, and those taking immunosuppressant drugs for inflammatory conditions[350].

The formidable immunogenicity of Shingrix® raises interest in the properties of its adjuvant $AS01_B$, which appears to activate both the innate and adaptive immune systems in a coordinated manner[351]. It is a liposome-based adjuvant that contains 2 immunostimulants: 3-*O*-desacyl–monophosphoryl lipid A (MPL) and QS-21. MPL is the detoxified derivative of lipopolysaccharide from *Salmonella minnesota*. It directly stimulates APCs via activation of TLR4, enhancing the production of cytokines and co-stimulatory molecules. QS-21 is a saponin molecule extracted from the bark of the South American tree Quillaja saponaria Molina. It promotes antigen-specific antibody responses but also stimulates cytotoxic $CD8^+$ T cells, primarily in animal studies[352,353].

Animal studies showed that after intramuscular injection, AS01 rapidly induced local cytokine production and innate immune cell recruitment to the muscle site. AS01 and vaccine antigen were rapidly cleared from the muscle, ruling out a depot effect, and were detectable in the draining LN within 30 minutes. In the LN AS01 increased the recruitment of monocytes and DCs, and enhanced their expression of the co-stimulatory molecules CD86 and CD40. Activated DCs in the LN were efficient and necessary for the effective priming of $CD4^+$ T cells. MSL and QS-21 acted synergistically to produce the highest antigen-specific response. Interestingly, AS01 induced production of IFN-γ by NK cells within 4 hours, and this appeared to be essential for the activation of DCs and development of Th1-type immunity[351–353].

The unique features separating AS01 from other adjuvants is the lack of depot effect, the robust increase of a diverse population of activated APCs, and the effective synergy between the two active constituents. The consequences are a potent early induction of the innate immune system, leading to strong activation of antigen-specific Th1 $CD4^+$ T cells and B cells capable of producing specific

antibodies[351,353]. Shingrix® serves as a model for highly effective vaccine design aimed at the ageing population.

5 BLOCKING P38 MAPK TO ENHANCE VZV-SPECIFIC IMMUNE RESPONSES IN AGEING SKIN

5.1 Chapter Introduction

We have seen that antigen-specific immunity declines in the skin of older people, and this may be due in part to an inhibitory microenvironment in this peripheral tissue in the old. We have also examined how the vaccine Zostavax® enhances the secondary immune response to VZV in the skin in a proportion of older people, although the clinical efficacy and immunological response to the vaccine decline significantly in the very old[238,239]. It is clear that vaccination strategies are currently suboptimal, highlighting the importance of investigating other approaches to boosting immunity in the old.

In this chapter we investigate a novel strategy to boost cutaneous immunity in the old by the therapeutic manipulation of the p38 mitogen-activated protein kinase (p38 MAPK) signalling pathway. This pathway was chosen on the basis of observations that human senescent T cells demonstrate spontaneous, increased activity of p38 MAPK, and blockade of this pathway restored proliferative potential and telomerase activity in these cells[55–57].

Interestingly, injection of a physiological 0.9% saline solution led to an overexpression in the skin of multiple pro-inflammatory genes and genes associated with myeloid cell activation in the old at 6 hours (compared to normal skin) but not in the young (Figure 5-1A). Pathway analysis found a significant association between these genes and predicted p38 MAPK activation. 24 of the top 30 overexpressed genes are induced by, or regulators of, the p38 MAPK signalling pathway (Figure 5-1B, asterisks). Many of the overexpressed genes at 6 hours after saline injection in the old were similar to the induced genes after VZV antigen challenge at the same time point, suggesting that the early transcriptional response to VZV in the old comprises a significant non-specific inflammatory component that may impair the antigen-specific immune response[143].

Additionally, the expression of 384 genes regarded as positive regulators of the immune response was evaluated in saline-injected skin at 6 hours in young and old individuals, and was used to generate an “inflammatory index” score for each person. When the inflammatory index was plotted against the clinical score in

response to VZV antigen (Figure 5-1C), a highly significant inverse correlation was found (p=0.0007)[143].

Skin biopsies taken from the site of saline-injected skin at 6 hours demonstrated an early transient influx of mononuclear phagocytes into old but not young skin, and these cells were no longer present at 24 hours. A similar transient increase was also observed in VZV-injected skin at 6 but not 24 hours[143].

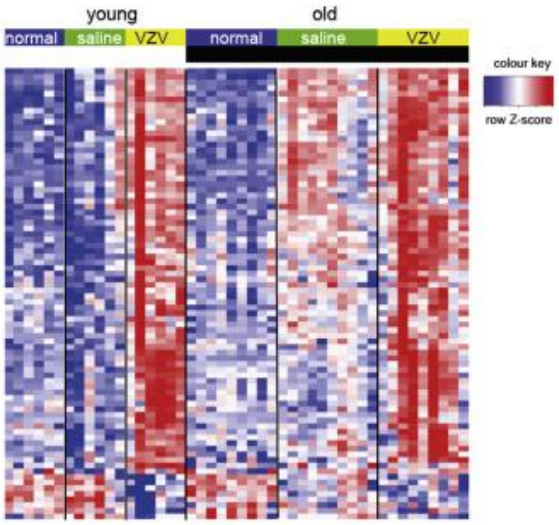
In summary from these experiments it appears that excessive sterile inflammation at an early time point in the old, associated with heightened activation of the p38 MAPK signalling pathway, is detrimental to generating a robust immune response to VZV antigen. This inflammatory response is likely to be driven by cells of the monocyte/macrophage lineage.

We therefore hypothesised that inhibition of p38 MAPK in the old would boost the antigen-specific immune response in the skin. To test this hypothesis, we measured the clinical and cellular response to VZV antigen in old individuals before and after treatment with the oral p38 MAPK inhibitor losmapimod for 4 days, in an experimental medicine trial. These individuals were selected for having a previously known poor clinical response to the VZV antigen.

5.2 Aims & Objectives

The aim of this chapter was to investigate whether pre-treatment of old individuals with losmapimod leads to an enhancement in the VZV-specific immune response in the skin.

A



B

	SYMBOL	FCH_Old Saline 6hrs vs Normal	FCH_Young Saline 6hrs vs Normal
*	FOSL1	51.5	21.63
*	FPR1	39.86	8.29
	PTX3	33.34	7.49
*	SERPINA1	24.27	1.04
*	IL6	23.67	4.03
	FCGR1A	23.29	5.47
*	MMP1	21.34	8.28
*	SELE	20.69	2.33
	FCGR1B	18.96	5.84
*	MMP3	18.63	22.16
*	CXCL1	18.01	4.51
*	CXCL8	17.67	4.09
*	IL1RL1	16.88	2.3
*	PROK2	15.75	5.61
	BCL2A1	14.06	1.88
*	CXCL2	13.86	2.49
*	IL1B	12.96	2.68
*	HAS3	11.94	18.49
*	S100A9	11.24	7.39
*	SELL	11.06	1.49
*	FCGR3B	10.9	2.44
*	NAMPT	10.74	2.56
*	CXCL10	9.85	-2.63
*	TNFAIP6	9.51	3.02
*	PTGS2	9.47	6.01
*	CH25H	9.44	2.36
	CYP27B1	8.81	6.12
*	CCL2	8.79	1.68
	ADAMTS8	8.42	1.66
*	THBS1	8.23	2.95

C

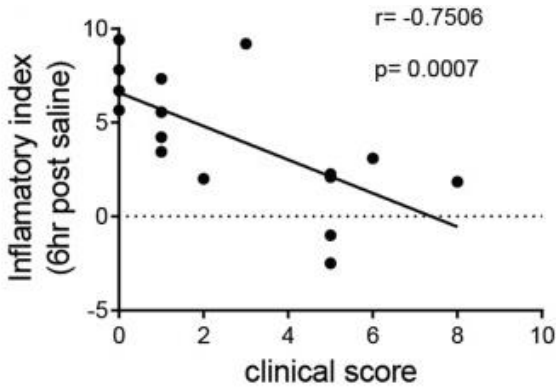


Figure 5-1 Comparison of global gene expression between normal, saline-injected, and VZV-injected skin

A: Heat map showing relative expression of DEGs (fold change > 2 and false discovery rate > 0.05) between normal skin, saline-injected skin and VZV-injected skin at 6 hours, in young (left) and old (right) individuals.
B: A table of the top 30 upregulated genes at 6 hours in saline-injected skin from old and young individuals compared with normal skin. Genes not reaching statistical significance are indicated in blue. Asterisks indicate genes related to p38 MAP kinase signalling.
C: An inflammatory index was calculated for each individual and plotted against VZV clinical scores at 72 hours (young n=6; old n=10). Regression line, R value and significance level are shown.
Akbar group data

5.3 Results

5.3.1 Losmapimod boosts the clinical response to VZV skin challenge

18 old volunteers (8 male and 10 female subjects; age range 65-77 years; median age, 69 years) were challenged with VZV skin test antigen before and immediately after losmapimod treatment (data shown in Table 5-1).

Donor	Age	Gender	Clinical score	
			Before losmapimod	After losmapimod
1	75	F	0	2
2	76	F	1	3
3	80	M	0	5
4	76	F	1	4
5	71	F	1	1
6	68	M	1	0
7	67	M	1	3
8	79	M	0	1
9	75	M	2	0
10	70	F	3	4
11	69	M	0	4
12	77	F	1	1
13	69	F	0	3
14	67	M	0	0
15	70	M	1	4
16	70	F	2	3
17	88	F	1	5
18	67	M	3	3

Table 5-1 Participant data for the losmapimod study

Overall there was a highly significant increase in the clinical score after treatment (mean score 2.6) compared to before treatment (mean score 1.0), as depicted in Figure 5-2 (p=0.0027).

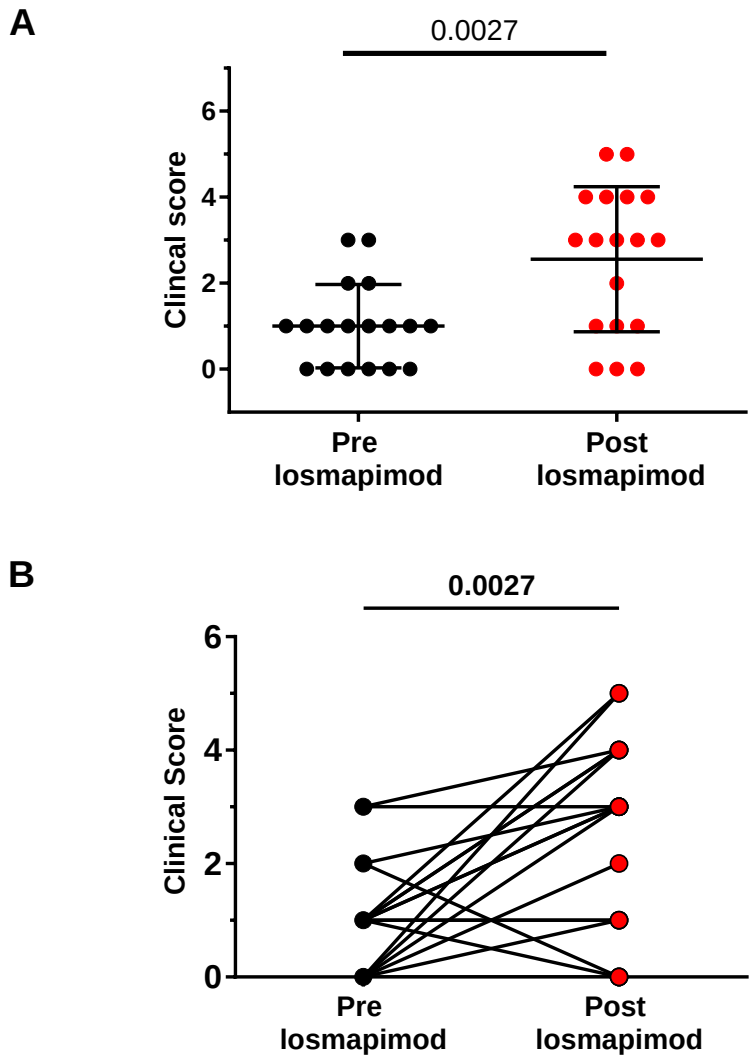


Figure 5-2 Effect of losmapimod on clinical response to VZV challenge

A & **B**: Graphs showing clinical scores before and after losmapimod treatment (n=18). The paired t test was used for analysis and p value is shown.

5.3.2 The effect of losmapimod on the CD4$^+$ T cell response to VZV skin challenge

5.3.2.1 Losmapimod boosted the CD4$^+$ T cell response in VZV-challenged skin

In a subset of individuals (n=7) taking part in the study, the mean numbers of perivascular CD4$^+$ T cells at Day 7 post-VZV skin challenge were enumerated by immunofluorescence microscopy, as described previously. Day 7 was chosen as this time point has previously been demonstrated to represent the peak in cellular response following VZV challenge. For analysis, individuals were grouped into those who had a good clinical response to VZV following treatment with losmapimod – defined by an increase in clinical score of at least 2 from baseline – and those who did not. These groups were termed “responders” and “non-responders” respectively. Losmapimod treatment was associated with a trend to an increase in CD4$^+$ T cell numbers in responders (Figure 5-3A; p=0.057), whereas there was no change in non-responders.

As there was some variation in the numbers of CD4$^+$ T cells pre-treatment between individuals, the mean increment in CD4$^+$ T cells was calculated (Figure 5-3B). The mean increment was significantly higher in responders (88.0) compared to non-responders (3.6); p=0.0067.

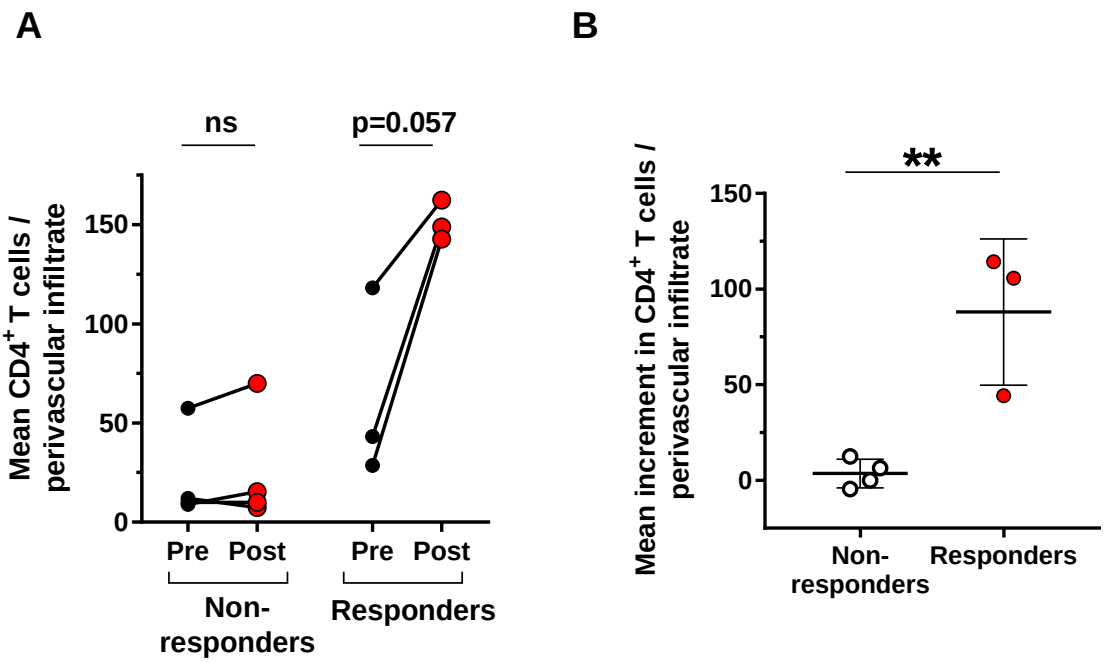


Figure 5-3 The effect of losmapimod on the CD4⁺ T cell response in VZV-challenged skin

A: Before-after graph showing the effect of losmapimod on the mean number of CD4⁺ T cells per perivascular infiltrate, separated into individuals who had a clinical response to the drug (responders, n=3) and those who did not (non-responders, n=4). The paired t test was used to compare the groups.
B: Graph showing the mean increment in CD4⁺ T cells per perivascular infiltrate, from before to after losmapimod treatment. Mean and standard deviations are shown; groups were compared using the unpaired t test.
Significance levels are shown.

5.3.2.2 The effect of losmapimod on CD4$^+$ T cell proliferation

The percentage of CD4$^+$ T cells staining positive for Ki67 was calculated to indicate the frequency of proliferating cells. There was a trend to increased proliferation after losmapimod in the responders (p=0.068), and there no change in the non-responders (Figure 5-4).

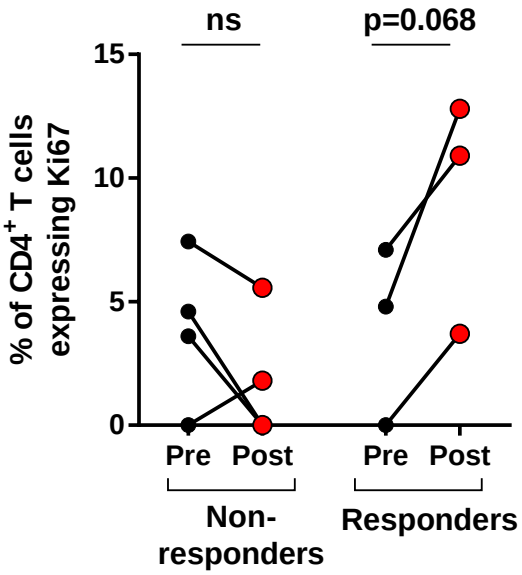


Figure 5-4 Before-after graph showing the effect of losmapimod on CD4$^+$ T cell proliferation

Data were analysed using the paired t test; significance levels are shown.

5.3.2.3 The boost in the CD4⁺ T cell response in VZV-challenged skin is correlated with the increase in clinical score

The mean increment of $CD4^+$ T cells after losmapimod was plotted against the change in clinical score for the non-responders and responders combined (Figure 5-5). Linear regression analysis demonstrated a significant positive correlation between the two variables (p=0.040).

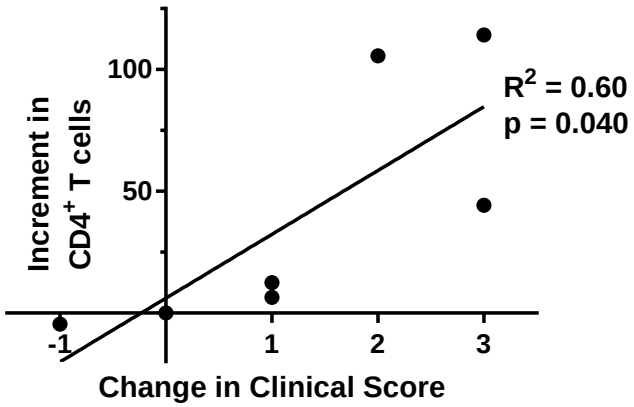


Figure 5-5 Change in clinical score plotted against mean increment in CD4⁺ T cells per infiltrate

Linear regression analysis is displayed; significance value and R-squared value are shown.

5.3.3 The effect of losmapimod on the CD8⁺ T cell response to VZV skin challenge

5.3.3.1 Losmapimod boosted the CD8⁺ T cell response in VZV-challenged skin

The CD8⁺ T cell response at the site of VZV antigen challenge was examined before and after treatment with losmapimod. Losmapimod treatment was associated with an increase in mean CD8⁺ T cell numbers (Figure 5-6A; p=0.047) in the responder group and there was no change in the non-responders. The mean increment in CD8⁺ T cells after treatment was significantly higher in responders (mean 37.6) compared to non-responders (mean -0.7), shown in Figure 5-6B (p=0.0034).

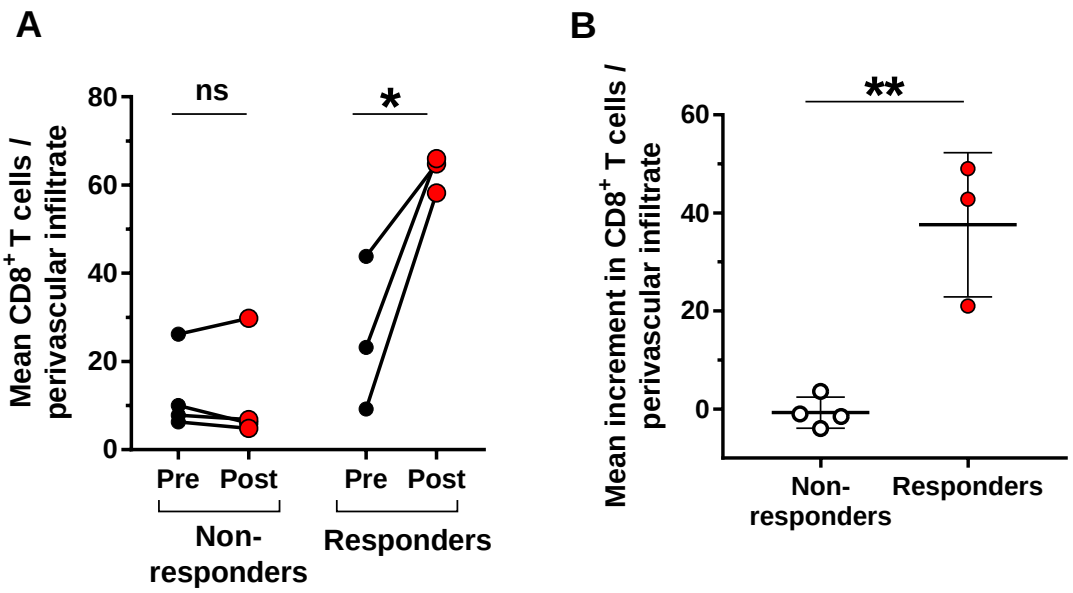


Figure 5-6 The effect of losmapimod on the CD8⁺ T cell response at the site of VZV antigen challenge

A: Before-after graph showing the effect of losmapimod on the mean number of CD8⁺ T cells per perivascular infiltrate, separated into individuals who had a clinical response to the drug (responders, n=3) and those who did not (non-responders, n=4). The paired t test was used to compare the groups.
B: Graph showing the mean increment in CD8⁺ T cells per perivascular infiltrate, from before to after losmapimod treatment. Mean and standard deviations are shown; groups were compared using the unpaired t test.
Significance levels are shown.

5.3.3.2 Losmapimod boosted CD8$^+$ T cell proliferation in VZV-challenged skin

The percentage of CD8$^+$ T cells that were proliferating was calculated (Figure 5-7). There was significantly increased CD8$^+$ T cell proliferation after losmapimod in the responders (p=0.0058), and there no change in the non-responders.

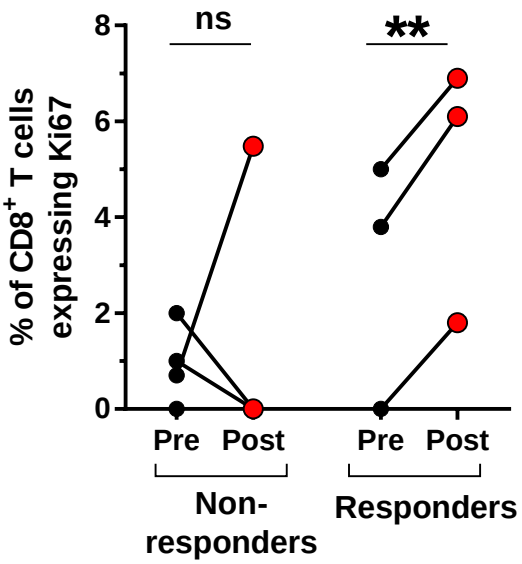


Figure 5-7 Before-after graph showing the effect of losmapimod on CD8$^+$ T cell proliferation

Data were analysed using the paired t test; significance levels are shown.

5.3.3.3 The boost in the CD8$^+$ T cell response in VZV-challenged skin correlated with the increase in clinical score

The mean increment of CD8$^+$ T cells after losmapimod treatment was plotted against the change in clinical score for the non-responders and responders combined (Figure 5-8), and there was a significant positive correlation between the two (p=0.029).

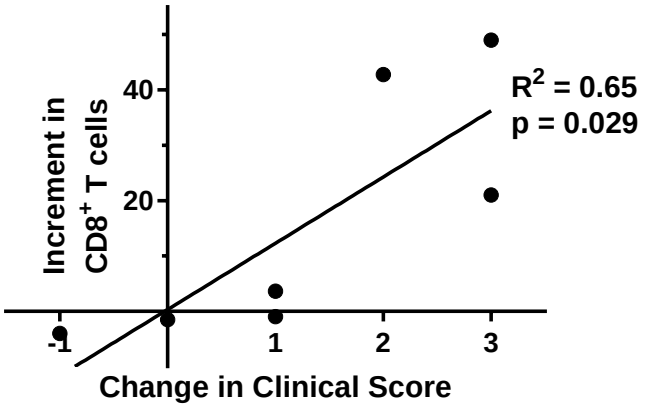


Figure 5-8 Change in clinical score plotted against mean increment in CD8$^+$ T cells per infiltrate

Linear regression analysis is displayed; significance value and R-squared value are shown.

5.4 Discussion

Pre-treatment with losmapimod led to a significant increase in the clinical response to VZV antigen. Contemporaneous experiments performed by the Akbar group showed that losmapimod treatment led to a reduction in serum C-reactive protein (CRP) levels and also decreased production of TNF-α, IL-6 and IL-8 by LPS-stimulated PBMCs. The increase in clinical score correlated with the reduction in CRP levels[143].

As shown in the Results section for this chapter, losmapimod treatment led to an enhanced $CD4^+$ and $CD8^+$ T cell response in the skin after VZV challenge, and these enhanced responses correlated with the boost in clinical score. There was also a significant increase in $CD8^+$ T cell proliferation in VZV-challenged skin after losmapimod. Together, these experiments have confirmed that blocking p38 MAPK-mediated inflammation in the old enabled the induction of a more robust VZV-specific immune response in the skin, at both the clinical and cellular level. The sample size presented here is small and will need to be extended to strengthen the findings.

The findings in this study are supported by a previous study in ageing individuals that demonstrated higher levels of systemic inflammation as measured by serum CRP were associated with poorer clinical DTH responses to Candida antigen[354]. It is perhaps counterintuitive that excessive early inflammation in the old prevents amplification of the VZV-specific immune response. The conventional dogma is that a robust innate immune response is an essential precursor to a strong antigen-specific T cell response; this is particularly apparent in vaccinology where the development of potent adjuvants for inducing a profuse inflammatory reaction is considered desirable. It is now proposed that excessive and dysregulated inflammation prevents innate immune cells from efficiently activating local endothelium early in the immune response, thereby preventing recruitment of antigen-specific T cells to the VZV-challenged site. It is proposed that blocking p38 MAPK reduces this excessive skin inflammation, facilitating the amplification of innate immune signals into a coordinated antigen-specific response – although the underlying mechanism for the immunosuppressive effect of exaggerated inflammation in this setting remains unclear.

Another previous study showed that pro-inflammatory macrophages from patients (mean age 69.9 years) with coronary artery disease, an ageing-related

disease, actively suppressed T cell activation and expansion, leading to impaired VZV-specific T cell immunity[355]. The mechanism involved was shown to be via the upregulation of PD-L1 on macrophages inhibiting PD-1$^+$ T cells[355]. It is also notable that macrophage chemokines, including MCP-1, are prominent components of the SASP, providing further evidence that macrophages are key cells involved in the age-related dysregulation of inflammation[64,356].

Several studies of ageing individuals have demonstrated an association between higher baseline levels of inflammation and poorer vaccine responses, for the shingles[357], influenza[358,359], hepatitis B[360] and yellow fever[361] vaccines. One could hypothesise, therefore, that suppressing baseline inflammation in old individuals before administering a vaccine would heighten the immune response to that vaccine[362].

This hypothesis is supported by a study in which the concomitant administration of oral aspirin alongside influenza vaccination in ageing individuals led to increased specific antibody responses compared to placebo given with vaccination[363]. Aspirin inhibits prostaglandin and thromboxane production through its irreversible inactivation of cyclooxygenase (COX). It has the advantage of being cheap and easily available, but this early study from 1994 has not been followed up by further trials investigating the use of this drug as an oral adjuvant. The use of paracetamol (mechanism of action unknown but does have weak anti-COX activity) before influenza vaccination[364,365], or indomethacin (COX inhibitor) before pneumococcal vaccination[366], in ageing individuals was not found to confer any boost in vaccine response. Further studies investigating the use of oral non-steroidal anti-inflammatory drugs (NSAIDs) as adjuvants prior to vaccination of the elderly are warranted.

In another study, inhibition of the mammalian target of rapamycin (mTOR) pathway using the mTOR inhibitor everolimus boosted the influenza vaccine response by 20% in elderly volunteers, and this was associated with a reduction in PD-1 expression by CD4$^+$ and CD8$^+$ T cells[367]. Aspirin[363] and everolimus[367] are therefore two potential anti-inflammatories that may boost vaccine responses in the ageing population, and it is possible that other drugs may also be used in this manner by targeting alternate inflammatory pathways.

We have now seen that the p38 MAPK pathway is a key player in the dysregulated early inflammatory response in ageing skin, and this raises the

possibility of the same pathway being implicated in the inflammaging of other compartments including the circulation. Having demonstrated that blockade of this pathway using losmapimod can enhance an antigen-specific response in the skin, the question now remains whether the same p38 MAPK-blocking strategy can be used to stimulate vaccine responses in older people. It is likely this will be investigated in further work.

Adjuvants are used in non-live vaccines to boost immune responses and can be highly effective in the right setting – an example includes the adjuvanted Shingrix® vaccine which was 91.3% effective in the over-70s[338,348]. Adjuvants modulate the innate immune response by providing danger signals to APCs and inducing a highly specialised inflammatory profile; this provides the conditions for generating a specific effector response that correlates with protection against the target disease[362,368]. In the context of inflammaging, it may be the case that inflammation induced by certain adjuvants may be counter-productive in some ageing individuals with a dysregulated early inflammatory response. A future strategy for vaccinating the elderly may be to establish a baseline inflammatory profile on an individual level, and tailor the adjuvant accordingly in a personalised approach.

6 GENERAL DISCUSSION

A deeper understanding of the mechanisms of immunosenescence in peripheral tissues is required to facilitate the targeting of specific components of the immune system when designing immune therapies for older people. In this work, immunosenescence of human skin was investigated by focusing on the role of a central player in barrier and tissue immunity – the tissue resident memory T cell. Further to this, two distinct therapies targeting the immune system were administered separately to human volunteers – a vaccine and an anti-inflammatory – to investigate whether they could enhance an in vivo antigen-specific response in the skin, and to explore the immune mechanisms involved in their mode of action.

6.1 Ageing and Cutaneous T_{RM}

Skin resident memory T cells, identified by their expression of CD69, did not diminish numerically in ageing skin. This is consistent with mice studies showing T cells in peripheral tissues remain numerically constant with increased age. T_{RM} are known to be long-lived, and so in the skin it is presumed their numbers are maintained through low-level homeostatic proliferation and this is likely to be independent of antigen[113]. Mechanisms for survival of T_{RM} are poorly understood, but some of the factors that have been suggested in promoting the long-term maintenance of T_{RM} include local availability of the cytokines TGF-β, IL-15, TNF-α and IL-33 [83,102], as well as a metabolic switch to free fatty acid oxidation[120] .

There was no age-related accumulation of terminally differentiated T_{EMRA} cells, and no loss in the ability to produce IL-2, providing some evidence that skin T_{RM} in the old were not of a senescent phenotype. Despite the increased PD-1 expression in skin $CD4^+$ and $CD8^+$ T cells in the old, these cells were fully functional in their ability to produce the pro-inflammatory cytokines IFN-γ and TNF-α, suggesting they were not functionally exhausted. Therefore, impaired skin immunity in the old does not appear to be due to defects occurring within the T_{RM} population, and other immune cell types in human skin require characterisation and investigation for age-related defects.

In addition to increased T cell PD-1 expression in the skin, the proportion of regulatory T cells in the skin was higher in the old, confirming the findings of previous work[135], and contributes to the impression that the skin is a tolerogenic

environment in the old. With ageing there is a loss of lipids from the stratum corneum in the skin, leading to a deterioration in skin barrier function[369]. Healthy skin is exposed to a multitude of environmental, commensal and pathogenic antigens, and the loss of barrier function in the old may therefore increase the antigenic load encountered by the skin immune system. The age-related transition to a more tolerogenic environment may therefore be a necessary adaptation to prevent allergy, autoimmunity, and exaggerated immune responses to microbes – and/or a response to the excessive and dysregulated inflammation seen when challenging the skin with a sterile or pathogen-derived antigen challenge, as discussed below. PD-1 inhibition or Treg depletion may be strategies to consider for boosting ageing immunity in the skin; however, this approach may carry an unacceptable risk of inducing a hyper-inflammatory skin environment.

6.2 Vaccination to boost cutaneous VZV-specific immunity – mechanisms

Vaccination with the live attenuated shingles vaccine Zostavax® is known to boost circulating VZV-specific $CD4^+$ T cells and VZV-specific antibody, but its immune effects on the skin – the site where shingles infection manifests – have not previously been studied. Here, a boost in VZV-specific $CD4^+$ T cells in the blood was confirmed after vaccination, but no parallel increase in the skin was observed. On VZV skin antigen challenge, vaccinated individuals had an enhanced clinical response as well as improved local $CD4^+$ and $CD8^+$ T cell responses – demonstrating the vaccine's ability to boost an in vivo VZV-specific immune response in the skin. Further work is required to confirm the vaccine's mechanism of protection, but the findings here suggest that VZV-specific memory T cells in circulation are critical in mediating immune protection against VZV reactivation, perhaps more so than VZV-specific T_{RM} in the skin. The absence of VZV-specific T_{RM} from sensory nerve ganglia latently infected with VZV implies that T_{RM} may not play an active role in suppressing VZV reactivation, and perhaps innate immune mechanisms take on this role. Dense $CD4^+$ and $CD8^+$ T cell infiltration of ganglia[196] and elevated circulating VZV-specific $CD4^+$ T cells[218] during acute shingles infection raises the possibility that VZV-specific T cells recruited from the blood are required for the control of already-reactivated VZV in the ganglia.

Zostavax® loses its ability to boost VZV-specific immunity in the very old, however, and the accumulation of defects in circulating VZV-specific memory T cells may account for this. These include the age-related reduction in the frequency of circulating VZV-specific $CD4^+$ T cells[186,321], narrowing of the TCR repertoire, increased PD-1 and CD57 expression on VZV-specific $CD8^+$ T cells[327], and loss of VZV-specific $CD4^+$ T cells following peak expansion after vaccination with an impairment in long-lived memory responses[241]. Contrastingly, Shingrix® is highly effective in older adults, and must therefore have the ability to circumvent some of these defects.

As there is now copious evidence for the protective role of T_{RM} in barrier tissues, some investigators have focused on using vaccines to enhance T_{RM} formation[315,370]. Mathematical modelling predicted that boosting $CD8^+$ T cells in human vaginal mucosa by 50% would achieve better control of HSV shedding for at least a year[314]. A strategy termed “prime and pull” has been developed, whereby parenteral vaccination is administered in combination with topical chemokine application, aiming to recruit activated effector T cells into a desired site and persist as local T_{RM} [313]. In humans the aim is that this strategy could be applied in some form to the development of vaccines for HSV and HIV[313]. In this work Zostavax® did not boost the VZV-specific T cell frequency in the skin, and this may be partly due to the subcutaneous route of vaccination, which bypasses the dense network of APCs in the dermis. The intradermal or skin scarification routes may have boosted VZV-specific T cells in the skin and this should be explored further. It is interesting to speculate that if an alternate vaccine could achieve such a boost, then perhaps this would confer an increased level of protection against VZV reactivation. Investigating the effect of the new shingles vaccine Shingrix® on skin VZV-specific T cell frequency remains an appealing prospect.

6.3 Dampening inflammation to enhance VZV-specific immunity in the skin

Increased p38 MAPK-associated inflammation in old skin at 6 hours after sterile saline injection was inversely correlated with the robustness of clinical response to VZV recall antigen challenge[143]. Here it was shown that experimental blockade of this pathway using the p38 MAPK inhibitor losmapimod enhanced clinical and cellular responses to VZV skin challenge, confirming that excessive early non-

specific inflammation impairs VZV-specific immune responses in the skin of old people. This novel finding adds to the existing evidence that inflammaging is detrimental to immunity in older adults. Transcriptional, flow cytometry and immunofluorescence data by lab colleagues implicated cells of myeloid lineage as the source of this excess inflammation in challenged skin, and further work is needed in characterising macrophages and dendritic cells in old compared to young human skin to further elucidate the mechanisms involved. Identifying the responsible cell type would facilitate more targeted therapies in boosting immunity in the old.

Human senescent T cells in circulation demonstrate spontaneous, increased activity of p38 MAPK, and blockade of this pathway restored proliferative potential and telomerase activity in these cells[55–57]. To extend the findings from this work, it is planned to employ p38 MAPK inhibition before administering the influenza vaccine to old individuals, with the intention of boosting influenza-specific immunity in an age group that is characterised by its typically poor immune responses to this intervention. Unfortunately, the use of losmapimod has not demonstrated clinical benefit in the treatment of a variety of medical disorders where inflammation plays a central role, including chronic obstructive pulmonary disease (COPD)[371], acute myocardial infarction[372], neuropathic pain from lumbosacral radiculopathy[373], and major depressive disorder[374]. These pathological disorders represent a distinct setting from vaccinating in order to reverse putatively physiological immunosenescence mechanisms, however, and so it is hoped that the use of this drug will be beneficial for the latter use.

7 FUTURE WORK

7.1 Further characterisation of T_{RM} and other skin resident immune cells during ageing

As skin T_{RM} represent a relatively newly discovered population, they remain incompletely characterised. Transcriptional analysis has been performed and phenotypic markers of differentiation and residency have been identified for skin T_{RM}; further characterisation in the context of ageing would include senescence markers such as KLRG1, CD57, telomere length, telomerase expression, γH2AX, and exhaustion markers such as CTLA4, LAG3, TIM3, Blimp1 and BIM. The ability of $CD4^+$ T_{RM} to produce effector cytokines has been investigated, but the ability of $CD8^+$ skin T_{RM} to produce cytotoxic proteins requires clarification.

As it has been shown that excessive early inflammation in challenged old skin is likely to be of myeloid cell origin, it would be interesting to investigate how macrophages and DCs in healthy skin change with regards to their phenotype, transcriptional analysis and function during the ageing process. This may yield clues as to why the change to a pro-inflammatory state occurs.

7.2 Effects of Shingrix® on cutaneous immunity

Shingrix® is clearly superior to Zostavax® at providing the elderly with long-lasting protection against shingles, and it will therefore likely supersede the latter in UK clinical practice in the near future. The reasons for the superiority of this adjuvanted non-live vaccine are unclear. It has been shown to induce long-lasting VZV-specific $CD4^+$ T cell memory in the circulation and this was reviewed in Section 4.4.10. It would be interesting to establish what effects this vaccine has on resident immune cell populations in the skin, the extent to which it can enhance the VZV-specific DTH response in the skin, and whether there is clear evidence of an advantage over Zostavax® in this regard. If there was evidence of an enhanced VZV-specific T_{RM} population in the skin, then this would confirm the validity of attempting to boost local T_{RM} in the barrier tissues to enhance immunity against pathogens invading via this route.

7.3 Implications for vaccination strategy in an ageing population

The advent of the highly effective Shingrix® is a major advance for vaccination strategy in the elderly. However, the currently recommended influenza and pneumococcus vaccines for the old are sub-optimal in their efficacy, and improved preparations would be welcome. Investigating the effect of adding $AS01_B$, or similar adjuvant systems, to these vaccines would be worthwhile. The following factors may be targeted in an attempt to achieve higher vaccine response rates:

- Vaccine Factors[223]
    - Higher dose of vaccine antigens[5]
    - Booster doses[375]
    - Considered selection of vaccine route eg. intradermal vaccination[375]
    - Vaccination at an earlier age
    - Adjuvants[5,351,375,376]
    - Viral vectors[5]
- Environmental factors[375]
    - Time of day of vaccine administration
    - Modifying psychological stress
    - Increased physical exercise

The $AS01_B$ adjuvanted preparation of Shingrix® was shown to be significantly more immunogenic than the unadjuvanted vaccine[343], and so it will be important to investigate the effects this adjuvant has on the ageing immune system, to explain its high efficacy in the old. $ASO1_B$ induces early (within hours) activation of the innate immune system[353], and perhaps this is at odds with the observations in this work that blocking innate-derived inflammation led to an enhanced VZV-specific response in old skin. It seems likely that multiple inflammatory pathways are involved in inducing a vaccine response; blocking excessive and dysregulated p38 MPAK-mediated inflammation may be as valid a strategy as stimulating detrimentally underactive inflammatory pathways with adjuvants. Thus, $AS01_B$ may mitigate dysregulated inflammation in the old and restore an

appropriate inflammatory response in these individuals. It would be of interest to investigate the efficacy of Shingrix® in a subset of individuals with high baseline inflammation, for example identified by biomarkers such as CRP, TNF-α and IL-6, or in individuals affected by a specific chronic inflammatory disease such as rheumatoid arthritis. Reduced efficacy in such individuals would strengthen the case for employing anti-inflammatory therapy as a therapeutic strategy for certain groups.

Increased baseline monocyte activation has been associated with poorer immune responses to the influenza vaccine[359]; the next challenge will be to investigate whether pre-treatment with losmapimod can boost influenza vaccination responses.

Due to the limitless variability between individuals' immune systems, future vaccination strategy may benefit from adopting an approach that is customised to the baseline immune profile of the individual. Employing a systems biology approach could identify biomarkers of vaccine efficacy and hyporesponsiveness, enabling targeted immune therapies at individuals who are prone to poor vaccine responses[351]. Examples of these may include:

- Counteracting inhibitory pathways[362]
    - Inhibitory cytokine blockade eg. anti-IL-10 and anti-TGF-β
    - Inhibitory receptor blockade[376] eg. anti-PD-1 and anti-CTLA-4
    - Inhibitory cell depletion[376] eg. Treg, myeloid derived suppressor cells (MDSC)
- Anti-inflammatory therapy[362]
    - Blockade of specific inflammatory pathways[376] eg. p38 MAPK inhibitors, COX inhibitors, rapamycin (targeting mTOR)
    - Metabolic anti-inflammatories eg. metformin, statins
    - Microbiome modulation[376]

More work is needed in characterising the ageing immune system and the detrimental role of inflammaging before the effectiveness of such targeted therapies can be demonstrated. Individualised strategies are likely to be expensive and time-consuming, however, and it may be some time before we see these methods reach clinical practice.

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