Investigating patterns of T cell differentiation in the blood and skin of patients with melanoma

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

Signed:

Abstract

Melanoma progression occurs despite evidence of melanoma-specific T cell activation. Chronic or repeated antigen stimulation can cause dysregulated T cell differentiation through upregulation of inhibitory receptors (immune exhaustion) or end-stage differentiation (immune senescence). This thesis therefore investigated the hypothesis that blood and skin derived T cells of melanoma patients are driven towards immune exhaustion and senescence. An increase in senescent $CD8^+$ T_{EMRA} cells was detected in the blood of old melanoma patients. These cells had high cytotoxic but low proliferative potential. Whilst it could not be determined whether they were melanoma specific, the T_{EMRA} expansions occurred independently from persistent viral infections such as CMV, and their function could be boosted through p38 signalling blockade. Skin resident T cells of melanoma patients showed no increase in T cell differentiation but instead upregulation of exhaustion markers PD-1 and CTLA-4. Granzyme B and perforin, essential for granule mediated cell killing, remained low in these cells, suggesting insufficient cytotoxic function. Skin derived T cells from healthy individuals also expressed high levels of PD-1 and low levels of cytotoxic granule components. Exposure to IL-2, IL-15 and CD3/CD28 boosted perforin and granzyme expression in healthy skin cells. Conversely, PD-1 signalling blockade during CD3 stimulation increased granzyme B expression. In summary, melanoma associated immune dysfunctions were of a different nature in blood and skin T cells. Immunotherapies designed to boost immune function in patients might therefore have different efficacies in both organs.

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Dedication

This work is dedicated to Anette, Walter and AJ.

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Abbreviations

AP-1	Activator protein 1
Blimp-1	B lymphocyte-induced maturation protein-1
BSA	Bovine Serum Albumin
CAD	Caspase-activated deoxyribonulcease
CFSE	5- (and 6-) carboxyfluorescein succinimidyl ester
CLA	Cutaneous leukocyte antigen
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAG	Diacylglycerol
EBV	Epstein-Barr Virus
Eomes	Eomesodermin
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
flow-FISH	Flow cytometric detection of fluorescence *in situ* hybridization
FoxP3	Forkhead box p3
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
ICAD	Inhibitor of CAD
IFN	Interferon
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
JNK	C-Jun-N-terminal kinase
kbp	Kilo base pairs

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
MHC	Major histocompability complex
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor κB
NLR	NOD-like receptor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD-1	Programmed cell death 1
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLC	Phospholipase C
PRR	Pattern recognition receptor
S1P	Sphingosine 1-phosphate
SEB	Staphylococcal Enterotoxin B
T-bet	T-box expressed in T cells
TAA	Tumour associated antigens
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
T_{EMRA}	Effector memory T cell reexpressing CD45RA
TGF	Transforming growth factor
TIM-3	T-cell immunoglobulin and mucin domain 3
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α

TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
T_{RM}	Tissue resident T cell
VLA-4	Very late antigen 4
VZV	Varicella-Zoster virus
WLE	Wide local excision
ZAP-70	Zeta-chain-associated protein kinase 70

1 Introduction

Melanoma is a cancer that arises from melanocytes. Although it can be easily cured in the early stages of disease through surgical removal, advanced disseminated melanoma often resists conventional therapies such as chemotherapy, resulting in high mortality. Despite being derived from self, melanoma can be recognized by the adaptive arm of the immune system and may lead to spontaneous regression (Quaglino et al. 2010). Unfortunately, in most melanoma patients tumour specific immune responses occur in the absence of disease amelioration, raising interest in identifying and understanding the role of T cell differentiation in the blood and skin of the patients affected.

1.1 T cell activation and effector functions

T cells constitute the cellular arm of the adaptive immune system that allows identification and killing of infected or malignant cells. The T cell receptor (TCR) recognizes antigen in form of a peptide presented on a major histocompability complex (MHC) molecule. The majority of cells in the body express MHC class I. MHC class II is expressed only by a few cell types which can act as antigen presenting cells that have specific immunological functions. $CD4^+$ T cells recognize antigen presented on MHC class II, and can differentiate into helper cells (TH1, Th2, Th17, Th22, follicular helper T cells) that can mediate downstream activation of an array of immune cells or regulatory T cells (Tregs) that suppress immune processes (Nakayamada et al. 2012). $CD8^+$ T cells recognize antigen presented on the MHC class I molecules and are able to differentiate into cytotoxic effector cells that can recognize and kill infected or malignant target cells.

1.1.1 T cell development

T cells arise from multipotent hematopoietic stem cells in the bone marrow, which migrate from the blood into the thymus where they proliferate, generating a large population of immature thymocytes. Thymocytes undergo gene rearrangement of the TCR, generating T cells of potentially any reactivity. Therefore, cells that cannot recognize the MHC molecule or are auto-reactive

must be removed**:** During positive selection, cells that are able to bind to MHC**:**self peptide presented by thymic stromal cells receive a signal to survive whilst cells that are not die by neglect. Depending on whether a cell recognizes MHC class I or class II, it will commit to the CD8 or CD4 lineage at this point. Thymocytes that survive positive selection migrate to the thymic medulla where they interact with the local MHC molecules. If they bind too strongly (i.e. are reactive to self), they will undergo apoptosis and die. This process is known as negative selection. Whilst the majority of thymocytes die during these selection processes, the cells that do survive emigrate from the thymus and mature into naïve cells once entering the blood stream. Thymic T cell selection and maturation therefore enable production of cells with intermediate reactivity, that are able to recognize MHC molecules and are potentially reactive against any pathogen or tumour antigen, without being reactive to self (Zippelius et al. 2002).

1.1.2 **T cell activation**

Naïve T cells circulate between peripheral lymphoid organs and sample MHC**:**peptide complexes presented on the surface of dendritic cells until finding their cognate antigen. Activation of naive $CD4^+$ and $CD8^+$ T cells is similar and can be divided into four phases: 1) activation 2) clonal expansion 3) contraction and 4) memory. However, activation of the innate arm of the immune system is a pre-requisite to trigger T cell activation. This includes professional antigen presenting cells, in particular dendritic cells.

1.1.2.1 **Activation of dendritic cells**

Dendritic cells are a diverse population of cells that can differ in lineage, phenotype, function and anatomical location. Dendritic cells sample antigen in peripheral tissues, which they then carry back to lymphoid organs to present to the T cells (Banchereau and Steinman 1998). In order to successfully activate T cells, immature dendritic cells themselves must become activated. This occurs through sensing of danger signals during antigen uptake, such as recognition of pathogen-associated molecular patterns (PAMPs) through conserved pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and NOD-like receptors (NLRs). Inflammatory cytokines such as Interleukin (IL)-1, GM-CSF and tumour necrosis factor (TNF)-α may also promote dendritic cell maturation

(Banchereau and Steinman 1998). These signals lead to dendritic cell migration by upregulation of lymph node homing receptors as well as maturation. Maturation involves increased antigen processing and upregulation of surface molecules involved in antigen presentation, including MHC, and of costimulatory molecules CD80 (B7.1) and CD86 (B7.2). Inhibitory signals such as IL-10 can block dendritic cell maturation (Buelens et al. 1997). Once migrated to the T cell areas of a lymphoid organ such as the lymph node, dendritic cells further release chemokines to attract T cells to sample their antigen. Only mature dendritic cells can mediate T cell activation, whilst immature dendritic cells promote immune tolerance.

1.1.2.2 **Migration of naïve T cells into the lymph nodes**

Naïve T cells circulate between the blood and the T cell areas of the secondary lymphoid organs in order to sample antigen presented by the local dendritic cells and maximize the possibility of meeting their respective antigen. Migration of the naïve T cells in this manner is facilitated by their expression of lymph node homing receptors. This includes CD62L, which binds GlyCAM-1 expressed on high endothelial venules and CCR7, a chemokine receptor that binds CCL19 and CCL21 produced by fibroblastic reticular cells and antigen presenting cells in the lymph node (Denton et al. 2014).

1.1.2.3 **T cell activation**

As they migrate through the cortical region of the lymph node, naïve T cells bind transiently to each antigen presenting cell they encounter with the help of adhesion molecules such as lymphocyte function-associated antigen 1 (LFA-1). When finally meeting its cognate antigen on the surface of a mature antigen presenting cell, the naïve T cells will become activated, divide and mature. It is essential that the naïve T cell obtains the appropriate signals for this to occur. These signals are commonly known as signal 1, 2 and 3.

Signal 1 involves the initial antigen-specific ligation of the TCR with the peptide:MHC complex on the surface of the antigen presenting cell. This interaction is highly specific and has a short half-life and therefore needs to be stabilized by co-receptors. This includes the CD3 complex that stabilizes the TCR on the cell surface and contains immunoreceptor tyrosine-based activation motif (ITAM) motifs that participate in the signal transduction. TCR binding to

MHC is also strengthened by the co-receptors CD4 or CD8, which bind conserved regions on MHC class II and class I respectively. CD4 and CD8 also recruit lymphocyte-specific protein tyrosine kinase (Lck) on their cytoplasmic tail into proximity of the CD3 complex. There, Lck can phosphorylate the CD3 ITAM motifs. This allows recruitment of zeta-chain-associated protein kinase (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 and cell growth (Smith-Garvin et al. 2009).

Signal 2 involves costimulatory receptors and ensures the survival of the activated T cell. The receptors involved generally belong either to the CD28 receptor family or the TNF receptor families and include CD28, CD27, CD40L, 4.1-BB and OX-40. The roles and functions of CD27 and CD28 will be discussed in detail in section 1.2. Costimulatory signals are important for proliferation, survival and differentiation. TCR activation in the absence of costimulation, in particular CD28, leads to low IL-2 production and to apoptosis or a hyporesponsive state known as clonal anergy (Powell et al. 1998). Signalling through inhibitory molecules such as programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) can counterbalance costimulation during T cell activation.

Signal 3 is mediated by cytokines produced by both, the antigen presenting cell as well as the stimulated T cell. IL-2 is the most important of these cytokines and IL-2 receptor signalling in the activated T cell allows cell cycle progression and differentiation. IL-2 is secreted by the activated T cell and acts in an autocrine fashion. Cells stimulated in the absence of IL-2 are therefore not able to proliferate and undergo cell death. Other cytokines can be produced by antigen presenting cells which may induce a variety of differentiation programs (depending on the specific combination of cytokines) in the stimulated T cells, determining $CD4^+$ T cell commitment to the various T helper lineages including Th1, Th2, Th17, Th22 and Treg (Boyman and Sprent 2012).

1.1.3 **Effector cell expansion and contraction**

The association between an activated T cell with the antigen presenting cell can last several days during which the T cell proliferates and the daughter cells also

bind to the dendritic cell. After 4-5 days of proliferation, naïve T cells therefore expand and differentiate into cells with specialized helper or cytotoxic functions. These short-lived effector cells then leave the secondary lymphoid organs to migrate to the sites of infection. These cells no longer require costimulation upon activation and express high levels of LFA-1 and very late antigen (VLA)-4, allowing binding to stressed endothelial cells in order to enter inflamed tissues. Experiments in mice have shown that recently activated or reactivated $CD8^+$ effector cells have the ability to enter most peripheral tissues, including those that are uninfected (Masopust et al. 2004). Following this initial expansion phase and disease resolution, the T cells enter the contraction phase, during which 90-95% of the cells die by apoptosis (Strasser and Pellegrini 2004). The remaining cells adopt a memory phenotype and persist in the circulation and tissues.

1.1.4 T cell effector functions

Cytotoxic T cells are antigen specific killers which can induce apoptosis in their target cells. T cell mediated target cell killing can be mediated in a receptor- or a cytotoxic granule-dependent manner.

1.1.4.1 Cytotoxic granules

Cytotoxic T cells readily carry pre-formed cytotoxic granule components in order to kill target cells quickly upon recognition through MHC-TCR ligation. Cytotoxic granules are formed from lysosomes and are surrounded by a lipid bilayer (de Saint Basile et al. 2010). They contain perforin, granzymes and granulysin, which are kept in an inactive state through a low pH (Ménager et al. 2007).

Upon release, perforin polymerizes and creates pores in the target cell membrane, enabling entry of other cytotoxic molecules into the cytoplasm of target cells (Browne et al. 1999; Pipkin et al. 2010). Perforin is thought to be essential for granzyme mediated killing, as perforin deficient mice have a severe defect in immune responses against the majority of viruses and are susceptible to spontaneous and chemically induced tumours (Kägi et al. 1994; Lowin et al. 1994; Broek et al. 1996). Similarly, polymorphisms in the perforin gene have been associated with melanoma development at young age (Trapani et al. 2013).

Granzymes are a family of serine proteases that has five members in humans: Granzyme A, B, H, K and M. The various granzymes are all thought to be involved in inducing apoptosis in the target cells by different mechanisms, but have redundant functions in immunity as mice lacking individual granzymes do not generally show more severe disease outcomes (Cullen et al. 2010). The role granzymes play in tumour rejection is therefore not well defined, as mice lacking these molecules did not have impaired immune responses to various transplanted tumours compared to wild type mice (Smyth et al., 2003).

Granzymes A and B are the most widely expressed and are also the most studied granzymes. Upon release into the target cell cytosol, granzyme B cleaves and activates caspase 3, which in turn activates a caspase proteolytic cascade that leads to the inactivation of ICAD (inhibitor of CAD), thereby releasing caspase-activated deoxyribonulcease (CAD), which finally degrades the target DNA. Granzyme B and caspase 3 also both cleave BID, triggering disruption of the outer mitochondrial membrane and the release of inner membrane proteins such as pro-apoptotic cytochrome c (Alimonti et al. 2001).

Granzymes can also be released into the extracellular space and can be expressed in the absence of perforin in a number of cell types including B cells and mast cells, suggesting additional roles of these proteins to killing. Indeed, granzymes can directly induce inflammation, by acting as a chemoattractant to other immune cells and by promoting the release of inflammatory cytokines. Granzymes can also cleave inflammatory cytokines such as IL-1b and IL-18 into more inflammatory forms (Boivin et al. 2009; Hiebert and Granville 2012).

Eomesodermin (eomes) and T-box expressed in T cells (T-bet) are known transcription factors involved in promoting a cytotoxic phenotype in T cells. Ectopic expression of eomes or Tbet were shown to induce perforin and granzyme B expression in Th2 cells that do not normally express these proteins (Pearce et al. 2003). Extracellular signals that promote *de novo* perforin and granzyme expression include TCR ligation and exposure to high levels of IL-2 and IL-15 (Janas et al. 2005; Salcedo et al. 1993; Leger-Ravet et al. 1994; Gamero et al. 1995). Conversely transforming growth factor (TGF)-β was shown to inhibit perforin and granzyme B expression in mice *in vivo* and in human T cell *in vitro* (Thomas and Massagué 2005; van Houdt et al. 2009)

Degranulation can be induced by TCR crosslinking and subsequent release of effector molecules must be tightly regulated in order to avoid bystander cell death. Indeed, cytotoxic granule mediated killing is highly focussed and involves the formation of an immunological synapse that encompasses integrins to stabilize T cell:target cell binding. The cytotoxic granules, anchored to microtubules, migrate towards the synapse and are released onto the target cell in a highly polarized manner (de Saint Basile et al. 2010).

1.1.4.2 **Death receptor mediated killing**

Other mechanisms exist that can induce target cell death but are granule exocytosis independent. Fas/FasL mediated killing for example involves ligation of effector cell FasL (CD95L) with Fas (CD95) on the target cell, leading to trimerization of the latter and the induction of caspase-dependent intracellular cell death pathways (Villa-Morales and Fernández-Piqueras 2012). Fas/FasL mediated induction of cell death is thought to be primarily involved in immune regulation, rather than tumour cell killing, as Fas deficiency in mice and humans leads to lymphoproliferative disorders and immunopathology (Griffith et al. 1995; Le Deist et al. 1996). However, FasL-mediated signalling can also induce T- and NK-cell induced tumour rejection in certain settings (Villa-Morales and Fernández-Piqueras 2012; Morales-Kastresana et al. 2013; Eberle et al. 2003; Liu et al. 2005).

TNF-related apoptosis-inducing ligand (TRAIL) is an alternative pathway of receptor mediated cell death. TRAIL can be excreted or membrane bound and induces trimerization of its receptors TRAILR-1 or -2 upon ligation, initiating a caspase-8 dependent signalling cascade that will induce apoptosis in the target cell. Like FasL, TRAIL mediated killing is involved in both, regulation of immune cells as well as tumour cell killing (Thomas and Hersey 1998; Dimberg et al. 2013).

1.2 Receptors controlling T cell behaviour

T cell activation is a complex process and its outcome depends on a balance of positive and negative signals. The function of each stimulatory and inhibitory molecule is strongly related to the timing and context of its ligation. Therefore, expression of each of these receptors and/or its ligand is tightly regulated and

dependent on the activation and differentiation status of the cell. The following section will examine in more detail the features of the costimulatory molecules CD27, CD28 and CD45 and the inhibitory receptors PD-1 and CTLA-4.

1.2.1 **CD28**

CD28 can act as costimulatory molecule upon binding its ligands CD80 (B7.1) and CD86 (B7.2) on the surface of antigen presenting cells. CD28 ligation during concomitant TCR stimulation recruits phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) to the TCR complex. PI3K converts the phosphoplid PIP_2 to PIP_3, allowing recruitment of phospholipase C (PLC)γ to the plasma membrane, which converts PIP_2 into diacylglycerol (DAG) and IP3. DAG in turn initiates downstream NFκB signalling, whilst IP3 induces downstream calcium release and subsequent NFAT pathway activation. PIP3 also allows recruitment of Akt (Rudd et al. 2009).

CD28 signalling therefore decreases the threshold for TCR activation, induces entry into the G1 phase of the cells cycle and promotes survival via upregulation of Bcl-xL (Boise et al. 1995). Further, CD28 signalling promotes IL-2 production (at both protein and mRNA level) and causes upregulation of the high affinity IL-2 receptor CD25 (Powell et al. 1998). CD28 knock-out mice showed a reduction in certain T cell functions such as induction of B cell class switching but were able to generate cytotoxic T cells (Shahinian et al. 1993).

CD28 can be detected on naïve cells and certain memory T cells, but is lost with differentiation. Indeed, long term stimulation with allogeneic cells or IL-2 causes CD28 downregulation *in vitro* (Effros et al. 1994; Borthwick et al. 2000). The frequency of CD28 expressing cells declines with age in humans (Effros et al. 1994; Boucher et al. 1998).

1.2.2 **CD27**

CD27 belongs to the TNFR superfamily and acts as a costimulatory molecule to enhance T cell function upon binding to its unique ligand CD70 during TCR activation. Ligation induces CD27 trimerization, engaging TNFR-associated factor 2 (TRAF2) and TRAF5 to its cytoplasmic tail and inducing downstream NFκB and c-Jun-N-terminal kinase (JNK)-signaling cascades. CD27 signalling therefore promotes survival and enhances proliferation, thereby leading to increased effector functions such as cytotoxicity and promoting memory

formation (Duttagupta et al., 2009). CD27 deficient mice develop similarly to wild-type mice, but show a reduction in cell numbers during primary and recall responses (Hendriks et al. 2000). Conversely, mice overexpressing CD70 show increased effector functions against certain pathogens but also display B cell depletion due to high levels of T cell derived interferon (IFN)-γ, leading to the animals becoming immunocompromised (Arens et al. 2004).

CD27 is expressed by naïve and early-differentiated memory T cells and is further upregulated upon CD3-mediated stimulation (Hintzen et al. 1993). However, after prolonged activation *in vitro* and *in vivo*, CD27 expression is gradually reduced and eventually lost irreversibly. CD70 expression is highly regulated and normally only occurs transiently in T cells, B cells, NK cells and dendritic cells upon activation (Nolte et al. 2009).

Constitutive signalling through CD27 during CD70 overexpression in T cells has also been shown to lead to an exhaustion-like phenotype in $CD8^+$ but not $CD4^+$ T cells, by causing reduced IL-2 and TNFα production and increased expression of PD-1, IL-10 and B lymphocyte-induced maturation protein-1 (Blimp-1) (van Gisbergen et al. 2009). CD27 signalling in the tumour setting can be both beneficial and detrimental to anti-tumour immune responses, depending on the context. Studies involving injection of tumour cell lines into mice have shown that CD27 signalling can mediate anti-tumour immunity by promoting effector T cell function (Arens et al. 2004; Roberts et al. 2010). However, CD27 signalling in mice with solid transplanted tumours was shown to be detrimental to the host by promoting Treg development and therefore immunosuppression (Claus et al. 2012). CD27 signalling therefore has a variety of outcomes, which may depend on the context and duration of CD70 ligation.

1.2.3 **CD45**

CD45 (lymphocyte common antigen) is a transmembrane receptor that is expressed on all haematopoietic cells and contains a protein tyrosine phosphatase on its cytoplasmic tail. The ligand for CD45 has not been identified to date. A number of CD45 isoforms exist that are generated through alternative splicing and differ in the length of their extracellular domain. On T cells, high molecular weight CD45RA was originally attributed to naïve T cells, whereas low molecular weight CD45RO was associated with antigen experienced cells

(Akbar et al. 1988). Subsequently, a population of antigen experienced and highly differentiated cells that do re-express CD45RA on their surface has also been identified (Hamann et al. 1997). However, the exact role of CD45 isotypes in T cell differentiation has not yet been established.

CD45 modulates early T cell signalling during antigen stimulation by removing an inhibitory phosphate group from the Src-family kinase Lck. Activated Lck in turn may phosphorylate the ITAMs on the CD3 complex, enabling further recruitment of components involved in CD3 signalling (Hermiston et al. 2003). Being the smallest CD45 isoform, CD45RO more readily associates with the T cell receptor and enables signal transduction than CD45RA. T cell development is heavily impaired in CD45 deficient mice (Byth et al. 1996).

1.2.4 **PD-1**

Programmed death-1 (PD-1; CD279) is an inhibitory cell surface receptor homologous to CD28. It is comprised of an extracellular Immunoglobulin-like domain and two tyrosine-based signalling motifs in the intracellular tail (Sheppard et al. 2004). Engagement of PD-1 to its ligands inhibits T-cell activation and induces peripheral tolerance.

The majority of T cells in the circulation do not express PD-1, but can be induced to do so upon TCR-mediated activation or exposure to cytokines such as IL-2, IL-7, IL-15, IL-21 and TGF-β (Agata et al. 1996; Kinter et al. 2008). Some T cell populations constitutively express PD-1, such as follicular helper T cells (Sage et al. 2013).

PDL-1 (B7-H1; CD274) and PDL-2 (B7-DC; CD273) are the ligands to PD-1 and are differentially expressed on a variety of cells in the circulation: PD-L1 is constitutively expressed by a number of haematopoietic cells including T cells and antigen presenting cells and is expressed in various lymphoid and non-lymphoid tissues (Freeman et al. 2000; Brown et al. 2003; Peña-Cruz et al. 2010; Bankey et al. 2010). PDL-2 expression is thought to be restricted to dendritic cells and macrophages (Freeman et al. 2006). IFNγ in particular causes PDL-1 and PDL-2 upregulation in various cell types (Brown et al. 2003).

The tyrosine residues in the cytoplasmic tail of PD-1 mark the presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) motif (Chemnitz et al. 2004). Upon PD-1

engagement with its ligands, the tyrosine residues in the cytoplasmic tail are phosphorylated, recruiting Src homology 2-domain-containing tyrosine phosphatase 2 (SHP-2) and SHP-1 (Chemnitz et al. 2004). SHP-1 and SHP-2 in turn lead to the dephosphorylation of TCR proximal signalling components, thereby inhibiting certain TCR/CD28-mediated signals (Parry et al. 2005; Patsoukis et al. 2012). Because PD-1 transduces a signal when engaged in combination with T cell receptor (TCR) ligation only, the extent of PD-1 mediated inhibition is dependent on the strength of the stimulation and will have the greatest impact under conditions of relatively weak stimulation (Freeman et al. 2000; Latchman et al. 2001; Brown et al. 2003).

Both CD28 and IL-2 promote T cell proliferation and cell cycle progression, survival and cytokine expression and PD-1 exerts its effects directly by inhibiting early activation events induced by CD28 or indirectly through undermining production of IL-2 (Carter et al. 2002). Therefore, PD-1 signalling undermines TCR-signalling mediated upregulation of cytokines such as IL-2, IFNγ and TNFα, cell cycle progression, upregulation of the survival gene Bcl-xL and leads to a reduced expression of transcription factors involved in effector functions such as Tbet and Eomes (Chemnitz et al. 2004; Patsoukis et al. 2012; Nurieva et al. 2006).

Originally discovered on murine T cell hybridoma and thought to be involved in cell death (Ishida et al. 1992), it is now clear that the major function of PD-1 involves inhibitory signalling and immune regulation. This is supported by direct and indirect evidence in mice deficient in PD-1 displaying a breakdown in peripheral tolerance and exacerbated features of autoimmunity (Nishimura et al. 1999; Salama et al. 2003) and humans where genetic polymorphisms in the PD-1 locus have been linked to various autoimmune diseases (Nielsen et al. 2003; Velázquez-Cruz et al. 2007).

PD-1 mediated signalling is not only important in mediating tolerance to self, but also in preventing exacerbated immune responses. During acute infection PD-1 is upregulated on activated T cells, but is not necessarily associated with impaired immune responses to the pathogen (Erickson et al. 2012; Phares et al. 2010). PD-1 is also constitutively upregulated by T cells through chronic antigen exposure during persistent disease. This mechanism may prevent further immunopathology during chronic antigen stimulation, but may also cause

immune dysfunction, and lead the T cells to adopt a state known as exhaustion (Speiser et al. 2014). This may occur in chronic viral infections as well as cancer. T cell exhaustion will be discussed in more detail in section 1.4.4.

PD-1 ligation may also suppress T cell responses to cancer. The inhibitory receptor can be upregulated on tumour infiltrating lymphocytes as well as tumour specific T cells in the circulation (Ahmadzadeh et al. 2009; Baitsch et al. 2011; Chapon et al. 2011). Expression of PD-1 in these cells was associated with decreased T cell function in humans and mice (Saito et al. 2013; Curran et al. 2010). PDL-1 can be expressed on tumour cells and correlated with poor prognosis in certain cancers, but not melanoma metastases (Hamanishi et al. 2007; Inman et al. 2007; Ahmadzadeh et al. 2009; Gadiot et al. 2011).

1.2.5 **CTLA-4 (CD152)**

CTLA-4 is a B7/CD28 family member that inhibits T cell function. Like CD28, CTLA-4 binds CD80 and CD86, but does so with higher affinity (Rudd et al. 2009). CTLA-4 is commonly expressed by Tregs and can be induced in other T cell subsets upon activation (Chan et al. 2014). In resting cells CTLA-4 is located in intracellular vesicles. Upon activation, it is transiently expressed in the immunological synapse before being rapidly endocytosed (Leung et al. 1995).

CTLA-4 can regulate T cells intrinsically or in a bystander-cell mediated fashion through a number of mechanisms (Wing et al. 2008). CTLA-4 is in direct competition with CD28 for their common ligands CD80 and CD86. Because CTLA-4 binds these with greater affinity, CD28 signalling is reduced in the presence of the inhibitory receptor. CTLA-4 has also been shown to oppose CD28 signalling by removing CD80 and CD86 (including their cytoplasmic domain) from the antigen presenting cell via trans-endocytosis (Qureshi et al. 2011). The cytoplasmic tail of CTLA-4 does not contain a classic ITIM motif, but can recruit the phosphatases SHP-2 and PP2A (Marengère et al. 1996; Chuang et al. 2000), which may interfere with TCR signalling, although the exact mechanism for this remains to be elucidated (Parry et al. 2005).CTLA-4 therefore works by opposing CD28 and TCR-mediated signalling during activation, leading to reduced expression of IL-2 and cell cycle arrest.

CTLA-4 signalling increases the activation threshold during T cell activation, thereby reducing the pathogenicity of effector T cells. CTLA-4 therefore plays a crucial role in immunological tolerance. This has been highlighted in mice lacking CTLA-4 globally or selectively in the FoxP3$^+$ Treg compartment, which develop a lymphoproliferative disorder and die at young age (Waterhouse et al. 1995; Wing et al. 2008). Similarly, polymorphisms within the CTLA-4 gene have been associated with autoimmune diseases in humans (Gough et al. 2005). CTLA-4 signalling has also been implicated with reduced immune responses during disease in infection and tumour settings. This was particularly noted in the context of immune exhaustion, discussed in more detail in section 1.4.4..

1.3 T Cell Memory

The generation of immunological memory is the hallmark of the adaptive immune response. Memory cells are more sensitive to antigen and are generally found in higher frequencies than their naïve precursors, allowing more rapid proliferation and effector functions upon antigen re-encounter. Memory T cells are not a uniform population of cells but are generally thought to come in three distinct classes: central memory (T_{CM}), effector memory (T_{EM}) and effector memory cells that re-express CD45RA (T_{EMRA}), which can be distinguished by their homing, proliferative and functional properties (See Fig Table 1-1, Sallusto et al. 1999; Faint et al. 2001). This is true for both, CD4$^+$ and CD8$^+$ T cells.

1.3.1 Markers used to identify subpopulations

Various markers and combinations of markers have been used in literature to define memory T cell subsets and there is currently no consensus on which is the most appropriate (Appay et al. 2008). The markers most commonly used for this purpose are CD45RA in combination with CD62L, CCR7 or CD27, although there is generally high consistency in the expression patterns of these markers in the subsets, meaning differently defined populations using these markers are comparable (Sallusto et al. 1999; Appay et al. 2008).

In this work, the combination of CD45RA and CD27 was used to define naïve, T_{CM}, T_{EM} and T_{EMRA} subsets, as CD27 expression was found to generate more discrete populations than CCR7 or CD62L and less likely to be re-expressed. Using these markers is also in accordance with previous work published by our

group and others (Hamann et al. 1997; Di Mitri et al. 2011; Henson et al. 2014). It should be noted that the identification of memory differentiation subpopulations based on surface receptor expression applies to resting cells only, as markers can be altered upon T cell activation. T_{EM} cells for example have been shown to upregulate the T_{CM} marker CD62L after stimulation, although it should be noted that these cells did not display equal proliferative functions compared to cells that expressed CD62L prior to stimulation (Sallusto et al., 2004).

Other marker combinations used to identify T cells of various differentiation stages are coexpression of CD27 and CD28 with double expressing cells being the least differentiated and double negative cells being the most differentiated (Henson et al. 2009). Alternatively, loss of CD28 or expression of CD57 or KLRG1 by themselves have been used to identify highly differentiated cells (Brenchley et al. 2003; Voehringer et al. 2002; Speiser et al. 1999).

Research in identification and characterization of T cell subsets has largely been based on blood derived T cells. Whether the $T_{CM}/T_{EM}/T_{EMRA}$ dogma can be applied to tissue resident T cells remains to be established.

		Naïve	T_{CM}	T_{EM}	T_{EMRA}
Receptors associated with homing	CCR7	+	+	-	-
	CD62L	+	+	-	-
	CLA	-	+	+	-
	CCR4	-	+	+	-
	CXCR1	-	-	+/-	+
Stimulatory and Inhibitory coreceptors	CD27	+	+	+/-	-
	CD28	+	+	+/-	-
	CD45RA	+	-	-	+
	CD45RO	-	+	+	-
	PD-1	-	+/-	+/-	-
	KLRG1	-	-	+	+
Cytokines and cytotoxic effector molecules	IL-2	-	+++	++	+
	TNFα	-	+	+++	+++
	IFNγ	-	+	+++	+++
	Granzyme B	-	+	++	+++
	Perforin	-	+	++	+++
Senescence and differentiation associated features	Telomere length	+++	++	+	++
	Proliferative capacity	+++	+++	++	+
	γH2AX	-	-	+/-	+
	CD57	-	-	+/-	+

Table 1-1: Phenotypic properties of T cell subsets

1.3.2 **Central memory T cells (T_{CM})**

T_{CM} are named after their homing pattern and express distinct surface receptors which cannot be found on effector memory cells (T_{EM}). This includes CD62L (L-selectin) and CCR7, which are necessary for migration to the T cell areas of secondary lymphoid organs, such as the lymph nodes or mucosal lymphoid organs (e.g. tonsils) (Sallusto et al. 1999). T_{CM} express high levels of the costimulatory molecules CD27 and CD28, which bind ligands generally found on mature dendritic cells. Upon activation, T_{CM} express high levels of CD40L, which can in turn stimulate dendritic cells, B cells and macrophages and provide $CD8^+$ T cell help (Sallusto et al. 1999).

Activated T_{CM} have little or no immediate effector functions and do not produce many cytokines apart from IL-2. However, they are able to proliferate extensively and produce effector cytokines much later as they lose CCR7 and differentiate into effector cells that produce large amounts of IFN-γ or IL-4 (de Jong et al. 1992; Sallusto et al. 1999). T_{CM} are therefore considered by some researchers to have memory stem-cell like attributes such as high proliferative capacity and high IL-2 production that can provide long term memory and contribute to the T_{EM} cell pool that mediates effector functions (Lanzavecchia and Sallusto 2005).

1.3.3 **Effector memory T cells (T_{EM})**

T_{EM} have lost the expression of lymphoid homing receptors such as CCR7 and may instead express homing receptors that facilitate migration to nonlymphoid tissues, particularly during inflammation. This includes high expression of integrins such as LFA-1 and inflammatory chemokine receptors including CCR1, CCR3, CCR5 and CXCR3, as well as the tissue homing receptors cutaneous leukocyte antigen (CLA) and CD103 (Sallusto et al. 1999; Willinger et al. 2005).

T_{EM} have a low activation threshold to CD3 stimulation and express low levels of the costimulatory receptors CD27 and CD28. At potential sites of inflammation, T_{EM} can produce IFN-γ, TNFα, IL-4 and IL-5 within hours of TCR restimulation and readily release prestored effector molecules such as perforin and granzyme A, B and H (Sallusto et al. 1999; Willinger et al. 2005). T_{EM} can also express high levels of TRAIL and FasL, involved in perforin independent

killing (Willinger et al. 2005). Consistent with expression of these molecules, T_{EM} express high levels of the transcription factors involved in effector T cell functions, such as T-bet and eomes (McLane et al. 2013). Compared to T_{CM}, T_{EM} have decreased proliferative function but markedly increased effector capacities, allowing them to mediate immediate immune protection upon challenge (Sallusto et al. 1999).

1.3.4 Effector memory T cells re-expressing CD45RA (T_{EMRA})

A third class of memory cells have a greatly reduced proliferative potential compared to the other subsets and accumulates with age (Koch et al. 2008). Similarly to the T_{EM}, these cells express particularly large amounts of effector cytokines, cytotoxic granule components and receptors that allow migration to inflammatory sites, whilst lacking the costimulatory molecules CD27 and CD28 (Hamann et al. 1997; Faint et al. 2001; Willinger et al. 2005). These cells are known as T_{EMRA}, as they can be distinguished from T_{EM} through the expression of CD45RA, a marker normally found on naïve T cells only (Faint et al. 2001).

T_{EMRA} are thought to be the most differentiated of all T cell subpopulations, as they have the highest levels of DNA damage associated marker γH2AX and lowest levels of telomerase activity, although it should be noted that their telomeres are longer than in T_{EM} (Di Mitri et al. 2011). T_{EMRA} also express the highest levels of the senescence associated receptors CD57 and KLRG1 (Di Mitri et al. 2011; Libri et al. 2011). The senescent nature of T_{EMRA} cells will be discussed in more detail later (section 1.4.1). T_{EMRA} cells also express high levels of inhibitory receptors commonly associated with NK cells, such as CD94, NKG2A, CD158a and NKB-1 (Baars et al. 2000).

T_{EMRA} are though to be the most differentiated of all the memory subsets and whilst they have very low proliferative function, their effector-like nature allows them to rapidly kill target cells upon challenge.

1.3.5 Lineage relationship and differentiation of T cell subsets

Transfer models in mice have shown that a single naïve T cell is multipotent and can give rise to any memory subset (Stemberger et al. 2007; Gerlach et al. 2010). It is generally accepted that T_{CM} are the least differentiated out of all the memory T cell subsets, whilst T_{EMRA} have reached an end stage (Di Mitri et al. 2011; Willinger et al. 2005). However, the exact generation and lineage

relationships between these cells are still a matter of debate. Certain models propose a linear differentiation from naïve→ T_{CM}→ T_{EM}→T_{EMRA}, determined by repetitive stimulation or signal strength. Alternatively, asymmetric division may generate T_{CM}, T_{EM} or T_{EMRA} independently from each other (Chang et al. 2007; Kaech and Cui 2012).

1.3.6 **Antigen specificity of T cell subsets**

Cells specific against various pathogens and of different clonotypes can be preferentially driven towards certain memory phenotypes rather than others and this can vary with anatomical location of the cells and period of exposure to the pathogen. Influenza specific T cells for example preferably adopt a T_{CM}-like phenotype ($CD62L^{hi}$) (Kedzierska et al. 2006). In Epstein-Barr virus (EBV) positive individuals, $CD8^+$ T cells specific against latent antigens show a T_{CM}-like $CD45RO^+CD28^+$ phenotype, whilst T cells against lytic epitopes were heterogenous but contained T_{EMRA}-like ($CD45RA^+CD28^-$) cells (Hislop et al. 2002). Cytomegalovirus (CMV) specific cells typically are skewed towards a T_{EM} or T_{EMRA} phenotype (Griffiths et al. 2013). Indeed, the TCR repertoires of circulating human $CD8^+$ T_{CM} and T_{EM} are largely distinct (Baron et al. 2003).

1.3.7 **Maintenance of memory**

All T cell memory subsets are stable T cell populations that can persist in the absence of antigen for a lifetime. Maintenance of memory T cells in the absence of antigen stimulation is a dynamic process during which the cells proliferate and die. Radioactive isotope labelled $CD4^+$ T cells were followed up in human volunteers *in vivo* and displayed an estimated doubling time of 1 year for naïve T cells, 48 days for T_{CM} and 15 days in T_{EM} cells (Macallan et al. 2004). Simultaneously, T_{EM} also displayed the highest disappearance rates, suggesting that homeostasis is maintained through occasional cell death as well as proliferation of the surviving cells (Macallan et al. 2004).

Long-term persistence of the memory cells is dependent on homeostatic proliferation driven by common γ-chain cytokines such as IL-7 and IL-15, which also promote survival (Sprent et al. 2008). Indeed, T_{EMRA} are thought to be predominantly generated by cytokine exposure rather than by antigen-dependent stimulation (Geginat, Lanzavecchia, and Sallusto 2003). Excess cells (generated during homeostatic proliferation or during expansion following

antigenic stimulation) undergo apoptosis due to cytokine deprivation or programmed cell death (Jameson 2002; Prlic and Bevan 2008). These cells display “eat-me” signals and are cleared by phagocytic cells such as macrophages or immature dendritic cells in an anti-inflammatory manner (W. Chen et al. 2001; Savill et al. 2002).

1.3.8 Tissue resident T cells

Various studies in the past have analysed cells in peripheral tissues and classified them into the three classic subsets described above based on the detection of CD45RA and CCR7 (Clark, Chong, Mirchandani, Brinster, et al. 2006; Sathaliyawala et al. 2013). However, it has become apparent that T cells in the skin, blood, lung and many other peripheral organs do in fact comprise an additional independent memory subset, as the cells differ in phenotypes and functions from their circulating counterparts. These cells are therefore now termed tissue-resident memory cells, or T_{RM} (Jiang et al. 2012; Purwar et al. 2011; Zhang and Bevan 2013).

T_{RM} are thought to act as immune sentinels in the peripheral tissues, as they are able to not only kill target cells but also secrete proinflammatory cytokines and chemokines to attract other immune cells. Indeed, T_{RM} have been shown to confer increased protection upon pathogen re-encounter and their efficacy has been directly linked to the numbers of pathogen-specific cells before re-infection (Liang et al. 1994). The nature of T_{RM} with a focus on skin resident T_{RM} will be discussed in more detail in section 1.5.3.

1.3.9 $CD4^+$ $FoxP3^+$ Tregs

Forkhead box p3 (Foxp3) positive regulatory T cells (Tregs) are a suppressive subpopulation of $CD4^+$ T cells that control immune responses to self and foreign antigen. They can be induced in the thymus (natural Tregs) as well as in the periphery (induced Tregs) and are characterized by expression of the high affinity IL-2 receptor CD25 in absence of the IL-7 receptor CD127 (Sakaguchi et al. 2008). Further, circulating FoxP3 Tregs express CD27 and are heterogenous for CD45, placing these cells either into the naïve or the T_{CM} compartments (Booth et al. 2010; Shen et al. 2011). Tregs can be detected in both, the circulation and peripheral organs where they ensure immune tolerance and control excessive immune reactions (Akbar et al. 2007; Fulton et al. 2010).

Tregs regulate immune responses in a number of ways. This includes production of anti-inflammatory cytokines such as IL-10, TGF-β and IL-35, IL-2 depletion and inhibitory receptor mediated inhibition (e.g. via CTLA-4 and PD-1) (Tang et al. 2004; Wang et al. 2009). Through these mechanisms, Tregs can inhibit T cell effector functions directly or indirectly by modulating accessory cells such as dendritic cells (Sakaguchi et al. 2009).

1.4 T cell dysfunction: Exhaustion and Senescence

Disease and disease free episodes dictate expansion and resting phases in memory T cells. However, under conditions of prolonged antigen stimulation, for example during persistent or chronic viral infections, T cell populations can be driven into dysfunctional states, such as T cell senescence or exhaustion. Whilst the terms are often being used interchangeably, senescence and exhaustion are in fact two distinct processes characterized by different phenotypic markers and different underlying molecular pathways (Akbar and Henson 2011). Senescence is generally associated with persistent reactivating infections, whilst exhaustion is associated with chronic antigen exposure as a result of failure of disease containment.

1.4.1 T cell senescence

Senescence was first described in fibroblast cultures, where, after 50 passages, cells underwent growth arrest but were resistant to cell death (Effros and Walford 1984). This state, termed replicative senescence, is characterized by irreversible loss of proliferative capacity and has since been described in T cells. Senescence in memory T cells is associated with end-stage differentiation, lack of proliferative capacity and evidence of DNA damage but preservation of certain effector functions (Akbar and Henson 2011; Baars et al. 2000). Senescence occurs when telomere loss due to excessive cell division and general DNA damage (which can be induced by reactive oxygen species, ionizing radiation and other mechanisms) induce DNA damage responses, triggering intracellular signalling cascades involving p53 and p38 that lead to growth arrest and loss of telomerase activity (Plunkett et al. 2005; Iwasa et al. 2003).

Senescent T cells are characterized by a loss of their CD27 and CD28 costimulatory molecules, increased expression of the DNA damage associated marker γH2AX and upregulation of NK-cell associated inhibitory makers KLRG1 and CD57 (Henson et al. 2009; Di Mitri et al. 2011). The T_{EMRA} memory subset bears the hallmarks of cellular senescence, by showing low proliferative capacity and telomerase activity upon activation but high levels of γH2AX, reactive oxygen species and CD57 and KLRG1 expression (Di Mitri et al. 2011; Henson et al. 2014).

Cells with a senescence associated phenotype accumulate as a result of repeated exposure stimulation and with age (Griffiths et al. 2013; Derhovanessian et al. 2011; Koch et al. 2008). The accumulation of senescent cells is most commonly associated with cytomegalovirus (CMV) infection in healthy individuals (Ouyang et al. 2004; Kuijpers et al. 2003). After infections, this herpes virus establishes latency in a variety of cell types and persists for life in the immunocompetent host (Gandhi and Khanna 2004). Progressive long term antigen exposure during reactivation can lead to progressive T cell differentiation and large oligoclonal expansions of senescent and dysfunctional cells (Lachmann et al. 2012; Ouyang et al. 2004).

Pathogen specific cells may also have senescent features in other viral diseases such as Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), and hepatitis C (Hoare et al. 2013; Mojumdar et al. 2011). Senescent cells were also increased in individuals with autoimmune conditions, such as rheumatoid arthritis (Michel et al. 2007). Repeated T cell stimulation during these conditions may lead to senescent cells accumulating in large oligoclonal expansions (Di Mitri et al. 2011).

Whilst they show low proliferative capacity and lack of IL-2 production, most senescent cells are functional and express high levels of effector cytokines such as IFNγ and TNFα, as well as granzyme B and perforin, indicating high cytotoxic potential (Akbar and Henson 2011; Lachmann et al. 2012; Henson et al. 2014). Indeed, the fact that CMV is generally only reactivated during immunosuppression suggests that these cells are able to mediate effective immune surveillance (Gandhi and Khanna 2004). However, early stages of senescence can precede eventual loss of functionality, as some studies

suggest reduced *in vitro* cytokine production of CMV specific cells derived from old individuals compared to young donors (Ouyang et al. 2004).

The accumulation of large oligoclonal populations of senescent cells has been associated with detrimental effects in overall immune function and health in a number of studies. Studies in mice showed that large clonal expansions caused by herpes simplex virus (HSV)-1 caused reduced anti-viral function in old but not young mice (Messaoudi et al. 2004). Similarly, old rhesus monkeys showed reduced responsiveness to vaccinia virus *in vivo* and this was associated with a reduced naïve T cell pool due to persistent clonal expansions (Čičin-Šain et al. 2010). In humans, CMV infection and large oligoclonal T cell expansions have been associated with reduced responsiveness to EBV and increased mortality in old individuals (Khan et al. 2004; Wikby et al. 2005). In a different study, increased mortality in old individuals was associated with low overall telomere length of leukocytes, but not with CMV status (Deelen et al. 2014). Conversely, a number of studies exist that fail to show a negative impact of high T cell senescence in old animals (Cicin-Sain et al. 2011).

It has been demonstrated that T cell senescence can be manipulated, as inhibition of KLRG1 ligation rescued proliferation but not telomerase activity whilst p38 inhibition allowed the restoration of both in highly differentiated T cells *in vitro* (Henson et al. 2009; Di Mitri et al. 2011, 20)

1.4.2 **Senescence associated markers**

CD57 (beta-1,3-glucuronyltransferase 1 or NK-1) is a surface glycoprotein that binds L- and P-selectin and a fragment of laminin. On T cells, CD57 expression has been shown to inversely correlate with telomere length and physical and emotional stress can increase $CD57^+$ cells in peripheral blood of humans (Brenchley et al. 2003). Although its expression is associated with low proliferative function and end stage differentiation in T cells, the exact role of CD57 in T cell senescence is unknown.

Killer cell lectin-like receptor subfamily G, member 1 (KLRG1) is a transmembrane receptor belonging to the C-type lectin-like superfamily and contains an ITIM in its intracellular domain. Its ligands are E- (Epithelial), N-(neural) and R-(retinal) cadherin which comprise a family of transmembrane glycoproteins that mediate cell-to-cell adhesion. KLRG1 is upregulated on T

cells during infection and can be found on highly differentiated resting memory cells, including T_{EM} and T_{EMRA}. KLRG1 expression on T cells is often associated with low proliferative function and high levels of differentiation. However, rather than just being a marker, KLRG1 signalling (through recruitment of SHIP-1 and SHP-2 to its ITIM motif) has been shown to actively contribute to decreased proliferation in $CD8^{+}CD28^{-}CD27^{-}$ T cells (Henson et al. 2009).

1.4.3 Telomere length as marker of replicative history

Telomeres are non-coding DNA regions at the end of chromosomes that consist of hexanucleotide (TTAGGG) repeats. During chromosomal replication upon cell division, a portion (typically 50-200 bases) of the terminal DNA sequence is lost in each telomere. This leads to proliferation induced telomere erosion and ultimately results in DNA damage responses that induce cell cycle arrest or apoptosis (Boer and Noest 1998). Telomere length can therefore be used as an indicator of proliferative history and differentiation stage of a cell subset. Indeed, within the T cell subsets, telomeres are longest in the naïve subset, shorter in the T_{CM} subset and shortest in the T_{EM} subset, suggesting a stepwise differentiation along the telomere loss in these subsets (Sallusto et al. 1999). T_{EMRA} have longer telomeres than T_{EM}, suggesting that T_{EMRA} might not originate from T_{EM} direclty (Di Mitri et al. 2011; Plunkett et al. 2005).

1.4.4 T cell Exhaustion

Exhaustion in T cells occurs during conditions of high antigenic load, when upregulation of inhibitory receptors causes signalling that leads to growth arrest, decreased cytotoxic capacity and decreased cytokine production (Akbar and Henson 2011). Exhaustion occurs in a hierarchical manner with a loss in IL-2 and proliferation preceding reduced killing capacity, TNFα production and finally an inability to produce IFNγ, leading to functional impairment and deletion in some cases (Wherry et al. 2003).

Exhaustion is mediated by inhibitory receptors which are usually associated with maintenance of self-tolerance and prevention of autoimmunity such as PD-1, CTLA-4, T-cell immunoglobulin and mucin domain 3 (TIM-3) and lymphocyte-activation gene 3 (LAG-3) (Sharpe et al. 2007; Blackburn et al. 2009). Blocking experiments targeting receptors associated with exhaustion such as PD-1 and CTLA-4 or PD-1 and LAG-3 have been shown to increase proliferation and

functionality in T cells in a non-redundant manner (Blackburn et al. 2009; Parry et al. 2005).

Exhaustion has been shown to occur during chronic viral infections when the host fails to contain the pathogen. Such infections can be caused by LCMV in mice (Wherry et al. 2003) and HIV and Hepatitis B virus in humans (Wherry et al. 2003). Cells displaying exhaustion markers are mostly found within T_{EM} and T_{CM} cells that are of intermediate stages of differentiation (Akbar and Henson 2011; Henson et al. 2012). The chronic antigenic stimulation occurring during these infections drives upregulation of the exhaustion associated inhibitory receptors but also results in downregulation of IL-7 and IL-15 receptor components, making them insensitive to the homeostatic cytokines IL-7 and IL-15. Indeed, exhausted cells cannot be maintained in the absence of antigen in mouse models and are thought to be arrested in an intermediate stage of differentiation (Wherry et al. 2004).

The inhibitory receptors involved in immune exhaustion, such as PD-1 and CTLA-4, play independent roles in immune tolerance. Expression of individual inhibitory markers is indeed not necessarily a sign of immune exhaustion (Erickson et al. 2012). Exhaustion can also be seen as a physiological mechanism designed to limit immunopathology during persistent infection (Speiser et al. 2014). However, exhaustion mechanisms are also hijacked by viruses and tumour cells, which use exhaustion pathways to avoid immune recognition and clearance (Baitsch et al. 2011; Day et al. 2006).

1.5 The skin as an immunological organ

Despite being the site of melanoma induction, the skin is often neglected by studies assessing melanoma-associated immune responses. Indeed, the skin is not only the largest organ in the body but is also the home of a variety of immune cells, including high numbers of resident lymphocytes (Clark et al. 2006).

1.5.1 Anatomy of the Skin

The skin is a highly complex organ that, exposed to the environment, fulfils a variety of functions such as physical sensing, temperature control, barrier

function and immunity. The skin consists of several layers, whereby the epidermis is exposed to the environment and shields the underlying dermis, under which in turn there is a layer of subcutaneous fat. The skin is interspersed by a number of structures, including hair follicles, sweat glands (in humans but not mice), sebaceous glands, nerves, blood vessels and lymphatics. A variety of immune cells reside in the skin, including T cells, NK cells, mast cells, various dendritic cell subsets, innate lymphoid cells, αβ and γδ T cells, NKT cells and macrophages. Structural cells such as keratinocytes and fibroblasts may also actively be involved in immune responses (see Fig. 1-1; Bos et al. 1987; Nestle et al. 2009).

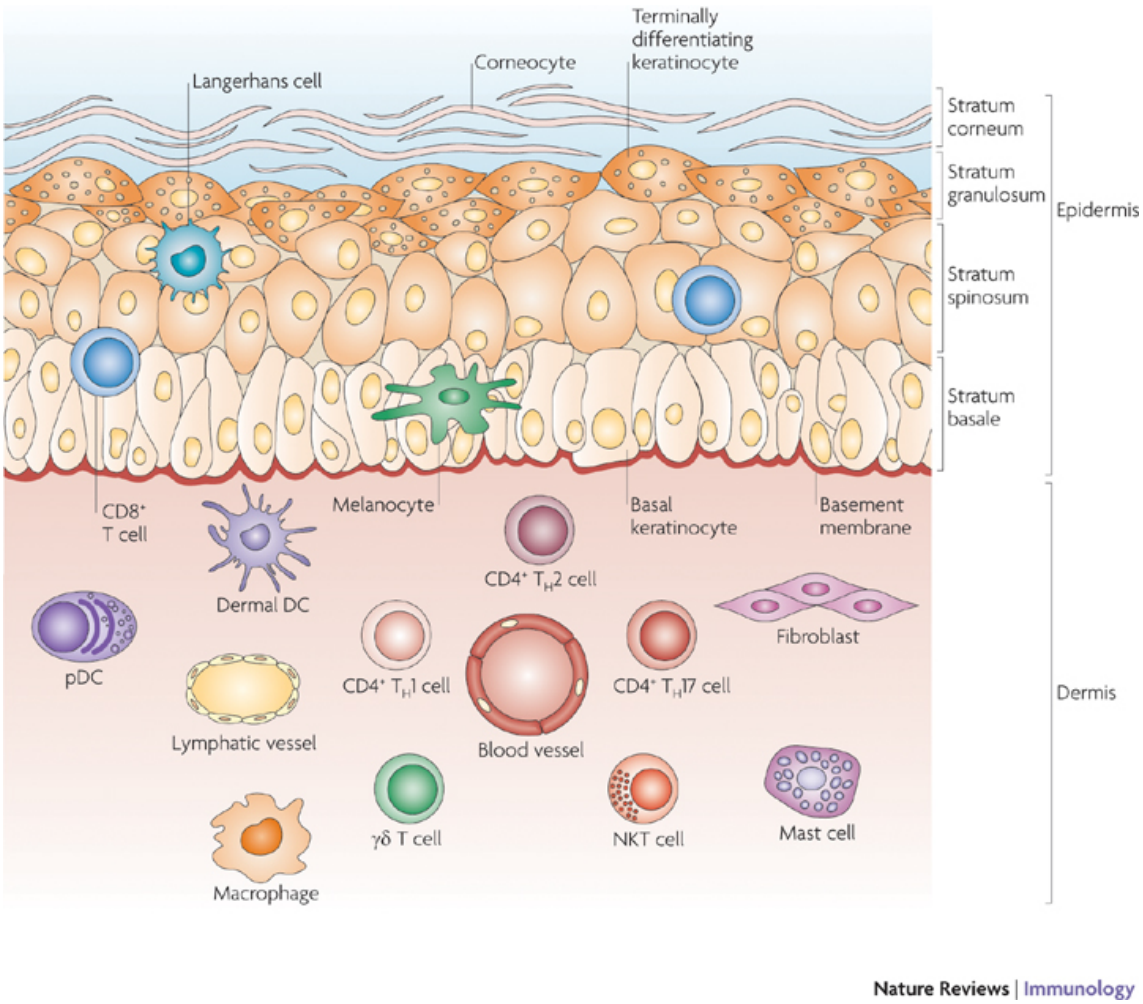


Fig. 1-1: Anatomy and immune cells of the skin

Schematic diagram showing the anatomic layers of the skin (epidermis and dermis, separated by the stratum basale) and the various cells involved in immune responses in the skin. DC= dendritic cell, pDC= plasmatycoid dendritic cell.
Adapted from (Nestle et al. 2009)

1.5.2 **Methods of studying immune cells in skin**

Whilst most research on immune processes heavily relies on blood derived immune cells, much less is known about tissue resident T cells. This is not only due to the fact that obtaining blood samples from a human donor is generally much less invasive than taking biopsies, but also to the fact that it is much more easy to isolate and manipulate cells from the blood than from solid organs.

Much research on skin immunity has therefore relied on imaging techniques that allow visualization of markers *in situ* using immunhistological techniques. However, immunofluorescence techniques are limited by the amount of detectors available (generally up to 4 colours) and interest is therefore high in using isolation techniques that allow multicolour flow cytometry in order to study more markers.

A number of techniques have been devised in order to obtain single cell suspensions from skin samples, which will be discussed in more detail in the material and methods and results sections. In general, these techniques rely on initial mechanical disruption followed by release of the cells from the skin matrix using EDTA or collagenase digestion (Clark et al. 2006). Alternatively, skin cells can also be recovered after emigration from skin explants in culture, although this entails extended periods of time between sampling and cell recovery and might therefore artificially select for and affect results obtained (Richters et al. 1995; Clark et al. 2006).

Because of the limitations of working with human skin, many studies have investigated skin T cells in animal models, leading to important observations. However, it should be noted that the mouse skin immune system is distinct from the human one in many respects, particularly as the dominant T cell population in mice are γδ T cells, whilst in humans αβ T cell prevail (Heath and Carbone 2013).

1.5.3 **Skin resident T cells**

The adult skin contains vast numbers of T cells and an emerging number of studies on their functions and phenotypes have yielded fascinating insights into the nature of these cells in humans and mice. Indeed, whilst skin resident T cells were initially thought to largely resemble the T_{EM} subset in the blood, recent studies in mice confirmed that skin resident T cells recognize different

targets and are transcriptionally distinct from their circulating counterparts (Mackay et al. 2013). T cells enter the skin during inflammation. After disease resolution, antigen specific T cells preferentially accumulate at the site of former infection, although they will also populate the entire skin (Jiang et al. 2012). However, parabiosis experiments have shown that skin resident memory T cells are not exchanged between animals sharing a common circulatory system (Jiang et al. 2012). Interestingly, skin $CD4^+$ T cells seem to be more mobile than their $CD8^+$ counterpart (Gebhardt et al. 2011).

Skin derived T cells are largely memory cells, as conventional naïve T cells are not thought to migrate into the peripheral organs until after activation in the lymph nodes. The majority of T cells in resting skin do not express the lymphoid homing markers CCR7 and CD62L and are $CD45RO^+$, which is why they were originally postulated to be T_{EM} cells (Clark, Chong, Mirchandani, Brinster, et al. 2006). In humans skin derived T cells almost exclusively express the skin homing markers CLA, CCR4 as well as high levels of CCR6 and CCR8 (Clark, Chong, Mirchandani, Brinster, et al. 2006). Skin resident $CD4^+$ T cells produce a variety of cytokines and are thought to be polarized towards a Th1 rather than a Th2 phenotype, although Th17 and Th22 have also been detected in the skin in various disease settings (Clark, Chong, Mirchandani, Brinster, et al. 2006; Segura et al. 2013; Fujita 2013).

Skin resident T cells can become activated, proliferate and carry out local immune responses. The importance of skin resident T cells in absence of T cell recruitment from the blood has been highlighted by an elegant study, which involved transfer of symptomless skin from psoriasis patients onto immunodeficient mice. This resulted in psoriasis-like inflammation in the skin transplant, which was caused by reactivation of skin resident T cells (Boyman et al. 2004). Cell transfer studies in mice have shown that skin derived T cells provided superior immune protection compared to T_{CM} upon localized vaccinia virus skin infection (Jiang et al. 2012). In humans, $CD8^+$ T cells were shown to infiltrate herpes simplex virus (HSV) infected genital skin and proliferate and display perforin expression during reactivation, confirming the role of skin resident $CD8^+$ T cells in immune surveillance in humans (Zhu et al. 2007; Zhu et al. 2013). Final evidence of the protective role of skin resident memory T cells comes from leukemic cutaneous T cell lymphoma patients who remain free from

skin infections despite being treated with alemtuzumab, a CD52-specific monoclonal antibody that depletes circulating T cells (Clark et al. 2012). Resident T cells can therefore protect the skin in the absence of T cell recruitment from the blood.

1.5.4 **Homing and tissue retention of skin T cells**

Whilst conventional T cells are abundant in adult human skin, they are virtually absent in the skin of neonates (Watanabe et al. 2015). T cells enter the skin during inflammation, where they eventually disappear or convert to a memory phenotype (Mackay et al. 2013). This occurs not only at the site of antigenic challenge, as T cells that have been activated in the draining lymph nodes also access and colonize uninvolved skin. Upon disease resolution, T cell trafficking to and from the skin ceases. The majority of T cells resident in healthy skin are therefore non-migratory, with only a fraction of cells immigrating into and emigrating from the skin during steady state conditions (Clark et al. 2012; Jiang et al. 2012). Interestingly, $CD8^+$ T cells are generally found to be static and resident in the epidermis, whilst $CD4^+$ T cells (and $CD4^+$ $FoxP3^+$ Tregs in particular) are more likely to localize to the dermis and traffic between the skin and circulation (Yawalkar et al. 2000; Tomura et al. 2010; Gebhardt et al. 2011; Watanabe et al. 2015).

T cells are able to home to different anatomical locations by bearing a variety of receptors that recognize various vascular addressins. For cells that home to the lymph nodes, this includes CD62L and CCR7. Conversely CLA, CCR4, CCR8 and CCR10 are thought to enable T cell homing into the skin. Expression of these receptors is thought to be imprinted during activation and can be influenced by the microenvironment of the local draining lymph node through local dendritic and stromal cells (Masopust and Schenkel 2013). T cell migration into healthy skin is low but enhanced during inflammation, thanks to upregulation of adhesion molecules such as VCAM-1 on the vascular endothelium and local expression of chemokines, such as CCL17 and CCL22 (both binding CCR4) and CCL27 (binding CCR10) (Mackay et al. 1992; McCully and Moser 2011).

CD69 and CD103 have been identified as markers for T cells resident in the skin and other peripheral tissues (Sathaliyawala et al. 2013). CD103 allows

binding to E-cadherin on epithelial cells and can be induced in skin infiltrating T cells through exposure with TGFβ (Mackay et al. 2013). The ligand for CD69 has not been identified, but CD69 is thought to mediate tissue retention by preventing surface expression of sphingosine 1-phosphate (S1P) receptor, which signals migration towards S1P in the blood (Lamana et al. 2011). Local cells, including keratinocytes and Langerhans cells, provide IL-15 to support T_{RM} development and survival (Mackay et al. 2013).

1.5.5 **Regulatory FoxP3$^+$ Tregs in the skin**

FoxP3$^+$ CD4$^+$ Tregs limit immune responses against self and foreign antigen. The majority Tregs in human blood express the skin homing receptors CLA and CCR4 and mice in which the Treg compartment is selectively deficient in either receptor develop severe autoimmune skin inflammation, testifying of the important role Tregs play in cutaneous immune regulation (Hirahara et al. 2006; Sather et al. 2007; Dudda et al. 2008). Indeed, Tregs are more abundant in the skin compared to the blood in both mice and humans and are recruited and proliferate further in the tissue during inflammation caused by antigenic challenge, autoimmune reactions or UVB-exposure (Vukmanovic-Stejic et al. 2013; Sanchez Rodriguez et al. 2014; Yamazaki et al. 2014). Interestingly, skin derived Tregs are highly mobile and show increased trafficking into the draining lymph nodes during inflammation (Tomura et al. 2010). FoxP3 Tregs are therefore abundant in healthy and diseased skin and crucial in preventing local immunopathology (Dudda et al. 2008).

1.5.6 **Innate immune cells in the skin**

Other cells present in the skin might modulate local immune responses. A variety of dendritic cell types occupy the healthy skin, including Langerhans cells and various dermal dendritic cells. These cells may have both, proinflammatory and immunomodulatory functions depending on the context in which they encounter antigen and may promote Treg formation or T cell activation (Seneschal et al. 2012; Bennett and Chakraverty 2012). Stromal cells such as keratinocytes or fibroblasts may also contribute to local immune responses (Barker et al. 1991; Rappl et al. 2001).

1.6 Melanoma

1.6.1 Melanoma: biology and epidemiology

1.6.1.1 Melanoma development

Healthy melanocytes normally reside in the basal layer of the epidermis with their main function being protection against UV radiation by increasing production of the melanin pigments. In healthy tissue, melanocytes are evenly distributed within the basal layer of the epidermis. Melanocytes may undergo mutations in genes regulating growth and cell adhesion, leading them to escape the control from surrounding keratinocytes. Subsequent proliferation can lead to a benign naevus (i.e. a common mole), and some melanocytes become morphologically atypical. When uncontrolled proliferation continues, radial growth phase and vertical growth phase melanoma might occur. Dissemination into other organs is the hallmark of the metastatic stage.

Melanoma incidence is associated with specific risk groups correlating with genetic background (i.e. in fair skinned populations, family history), behaviour (i.e. sun exposure and sunbed use), immunosuppression (e.g. treatment with methotrexate) and age and has been increasing worldwide more than any other cancer (Cancer Research UK 2010). Melanoma is especially prevalent among the elderly, who also suffer the greatest morbidity and mortality from this condition. However, this is thought to be at least in part due to diagnosis and treatment being delayed in the older age groups (Tsai et al. 2010).

Melanoma disease can be divided into four stages, with stage I and II patients having localized tumour, whilst stage III and IV patients display metastases in the draining lymph nodes and beyond (see Table 1-2). The fatal outcome of melanoma is generally caused by metastases in distant organs, such as the brain or the lung.

Stage	I	II	III	IV
Disease	Local to epidermis	Invasion of dermis	Lymph node metastases present	Metastases in peripheral tissues
5-year Survival	88-95%	43-79%	20-73%	5-22%

Table 1-2: Staging in cutaneous melanoma

Adapted from Cancer Research UK, 2012

Several features are independently linked with disease outcome. These include tumour stage as mentioned above, as well as sentinel lymph node status, Breslow thickness, melanoma mitotic rate, presence of ulceration and the location of the primary tumour (Teixeira et al. 2013). Positive prognostic factors include presence of infiltrating lymphocytes (van Houdt et al. 2008). Poor prognosis has been associated with lack of MHC class I expression on tumour cells and presence of infiltrating neutrophils and plasmatycoid dendritic cells (Jensen et al. 2012; van Houdt et al. 2009)

1.6.2 Melanoma immunogenicity

Melanoma is a highly immunogenic tumour and direct and indirect evidence exist highlighting the importance of naturally occurring immune responses against the malignant cells. Despite the pessimistic outlook of advanced disease, spontaneous complete and partial tumour regressions do occur. This is often associated with vitiligo, where autoimmune destruction of healthy melanocytes is indicative of active tumour-specific immune responses (Quaglino et al. 2010). Melanoma can develop during immunosuppression, which has been reported in rheumatoid arthritis patients receiving methotrexate (Buchbinder et al. 2008) and in transplant patients receiving organs donated by melanoma survivors thought to be disease free (Laing et al. 2006). Further evidence for the importance of immune responses in controlling melanoma disease includes the widely accepted correlation between the presence of tumour infiltrating lymphocytes and improved disease outcome, the detectable presence of tumour specific T cells and antibodies, and finally the vast array of active and passive immune evasion strategies displayed by the cancer,

including downregulation of genes coding for immunodominant proteins in the tumour (Aisner et al. 2005; McGovern 1975; Kaur et al. 2008; Jäger et al. 1996).

Although being derived from self, tumour cells often display antigens that are either distinct or overexpressed compared to the surrounding tissue and can therefore be targeted by the adaptive immune system. In many melanoma patients, a number of these tumour associated antigens (TAA) have been identified. These include proteins such as Melan-A (also known as MART-1), tyrosinase and gp100 which are also expressed in healthy melanocytes (Jäger et al. 1996). A different class of antigens overexpressed in melanoma cells are proteins which are not commonly found in healthy tissue but restricted to immunoprivileged germline cells. These antigens include NY-ESO-1, normally found in the testis (Velazquez et al. 2007). Melanoma cells may also acquire unique cancer antigens through genetic mutation (Robbins et al. 2013).

The discovery of immunodominant TAA epitopes has highly facilitated the identification and characterization of melanoma-specific T cells via the generation of fluorochrome-conjugated peptide containing MHC multimers (Maeurer et al. 2002). Whist the underlying genetic mechanisms of melanoma progression are different among patients, the same is true for anti-melanoma immune responses, which translates into a vast array of contradicting publications on T cell phenotypes in melanoma patients. It is generally agreed upon that detectable melanoma specific T cell populations are more likely to arise in patients with advanced disease only, as the disruptive nature of the cancer in the later disease stages is required to break tolerance and trigger an adaptive immune response (Dunbar et al. 2000; van Oijen et al. 2004).

It should be noted that Melan-A specific $CD8^+$ T cells, which are probably the most widely studied tumour specific T cells, can also be found at low but detectable frequencies of around 0.1-0.01% in healthy individuals where they are generally found to have a naïve-like ($CD45RA^+CD27^+$) phenotype (Pittet et al. 1999). Melanoma-specific $CD4^+$ T cells identified via fluorochrome conjugated MHC class II multimers have also been described but are not commonly used due to their low avidity nature that poses technical difficulties in using them in experiments reliably (Bioley et al. 2006).

1.6.3 **Melanoma Immune evasion**

Like any tumour, melanomas are the result of accumulated mutations in autologous cells leading to uncontrolled growth and proliferation, which do not only entail withdrawal of intra- and intercellular regulation mechanisms but also escape from immune regulation. Much research has been devoted to identifying tumour escape mechanisms such as secretion of immunosuppressive cytokines (e.g. IL-10 and TGFβ), increased frequencies of suppressive cells (e.g. Tregs, M2 macrophages or myeloid suppressor cells) upregulation of inhibitory receptors (e.g. PDL-1) or downregulation of HLA, target antigen, integrins and costimulatory molecule expression (Zitvogel, Tesniere, and Kroemer 2006).

Tregs are also known to play an important role in tumour immune responses. $CD4^+$ Tregs are increased in the circulation of some melanoma patients compared to healthy controls and enriched in primary melanoma and melanoma-infiltrated lymph nodes and metastases (Ascierto et al. 2010; Viguier et al. 2004). Tregs may contribute to a lack in T cell functionality in the tumour microenvironment (Read et al. 2000; Jacobs et al. 2012). Depletion of Tregs during treatment of melanoma has shown better outcome in mouse models as well as human trials (Sutmuller et al. 2001; Mahnke et al. 2007).

1.6.4 **The melanoma microenvironment**

The melanoma microenvironment is both, inflammatory and immunuppressive. Inflammatory mediators such as CCL-2, CCL-3, TNFα, IL-1β, IL-4 and IL-6 were shown to be increased in the tumour environment and can support tumour development by providing growth signals and angiogenesis (Umansky and Sevko 2012). This inflammatory environment leads to the accumulation of suppressive cell types such as myeloid derived suppressor cells, $CD4^+$ FoxP3 Tregs and M2 macrophages (Meyer et al. 2011). These, or the melanoma cells, secrete immunosuppressive factors such as VEGF, IL-10 and TGFβ leading to local immunosuppression. Dendritic cells in the tumour environment may have also regulatory/tolerogenic phenotypes or may be depleted locally (Molenkamp et al. 2005).

Cytotoxic T cells attracted to the tumour may not only be impaired by these local inhibitory factors but may also be simply denied access to the tumour microenvironment through lack of adhesion molecules, therefore accumulating

in the areas surrounding the tumour (Mempel and Bauer 2009). However, ectopic lymphoid structures may also form in melanoma lesions, attracting and potentially activating effector T cells on site (Messina et al. 2012; van Baren and Coulie 2013; Ladányi et al. 2014).

1.6.5 T cell senescence and exhaustion in melanoma

Since melanoma is highly immunogenic this malignancy, like persistent viral infections, may cause decreased T cell proliferation and functionality associated with chronic antigen exposure. This phenomenon is debated, as melanoma-specific T cells were found to be dysfunctional in some studies but not others (Mortarini et al. 2003). Some evidence further suggests that within the same person different clonotypes of melanoma specific $CD8^+$ T cells may be differentiated to different degrees. Similarly, melanoma specific T cells may adopt different phenotypic and functional properties in the melanoma lesion compared to the blood (Zippelius et al. 2004).

Evidence for T cell senescence in melanoma patients includes published data by two different groups who found that tyrosinase but not Melan-A specific $CD8^+$ T cells had a T_{EMRA}-like phenotype and low responsiveness to peptide pulsed target cells or PMA/ionomycin stimulation *in vitro* (Lee et al. 1999; Maczek et al. 2005). Increased frequencies of $CD8^+$ T_{EMRA}-like cells bearing NK cell markers have also been reported in melanoma patients compared to healthy age matched controls (Casado et al. 2005).

Evidence for inhibitory receptor upregulation and immune exhaustion in the context of melanoma has been shown in both mice and humans. Tumour infiltrating lymphocytes expressed high levels of PD-1 and TIM-3 and failed to proliferate and produce cytokines upon stimulation (Sakuishi et al. 2010). Similarly, melanoma specific NY-ESO-1 (but not Melan-A) specific cells coexpressed TIM-3 and PD-1 in humans and blocking both receptors showed increased function (Fourcade et al. 2010). In a different study, dual blockade of PD-1 and CLTA-4 was shown to increase melanoma rejection in murine models (Curran et al. 2010).

Encouraging clinical trials involving Ipilimumab, an anti-CTLA-4 blocking antibody, in advanced stage patients (Hodi et al. 2010; Cranmer and Hersh 2007; Gyorki et al. 2013) and anti-PD-1 trials (Brahmer et al. 2012; Brahmer et

al. 2010; Mkrtichyan et al. 2011) indicate the role of exhaustion-associated receptors in melanoma. However, whilst some patients do respond to anti-PD-1 and anti-CTLA-4 treatments, the effect is not always permanent and mortalities remain high, as highlighted in the survival curve of patients treated with anti-CTLA-4 shown in Fig. 1-2 (Prieto et al. 2012; Robert et al. 2011).

The need for improved immunotherapies for advanced stage patients therefore remains as important as ever and whilst most focus is concentrated on PD-1, CTLA-4 and other exhaustion-associated inhibitory receptor blockades to improve immune function, melanoma patients may also benefit from alternative interventions (Fourcade et al. 2010). Inhibition of p38 MAP kinase signalling for example has been shown to rescue proliferation and telomerase activity in highly differentiated and senescent T cells which are known to accumulate with age (Koch et al. 2008; Di Mitri et al. 2011; Akbar and Henson 2011). Such a treatment might therefore be especially beneficial to old patients, who additionally suffer from age-related immune defects but are generally underrepresented in melanoma related investigations and trials (Castle 2000; Hegde et al. 2010).

Another consideration in studying melanoma related immune defects and treatment efficacy is that there seems to be an imbalance between the function and phenotypes of melanoma associated T cells in the circulation and those infiltrating the tumour (Appay et al. 2006). When studying T cells in melanoma patients, one must therefore also consider T cells resident in the skin, as this is the site of tumour induction.

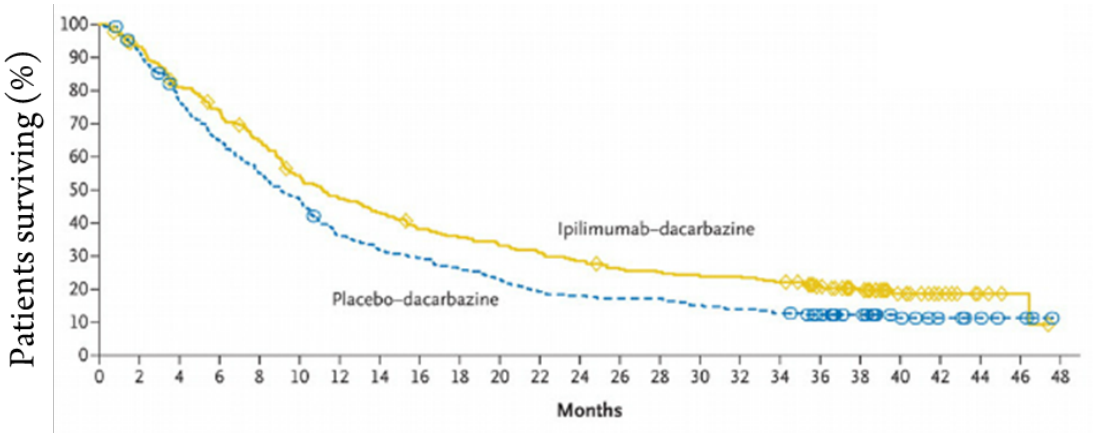


Fig. 1-2: Survival in patients with metastatic melanoma treated with dacarbazine alone or in conjunction with the anti-CTLA-4 blocking antibody Ipilimumab.

Adapted from (Robert et al. 2011)

1.7 **Aims**

The aim of this project was to characterize and define global and melanoma-specific T cell differentiation in patients with melanoma with the ultimate goal to identify means to reverse potential defects and to improve current melanoma therapies.

The objectives were as follows:

- Assess global and melanoma specific T cell differentiation in melanoma patients as compared to healthy controls
- Place particular emphasis on the older age groups which are the most affected by the disease
- Investigate T cell differentiation in uninvolved skin and sites of tumour invasion in the melanoma patients and compare it when possible to healthy controls
- Investigate the potential benefits of targeting receptors and intracellular pathways associated with senescence and exhaustion in T cells derived from melanoma patients

2 Material & Methods

2.1 Recruitment, Ethics and Exclusion Criteria

2.1.1 Healthy donors

Blood and blister samples were collected from consented healthy individuals from within UCL and the local community in accordance with the ethical committee of the Royal Free Hospital and University College London. Skin from healthy individuals was obtained from previously consented donors undergoing plastic surgery at the Guy's and St. Thomas' or Royal Free hospitals and approved by the Institutional Review Board of Guy's Hospital or the ethical committee of the Royal Free Hospital and University College London respectively. Skin samples from healthy donors were typically derived from breast or abdomen of individuals that underwent cosmetic or risk reducing (due to genetic predisposition to breast cancer) surgery. Individuals suffering from acute or chronic illness, with a history of cancer or on medication were excluded from this study.

2.1.2 Melanoma Patients

Consented cutaneous melanoma patients from Guys' and St Thomas Hospital provided fresh blood and skin samples. Blood was obtained from patients during their follow up visits. Skin was obtained in the form of several punch biopsies taken from local wide excisions extracted after positive melanoma diagnosis. Lymph node and primary tumour samples were obtained when there was excess tissue not needed for histological grading. Patients suffering from co-morbidities or on medication (including drugs for melanoma treatment) were excluded from this study. Frozen primary melanoma specimens were obtained from Professor Jim Kruger at the Rockefeller University, New York.

2.2 Sample preparation

2.2.1 PBMC isolation

Whole blood from melanoma patients and healthy controls was collected in 9ml EDTA or heparin tubes. Blood was mixed with an equal volume of Hanks

Balanced Salt Solution (HBSS) (Sigma-Aldrich, Gillingham, UK) and layered carefully onto 15ml Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK) in a 50ml Falcon tube to a total maximum volume of 50ml. The tubes were centrifuged at 2000 rpm for 20 minutes with the lowest brake setting. The resulting peripheral blood mononuclear cell (PBMC) layer between diluted serum and Ficoll was aspirated manually using a pasteurette and transferred into a new 50ml tube, in which it was washed twice in HBSS for 10 minutes, first at 1800rpm and subsequently at 1200rpm. Finally, the cells were resuspended in complete medium (RPMI 1640 complemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-Glutamine; all purchased from Sigma-Aldrich) and either frozen, kept in the fridge overnight or used immediately for further experiments.

2.2.2 **Isolation of cells from whole skin specimen**

2.2.2.1 **Enzymatic extraction of skin lymphocytes**

T cell extraction from the skin was performed using an enzymatic digestion protocol adapted from a method described by Haniffa and colleagues (Haniffa et al. 2009): Skin biopsies underwent removal of subcutaneous fat, followed by several washes in phosphate buffered saline (PBS). Skin was then cut into ~1-2mm^2 pieces in order to facilitate digestion. Pieces of skin were incubated in a 48-well plate overnight in complete medium with 0.8mg/ml of Collagenase IV (Life Technologies, Paisley, UK) with varying percentages of FBS (see results section). The following day, skin and supernatant were transferred to a 100µm cell strainer. Mechanical action with a syringe plunger and washes with PBS allowed transfer of cells through the mesh. The flow through was centrifuged and cells were resuspended in complete medium. Further experiments were performed on the same day.

2.2.2.2 **Devices for semi-automatic mechanical skin dissociation**

Two benchtop devices for mechanical disaggregation of skin were tested. The Medimachine (BD Biosciences, Oxford, UK) and GentleMACS (Miltenyi Biotec, Bisley, UK) both rely on disposable sterile plastic tubes, which contain either ~~a~~ stainless steel or plastic blades respectively. Tissue sample and PBS are added into the tubes and inserted into the machines that power the rotation of the blades at high speed. This process only lasts minutes but was found to be

inadequate for full skin disaggregation for obtaining tissue resident cells into suspension (see results section 4.3.1.1).

2.2.3 **Isolation of cells from suction blisters**

Applying negative pressure on a section of live skin over time leads to the physical separation of the epidermis from the dermis, creating a fluid-filled blister (Kiistala 1968). Immune cells accumulate in this fluid over time, making suction blisters an ideal tool to generate viable skin derived cells for research purposes. During this study, suction blisters were induced as previously described (See Fig. 2-1)(Akbar et al. 2013): A suction chamber with a round opening of 12.5mm in diameter (Medical Engineering, Royal Free Hospital, UK) was applied to the normal skin on the forearm of healthy volunteers, and kept under constant negative pressure of 25-40 kPa. Negative pressure in the chamber was provided from a clinical suction pump (VP25, Eschmann, Lancing, UK) connected via sterile disposable tubing and was maintained until a fully formed blister had been raised in the induration area of the chamber. This took typically 2-4 hours. The blister was then protected overnight through the assembly of a rigid adhesive dressing composed of a universal tube lid (Sterilin, Fisher Scientific UK Ltd, Loughborough, UK), Comfeel plus ulcer dressing (Coloplast, Peterborough, UK), Micropore tape (3M healthcare, Loughborough, UK) and Tubigrip bandaging (Seton Healthcare Group plc, Oldham, UK). The following day, blister fluid was aspirated using a 2ml syringe, collected in a 1.5ml Eppendorf tube and centrifuged at 650g (3000rpm) for 4 minutes. Blister fluid was removed gently and the cell pellet resuspended in 500µl complete medium for cell counting.

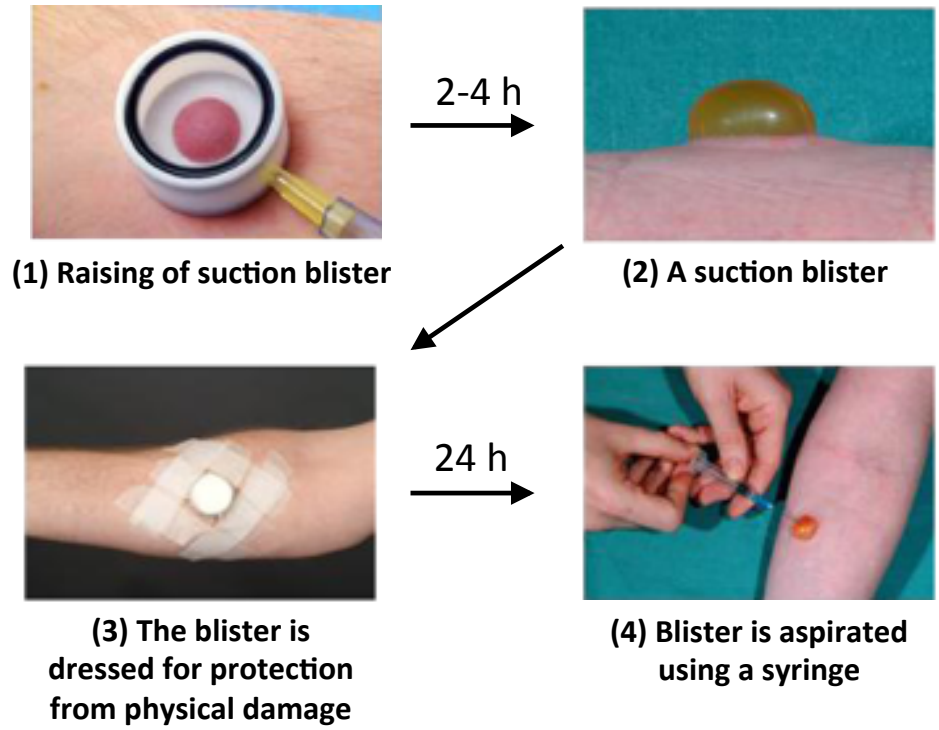


Fig. 2-1: Outline of the method for suction blister induction and collection of blister fluid

(1) A blister was induced by negative pressure through a suction chamber placed onto healthy skin.
(2) Representation of a fully formed suction blister
(3) Protective dressing was applied to the area of the suction blister
(4) The following day, blister fluid was aspirated using a syringe
Pictures were adapted from (Akbar et al. 2013)

2.2.4 **Freezing and thawing of cells**

When experiments could not be performed on the same or following day after extraction, cells were carefully resuspended in FBS with 10% DMSO at a concentration of 4-12 million cells/ml and aliquoted in 1ml volumes into cryovials (Nunc, Cramlington, UK). They were immediately placed into a “Mr Frosty” container (Nalgene/Thermo Fisher, Rochester, NY, USA) for gradual freezing at -80°C before being transferred into liquid nitrogen the following day for long-term storage. In order to recover frozen PBMCs, the cryovials were incubated at 37°C until most of the sample was thawed. Contents were swiftly transferred into pre-warmed complete medium and centrifuged, then resuspended in complete medium and finally counted using a haemocytometer before being used for further experiments. Freezing or storage in the fridge overnight of the PBMCs is routinely used in our lab and has not been found to significantly impair T cell cytokine production.

2.2.5 **Viable cell counts**

Cells were resuspended in a known volume of which 10µl were transferred to an equal volume of 0.4% trypan blue (Sigma-Aldrich). The cell:trypan blue suspension was applied to a Neubauer chamber (Weber Scientific International, Sussex, UK) mounted with a coverslip. Cells in the central chamber of both sides were counted, not including the dead cells that stained blue. The average of both counts were multiplied by the dilution factor of 2 and by 10 000, resulting in the estimated amount of cells per ml. If the counts of the two chambers differed considerably, this process was repeated to ensure an accurate enumeration.

2.2.6 **Isolation of $CD8^+$ T cell subsets from PBMCs**

In order to obtain purified $CD8^+$ T cell subset populations, $CD8^+$ T cells were isolated from total PBMCs using magnetic bead isolation, followed by FACS-based cells sorting:

2.2.6.1 **Magnetic cell isolation**

Magnetic cell isolation was performed using MACS columns (Miltenyi Biotec). The technique involves cell populations binding antibody-coated super-paramagnetic beads that are biodegradable and do not influence cell function.

Cells are applied to a separation column placed into a magnetic field in which labelled cells are retained, whilst unlabelled cells pass through. Labelled cells can be collected after removal of the column from the magnetic field. $CD8^+$ T cells were isolated by positive selection using CD8 microbeads, following the manufacturer's instructions: PBMCs were washed in cold MACS buffer (PBS, 0.5% Bovine Serum Albumin [BSA], 2mM EDTA) at 1200 rpm and labelled with anti-CD8 microbeads. The cells were incubated for 15 minutes at 4°C, before being washed again and resuspended in MACS buffer. The cells were then transferred to a pre-rinsed MACS column inserted into a magnetic holder. Columns were rinsed three times with cold MACS buffer in order to remove the CD8 negative fraction. Then, the column was removed from the magnetic field and MACS buffer was applied in order to remove the CD8 positive cells.

2.2.6.2 **FACS based sorting**

Some experiments required further sorting of CD27/CD45RA subsets. Magnetically purified $CD8^+$ T cells were stained with antibodies against CD8, CD27 and CD45RA for 30 minutes at 4°C in PBS with 1%BSA. Cells were subsequently washed in MACS buffer to avoid cell clumping, resuspended in MACS buffer and passed through a 35 µm nylon mesh into a new FACS tube (BD Biosciences). Cells were sorted into the four CD27/CD45RA subsets using a FACS Aria device (BD Biosciences) and collected in sterile tubes containing 50% FBS in RPMI. As soon as possible, cells were then centrifuged and resuspended at $2x10^6$ cells/ml in complete medium.

2.2.7 **Purification of immune cells from digested skin**

Purification using FACS sorting of immune cells from the skin was necessary for certain functional assays. For this purpose, skin cells extracted through the collagenase digestion method were stained with live/dead UV, CD3-FITC and a cocktail of APC-conjugated antibodies against CD11c, CD209, HLA-DR and CD34 for 30 minutes at 4°C, washed and resuspended in MACS buffer and filtered through a 35 µm nylon mesh. Samples were sorted into FITC positive T cells and APC positive antigen presenting cells that were used in a 1:1 ratio in subsequent functional assays.

2.3 Functional assays

2.3.1 Cell culture conditions

Cells were cultured in complete medium at 37°C in a humidified 5% CO_2 incubator unless otherwise stated.

2.3.2 Screening for CMV, EBV and VZV responders

Viral lysate stimulation as mean to identify carriers of the persistent virus CMV is routinely used in our laboratory as it has been found to reliably identify seropositive individuals (Fletcher et al. 2005). Fresh or thawed cells were incubated overnight in complete medium with CMV lysate (1:10). The CMV lysate was prepared in house as described by Fletcher and colleagues (Fletcher et al. 2005): Human embryonic lung fibroblasts were infected with Towne strain CMV for 5 days, after which cells were lysed by repeated freeze-thawing. For each batch prepared, titration was performed to determine the optimal concentration for cytokine production in PBMCs of CMV positive donors.

Screening for Epstein-Barr Virus (EBV; 1:200) and Varicella-Zoster virus (VZV; 1:25) was performed using commercially available viral antigen (both from Virusys, Taneytown, USA). The superantigen Staphylococcal Enterotoxin B (SEB; 1ng/ml; Sigma-Aldrich) was used as a positive control and no stimulant was added as a negative control in separate wells.

Stimulated cells were incubated overnight at 37°C. Brefeldin A (5µg/ml, Sigma-Aldrich) was added 2 hours after the start of incubation. Brefeldin A is a fungal metabolite that inhibits transport of vesicles from the Golgi apparatus to the endoplasmic reticulum, thereby inhibiting secretion and leading to intracellular accumulation of cytokines, facilitating their detection using flow cytometry.

The following day, cells were harvested and IL-2 and IFNγ levels were measured via intracellular flow cytometry as detailed below. Participants were considered to be CMV, EBV or VZV positive if above background production of cytokines was at least 0.01% of the total $CD4^+$ T cells.

2.3.3 Polyclonal T cell activation

Certain proteins of interest, such as markers of cytotoxicity, are only upregulated upon T cell activation. In order to detect their presence, or measure

proliferation, cells were stimulated polyclonally via incubation with immobilized anti-CD3 antibodies (0.5 µg/ml, clone: OKT3). If sorted cell subsets were stimulated with anti-CD3, 40Gy irradiated autologous APCs were added in equal numbers as a source of costimulation.

2.3.4 IL-15 stimulation assay

FACS sorted T cell subsets were incubated with 10ng/ml of rhIL-15 (R&D systems, Abingdon, UK) for up to 14 days. The complete medium used included HyClone (GE Healthcare) instead of RPMI. Half of the medium was replaced with fresh medium complemented with IL-15 every 3 to 4 days. Surface marker expression was measured by flow cytometry before incubation and at day 14.

2.3.5 P38 signalling blockade

Cells were incubated for one hour prior to stimulation with 500nM of the p38 inhibitor BIRB 796 (Selleck chemicals) or 0.1% DMSO as control. For cytokine detection following p38 blockade, cells were stimulated overnight in the presence of brefeldin A, added after 2 hours of stimulation, and harvested the following day for intracellular flow cytometry. For measurement of proliferation, cells were harvested 3 days after stimulation and stained intracellularly for Ki67.

2.3.6 PD-1 ligand blockade

In order to block PD-1 signalling, cells were incubated with functional grade purified anti-PDL-1 and anti-PDL-2 antibodies (specified in Table 2-1) or the isotype control antibody, for two hours before being stimulated with 0.05µg/ml immobilized anti-CD3. Cells were stimulated for 3 days before being harvested and analysed for intracellular granzyme B, perforin and Ki67 levels.

Target	Clone	isotype	Stock	Working concentration	Manufacturer
PDL-1	MIH1	Mouse IgG1, kappa	1mg/ml	10µg/ml	eBioscience
PDL-2	MIH18	Mouse IgG1, kappa	1mg/ml	10µg/ml	eBioscience
irrelevant	P3.6.2.8.1	Mouse IgG1, kappa	1mg/ml	20µg/ml	eBioscience

Table 2-1: Antibodies and isotype controls used for PD-1 signalling blockade

2.3.7 Measurement of cytotoxic degranulation via detection of surface CD107a

Pre-formed cytotoxic granules can be found in the cytoplasm of cytotoxic T cells. The granules are surrounded by a lipid bilayer and contain the cytotoxic effector molecules as well as lysosomal membrane proteins such CD107a (LAMP-1). Upon stimulation, degranulation occurs, releasing not only effector molecules but also exposing CD107a to the cell surface, thus making extracellular CD107a detection an ideal method for detection of cytotoxic degranulation.

CD107a based detection of degranulation required pre-incubation of PBMCs with the CD107a antibody (1:10, CD107a-APC, H4A3, BD Biosciences) before adding the cells to anti-CD3-coated plates. After 2 hours at 37°C, brefeldin A was added to allow simultaneous cytokine detection and monensin was added to prevent anti-CD107a antibody degradation. Cells were further incubated overnight and harvested the following day to be stained for flow cytometry.

Anti-CD107a antibody uptake was measured in all samples and flow cytometry gates were set according to unstimulated cells, allowing quantification and characterization of cells having undergone degranulation.

2.3.8 Perforin and granzyme expression in response to cytokine exposure

PBMCs or cells derived from collagenase digested skin were incubated for four days in the presence of no stimulant, 10ng/ml TNFα (PeproTech, London, UK), 5ng/ml IL-2 (PeproTech, London, UK), 10ng/ml IL-15 (R&D systems) or anti-CD3/CD28 coated beads (Dynabeads; Life Technologies). After this period, cells were harvested and stained for granzyme B, perforin and the appropriate extracellular antibodies as described below.

2.4 Flow Cytometry

Flow cytometry allows measurement of various properties of individual cells in suspension as they pass a laser. Size, granularity and fluorescence of the cells can be measured through various filters and detectors. Cells can be stained extracellularly and intracellularly with fluorescent dyes or fluorochrome-

conjugated antibodies, allowing measurements of various phenotypic and functional properties of cell populations.

2.4.1 **Surface staining**

Cells were centrifuged and washed in PBS. Fluorochrome-conjugated extracellular antibodies were added together with a UV live/dead stain (Invitrogen, Life technologies) at the appropriate concentration (see Table 2-2) and allowed to bind for 15 minutes at room temperature or 30 minutes at 4℃. After staining, cells were resuspended in PBSA (PBS, 1% bovine serum albumin, 0.01% sodium azide), spun down at 1800 rpm and fixed in 2% paraformaldehyde, unless further intracellular staining was needed.

If a biotin-labelled antibody was used, cells were incubated with streptavidin-Cy3 for 15 minutes at room temperature and washed again in PBSA before fixation.

2.4.2 **Cytoplasmic staining**

For detection of cytoplasmic proteins such as cytokines, perforin and granzyme B, cells first underwent surface staining as described above. However, before fixation, cells were treated using Fix & Perm Cell Permeabilization Kit reagents (An Der Grub, Buckingham, UK): cells were initially incubated for 20 minutes at room temperature with the fixation reagent A, followed by a wash with PBSA and 20 minutes incubation with the permeabilization reagent B together with the intracellular antibodies. After that, cells were washed in PBSA and resuspended in 2% paraformaldehyde until acquisition.

2.4.3 **Nuclear staining**

Nuclear antigens such as ki67 and FoxP3 were detected using the appropriate antibodies (listed in Table 2-2) using the intranulcear Miltenyi FoxP3 Staining Buffer Set according to the manufactures instructions (Miltenyi): First, cells underwent surface staining as described above. Then, cells were resuspended in the fix/perm solution (reagents A and B, diluted 1 to 3) for 30 minutes at 4°C, washed in cold perm buffer (stock diluted 1:10 in distilled water) twice, before being resuspended with the antibodies specific to Ki67 or FoxP3 for 30 minutes at 4°C. Finally, cells were washed in the perm buffer and resuspended in 2% paraformaldehyde.

2.4.4 **Phosphoflow staining**

Cells were stained extracellularly at room temperature as described above, washed in 1%FBS in PBS and fixed with pre-warmed cytofix buffer (BD Biosciences) for 30 minutes at 37°C. Cells were centrifuged and permeabilized with ice-cold Perm Buffer III (BD Biosciences) for 30 minutes on ice. Cells were subsequently washed twice with BD stain buffer and resuspended with the anti-phophorypated p38 (pp38) antibody and incubated for 30 minutes at room temperature. Finally, cells were washed in stain buffer and resuspended in stain buffer in order to be acquired on the flow cytometer.

	Target	Clone	Fluorochrome	Working Dilution	Manu-facturer
Extracellular targets	CD4	RPA-T4	APC-H7, BrilliantViolet421	1:20	BD
		SK3	PE-Cy7	1:10	BD
	CD8	SK1	PerCP	1:5	BD
		RPA-T8	Biotin	1:20	BD
	CD27	0323	APC	1:10	eBioscience
		M-T271	FITC ,PE, V500	1:10	BD
		L128	AlexaFluor786	1:20	BD
	CD25	M-A251	PE-Cy7	1:20	BD
	CD28	CD28.2	PE		BD
	CD45RA	HI100	PE-Cy7	1:20	BD
		M_{EM}-56	APC	1:10	Invitrogen
		HI100	BV605	1:50	BD
	CD127	HIL-7R-M21	AlexaFluor647	1:10	BD
	CD69	FN50	FITC	1:10	BD
	PD-1	EH12.2H7	PE-Cy7	1:20	Bioloegend
	CD3	UCHT1	ECD	1:50	Beckman Coulter
		SK7	FITC	1:10	FITC
	KLRG1	13F12	PE	1:100	(A kind gift from H.P. Pircher).
Cytoplasmic targets	IL-2	MQ1-17H12	FITC	1:10	BD
	IFNγ	B27	V450	1:100	BD
	TNFα	MAb11	APC	1:10	BD
	CTLA-4	BNI3	APC	1:10	BD
	Perforin	δG9	FITC	1:10	BD
	Granzyme B	GB11	AlexaFluor700	1:25	BD
Nuclear Targets	Ki67	MOPC-21	FITC or PE	1:10	BD
	FoxP3	PCH101	PE	1:10	BD
Phospho-Targets	p-p38 (pT180/pY182)	36	PE	1:20	BD

Table 2-2: Antibodies used for flow cytometry

2.4.5 **CFSE staining**

5- (and 6-) carboxyfluorescein succinimidyl ester (CFSE) stably incorporates into cells by covalently binding to intracellular molecules. It is used as a florescent dye to stain whole cell preparations and monitor proliferation through halving of florescence intensity with each cell division. For this purpose, cells were washed in PBS to remove any free protein in the solution. Cells were then resuspended in warm PBS with 0.5μM CFSE (Molecular Probes, Paisley, UK) and incubated at 37°C for 10 minutes. FBS was added to a final concentration of 10% and cells were placed on ice to stop the reaction. Cells were washed twice in complete medium and stimulated for 4 days before being harvested, stained and analysed via flow cytometry. CFSE labelled cells were protected from light at all times in order to prevent photo bleaching.

2.4.6 **Detection of Melanoma specific T cells**

Only HLA-A2 positive donors were used for MHC multimer screens. They were identified by extracellular staining using an anti-HLA-A2.1 antibody (clone BB7.2, AbD Serotec, Kidlington, UK). Initial experiments involved using an unconjugated Melan-A pentamer (loaded with ELAGIGILTV, Proimmune, Oxford, UK), but due to low rates of staining, the higher avidity dextramer system (Immudex, Sheffield, UK) was used. After optimisation, the following protocol was adopted**:** cells were stained in complete medium with peptide loaded MHC dextramers for 12 minutes at 37°C, followed by two washes with PBS with 10% FBS. Subsequent extra- and intracellular stains were performed on ice. Dextramers were loaded with a peptide derived from Melan-A (ELAGIGILTV) or NY-ESO-1 (SLLMWITQV) conjugated with PE or APC respectively. A PE or APC conjugated HLA-A2 Dextramer loaded with an irrelevant peptide was used as negative control.

2.4.7 **Measuring telomere length using Flow-FISH**

Flow cytometric detection of fluorescence *in situ* hybridization (flow-FISH) allows quantification of telomeric repeats using flow cytometry. The technique involves annealing fluorescently labeled nucleic acid probes with the complimentary telomeric DNA within fixed cells. The fluorescent signal can be measured via flow cytometry and signal strength correlates with telomere length. The technique can be used together with other flow cytometric staining

procedures in order to link telomere length with phenotypic and functional properties at a single cell level.

$2x10^6$ PBMCs were stained in FACS tubes with antibodies conjugated to fluorochromes that can withstand the heat and chemical treatment used during this protocol (CD3- FITC, CD4- BV421, CD8-Biotin$^+$Strept-Cy3, CD27-AF786 and CD45RA-BV605), as well as a live/dead stain, using the extracellular FACS staining method described in section 2.4.1.

Cells were then fixed in 1mM BS3 (Thermo Scientific) for 30 minutes at 4°C, which was then quenched using 50mM Tris pH7.2 for 20 minutes, before being washed in PBS.

Fixed cells were washed using hybridization buffer (70% formamide, 20mM Tris HCl, 1%BSA, 150mM NaCl) and resuspended in exactly 300µl hybridization solution to be spit into three separate tubes. An equal volume of 1.5ug/ml Cy5 conjugated telomeric (CCCTAA) peptide nucleic acid probe (PNA Bio, Daejon, South Korea) was added to each tube and cells were transferred to a 82°C water bath for 10 minutes before being snap cooled in an ice water bath. Samples were then left to rest in the dark at room temperature for 60 minutes, washed twice in post-hybridization buffer (70% Formamide, 10mM Tris HCl, 0.25% Tween20, 1.5mM NaCl and 22.5% H_2O) and finally washed twice in PBSA before being acquired on the flow cytometer.

During acquisition, voltages for the Cy5 detection channel were adjusted to the fluorescence of QuantumCy5 Molecules of Equivalent Soluble Fluorochrome beads (Bangs Laboratories, Fishers, Indianapolis, USA): The standard curve which allows the conversion of fluorescence intensity of the samples into the telomere length in kilo base pairs (kbp) was previously generated by members of our group using telomere lengths from PBMC samples of varying telomere length that were measured by both Flow-FISH and telomeric restriction fragment analysis (Riddell et al. 2014).

2.4.8 **Flow Cytometers**

Samples were acquired on LSRII or Fortessa (both from BD Biosciences, San Jose, CA, USA) flow cytometry machines. Both machines were equipped with blue (488nm), red (640nm) and yellow/green (561nm) and violet (405nm) lasers. The LSRII had an additional ultraviolet (355nm) laser. Up to 11 different

fluorochromes were detected simultaneously. Data was acquired using Diva software (BD Biosciences). Control samples stained with a single colour were used for compensation to account for emission overlap between different fluorochromes.

2.4.9 **Analysis of flow cytometry data**

Flow cytometry data were analysed using FlowJo (Tree Star Inc., Asland, USA). Lymphocytes were identified by forward/side scatter profiles and dead cells staining positive for the live/dead stain marker were excluded. Fluorescence minus one (FMO) control samples, containing all but one colour, were used to define positive populations and confirm the quality of the stain. Antibodies were titrated for optimal concentrations before routine use.

2.5 **Histology**

2.5.1 **Sample preparation and cutting**

5mm punch biopsies from young and old healthy donors were embedded with the epidermis facing up in OCT compound (VWR, Lutterworth, UK) on a cork disk and snap frozen in isopentane (Sigma-Aldrich) precooled in liquid nitrogen. Frozen skin samples were stored at -80°C until being cut into 6µm sections and mounted on poly-L-lysine coated glass slides (Sigma-Aldrich). Poly-L-lysine provides increased adhesion of biomaterial compared to uncoated glass. Cutting was performed using a Leica Biosystems $_{CM}$1950 cryostat. Two sections were mounted on each slide and left overnight at room temperature overnight to air dry, before being fixed in acetone for 10 minutes, and then for 10 minutes in 99% Ethanol. Sections were then air-dried for 10 minutes, wrapped in cling-film and stored at -80°C.

Primary melanoma sections from old patients were prepared by Professor James Krueger's laboratory at Rockefeller University. After cutting, these samples were dried but not fixed before being frozen. Instead, sections needed to be fixed in acetone for 5 minutes after thawing and before staining.

2.5.2 **Immunohistochemistry**

An indirect streptavidin-biotin immunoenzymatic antigen detection system was used to detect and quantify PDL-1 and PDL-2 expressing cells in frozen skin

sections. The technique involves using a primary antibody specific to the antigen of interest followed by a secondary detection antibody specific to the conserved FC portion of the primary antibody. The secondary antibody is biotinylated and will in turn bind an avidin-biotinylated peroxidase H complex that will catalyse 3-amino-9-ethylcarbazole (AEC) into a brown coloured product, thus staining the area that bound the primary antibody. Detection was performed using Mouse Vectastain Elite ABC kit: PK-602 (Vector laboratories, Burlingame, USA).

Skin sections from healthy individuals and primary melanoma patients were brought to room temperature and fixed if necessary, before being circled using a water repellent Dakopen (Dako, Stockport, UK) in order to contain any liquid applied to the sections. Samples were then washed twice for 5 minutes in a PBS water bath. Excess PBS was removed by gentle tapping and 10% horse serum in PBS was applied for 20 minutes to prevent unspecific antibody binding. Excess blocking solution was removed and the primary antibody was added in 1% horse serum in PBS in the optimised dilution as listed for each antibody in Table 2-3 and incubated overnight at 4°C in a humidified chamber. The following day, slides were washed twice in PBS and incubated for 30 minutes with the biotinylated horse anti-mouse secondary antibody in 1% horse serum, followed by two washes in PBS. Slides were then incubated for 10 minutes in 0.3% H_2O_2 in ddH_2O in order to block endogenous peroxidase activity, followed by 2 washes in PBS. Excess PBS was removed and freshly prepared Vectastain ABC solution (1:100 of both solution A, containing the avidin, and solution B, containing the biotinylated peroxidase, in PBS) was applied for 30 minutes onto the sections, before being washed twice in PBS. The Vectastain AEC solution (0.25ml of 40mM AEC in N,N-dimethyl formamide was diluted in 4.75 0.1M Acetic acid and then activated with 2.5µl H_2O_2) was applied to each section. Resulting colour change was monitored and slides were washed when the desired intensity developed. Sections were left to dry at room temperature and mounted with a coverslip.

Target	Clone (Animal)	Dilution	Company	Secondary antibody [dilution]
PDL-1	MIH1 (mouse)	1:50	eBioscience	Horse anti-mouse [1:200]
PDL-2	MIH18 (mouse)	1:200	eBioscience	

Table 2-3: Antibodies used for immunohistochemistry

2.5.3 Double Immunofluorescence

Double labelled indirect immunofluorescence involves several primary unconjugated antibodies being used for the simultaneous detection of several antigenic targets in frozen skin sections. Fluorochrome conjugated secondary antibodies raised against the FC portions of the primary antibodies are then used to amplify the signal and emit light of various colours that can be individually detected by fluorescent microscopy. This technique generally requires the primary antibodies to be raised in different animals to ensure differently coloured secondary antibodies bind their respective target only. As this was not possible for the simultaneous detection of CD8 with perforin, an additional step needed to be added to the protocol. This was possible because the anti-CD8 antibody was directly conjugated to FITC, which could be detected by a separate secondary antibody to the one previously used to detect perforin.

Frozen skin sections mounted on glass slides were brought to room temperature before being circled using a water repellent Dakopen (Dako). Slides were washed twice for 5 minutes in a container with PBS. PBS was removed by gentle tapping and 10% goat serum in PBS was applied for 30 minutes at room temperature in order to minimize unspecific antibody binding. Slides were again washed twice in PBS, which was removed before applying the primary antibodies (see Table 2-4) in 1% serum. Slides were incubated overnight at 4°C in a humidified chamber. The following day, unbound antibody was tapped off, followed by two washes with PBS in the dark. Then, the fluorochrome-conjugated secondary antibodies (see Table 2-4) was applied and incubated in the dark for 30 minutes at room temperature, before being washed off by two washes with PBS. Finally, sections were dried at room temperature in the dark before being mounted with coverslips and mounting reagent ProLong Antifade Gold (Invitrogen), which contains the nuclei staining DAPI.

As mentioned, staining for perforin and CD8 required an additional step during the staining procedure as the two antibodies were raised in the same species of animal. For this, only perforin but not CD8 was incubated during the first day. Instead, staining on day 1 used anti-perforin antibody only. The appropriate secondary antibody was applied on the second day, followed by two washes and 30 minutes blocking with 10% mouse serum in PBS. After blocking, the anti-CD8-FITC antibody was applied overnight, followed by two washes and addition of anti-FITC antibody for 30 minutes on the third day. Unbound antibody was washed off in two washes and slides were washed and mounted as described above.

Control slides were incubated with secondary antibodies only to ensure no unspecific staining occurred. Collagen fibres gave green autofluorescence, which could not be removed.

Target	**Conjugate**	**Clone (Animal)**	**Dilution**	**Company**	**Detection antibody** (all from Invitrogen)
CD8	FITC	SK1 (mouse)	1:50	BD	Goat-anti-FITC [1:250], AlexaFluor488
Perforin	none	δG9 (mouse)	1:50	BD	Goat-anti mouse [1:250], AlexaFluor468
Granzyme B	none	polyclonal (rabbit)	1:200	Abcam	Goat-anti-rabbit [1:25], AlexaFluor546

Table 2-4: Antibodies used in immunofluorescence

2.5.4 Microscopy and image processing

2.5.4.1 Immmunofluorescence

Immunofluorescent images were acquired for each antibody combination on the same day, using the appropriate filters of a Zeiss Axioplan 2I microscope. Images were taken with an emphasis on areas showing staining for $CD8^+$ T cells (in green). Images were overlaid and analysed using ImageJ software and colour threshold were adjusted for the whole image in order to reduce unspecific background fluorescence equally in all samples from the same batch.

2.5.4.2 **Immunohisotchemistry**

Skin sections stained by immunohistochemistry were acquired using a light microscope (Nikon Eclipse E600). Images generated were analysed using ImageJ software, allowing manual counting of positive staining cells per surface area.

2.6 **Statistical Analysis**

Graphs were drawn and statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, USA). Non-parametric data were identified using the D'Agostino and Pearson omnibus normality test. Statistical tests used included the Student's T-test or paired T-test when data followed a Gaussian distribution and the Whitney-Mann test or Wilcoxon test for non-parametric data. Correlations were calculated using Pearson correlation coefficient or Spearman correlation for non-parametric data. Lines of best fit were generated using linear regression. Fisher's exact test was used to compare prevalence of CMV between melanoma patients and healthy controls. If more than two groups were compared simultaneously, one-way ANOVA (for parametric data) or the Friedman test (for non-parametric data) were applied, followed by Holm-Sidak or Dunn's multiple comparison tests respectively for paired comparisons.

Differences were considered significant when $p<0.05$.

3 Investigating global and specific T cell differentiation in the circulation of patients with melanoma

3.1 Chapter Introduction

Melanoma is a highly immunogenic tumour, but despite the reported presence of melanoma specific T cells in patients with advanced stage, disease progression occurs (Jäger et al. 1996). Some persistent diseases are known to cause increased T cell differentiation and drive T cell dysfunction via chronic antigen exposure, especially with age (Ouyang et al. 2004). By virtue of its antigenicity and simultaneous lack of disease resolution, melanoma may therefore drive T cell differentiation in the patients affected.

T cell differentiation or senescence can be measured in a number of ways. The cell surface markers CD45RA and CD27 are often used to distinguish the less differentiated naïve ($CD45RA^+CD27^+$) and central memory (T_{CM}; $CD45RA^-CD27^+$) from the more differentiated effector memory (T_{EM}; $CD45RA^-CD27^-$) and the end-stage differentiated and senescent effector memory T cells re-expressing CD45RA (T_{EMRA}; $CD45RA^+CD27^-$) (Hamann et al. 1997; Sallusto et al. 1999). Surface CD57 and KLRG1 expression are also associated with highly differentiated cells (Brenchley et al. 2003; Henson and Akbar 2009). Expression of inhibitory markers such as PD-1 and CTLA-4 on the other hand is not usually associated with senescence, but instead with exhaustion which affects less differentiated cells (Libri et al. 2011; Akbar and Henson 2011). Despite showing low proliferative capacity, highly differentiated T cells often display high effector potential such as inflammatory cytokine production upon challenge or expression of cytotoxic granule components (Akbar and Henson 2011).

Known pathogens that drive T cell differentiation include Epstein-Barr virus (EBV) and human immunodeficiency virus (HIV) and most prominently Cytomegalovirus (CMV), which has been associated with a global increase in T cell differentiation in healthy appearing individuals (Ouyang et al. 2004; Fletcher et al. 2005; Lachmann et al. 2012). CMV induced global $CD4^+$ and $CD8^+$ T cell

differentiation is particularly apparent among the old, which is most likely due to the lifelong exposure to the virus (Koch et al. 2008).

Previous studies have reported an increase in highly differentiated T cells in a number of non-melanoma tumours such as myeloma and renal cell carcinoma (Sze et al. 2001; Characiejus et al. 2002). In the context of melanoma, no highly differentiated cells could be detected amongst T cells infiltrating the primary melanoma or tumour invaded lymph nodes (Mortarini et al. 2003; Anichini et al. 2010). Conversely, reports do exist confirming the presence of melanoma-specific T cells with a T_{EMRA} phenotype in the blood of patients with metastatic disease (Maczek et al. 2005). It should be noted that most studies have focussed on patients of intermediate age groups with the old cohorts (which present with the highest mortality) generally being underrepresented.

This first results chapter therefore investigates whether increased T cell differentiation occurs in old melanoma patients compared to healthy controls by measuring the differentiation patterns in circulating $CD4^+$ and $CD8^+$ T cells using relative expression of CD45RA/CD27 and other differentiation associated markers.

3.2 **Aims and objectives**

The aim of this chapter was to characterize and define global and melanoma-specific differentiation patterns in the blood T cells of patients with melanoma.

3.3 Results

3.3.1 Global CD4$^+$ T cell differentiation in the circulation of melanoma patients compared to healthy controls

Melanoma patients and healthy volunteers were assessed for their CD4$^+$ and CD8$^+$ T cell differentiation patterns based on the cells surface expression of CD45RA and CD27 using flow cytometry (Representative gating strategy shown in Fig. 3-1). Cells that are CD45RA$^+$CD27$^+$ are considered to be naïve, CD45RA$^-$CD27$^+$ cells are central memory cells (T_{CM}), CD45RA$^-$CD27$^-$ cells are effector memory cells (T_{EM}) and finally cells which are CD45RA$^+$CD27$^-$ are termed effector memory cells re-expressing CD45RA (T_{EMRA}). T_{EMRA} cells are considered to be the most differentiated (Di Mitri et al. 2011; Henson et al. 2014).

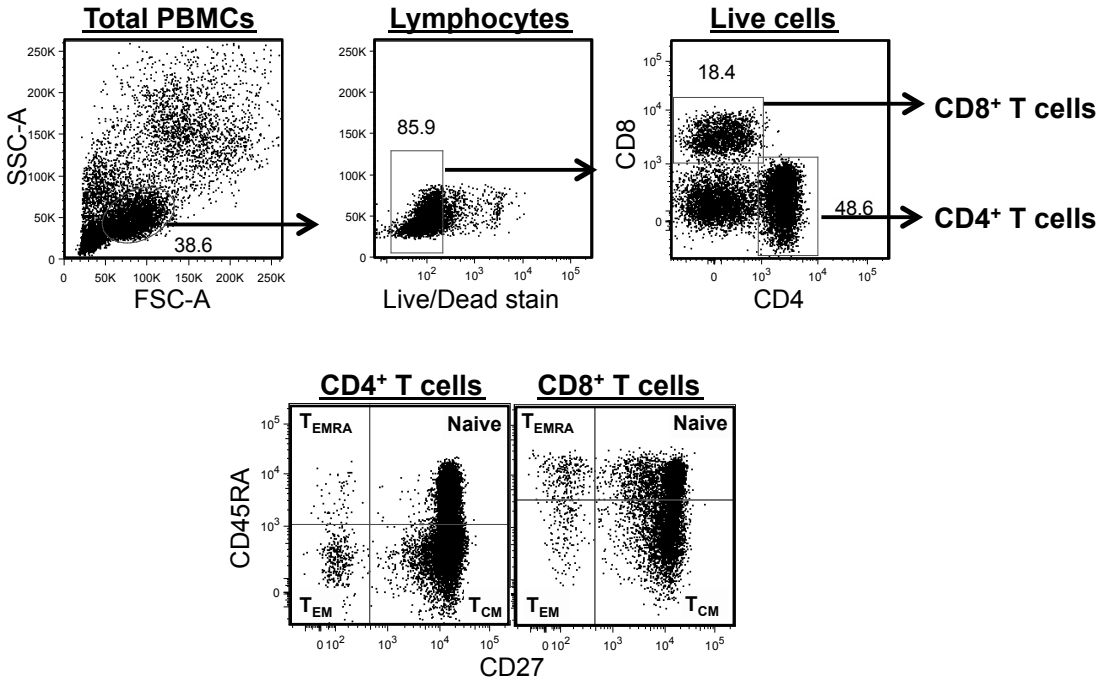


Fig. 3-1: Representative FACS plots showing gating of live CD4$^+$ and CD8$^+$ T cell subsets based on their CD27 and CD45RA surface expression in PBMCs derived from a healthy individual.

The T cell differentiation compartments are: CD45RA$^+$CD27$^+$ = naïve cells, CD45RA$^-$CD27$^+$ = central memory cells (T_{CM}), CD45RA$^-$CD27$^-$ = effector memory cells (T_{EM}), CD45RA$^+$CD27$^-$ = CD45RA re-expressing effector memory cells (T_{EMRA}).

3.3.1.1 **Differentiation patterns of circulating $CD4^+$ T cells are altered in melanoma patients**

The distribution of the CD45RA/CD27 subsets within the $CD4^+$ T cell compartment was stratified by age in both healthy individuals and melanoma patients (representative staining and cumulative data are shown in Fig. 3-2A,B). In both healthy and melanoma groups, the percentage of naïve cells decreased with age ($p=0.0193$ and $p<0.0001$ respectively) and the amount of T_{EM} increased ($p=0.0115$ and $p=0.0050$), although these trends were more pronounced among the melanoma patients. Further, the melanoma patients (but not the healthy controls) displayed a significant increase of T_{CM} with age ($p=0.029$). Direct comparison of $CD4^+$ T cell subset distributions between melanoma patients and healthy controls showed no difference in the younger age group (aged 35 or less), whilst a significant increase in T_{CM} could be confirmed among old (aged 60 and above) melanoma patients compared to the age matched healthy controls ($p=0.0393$; Fig. 3-2C). No increase of highly differentiated cells was therefore found in the $CD4^+$ T cell compartment of patients with melanoma.

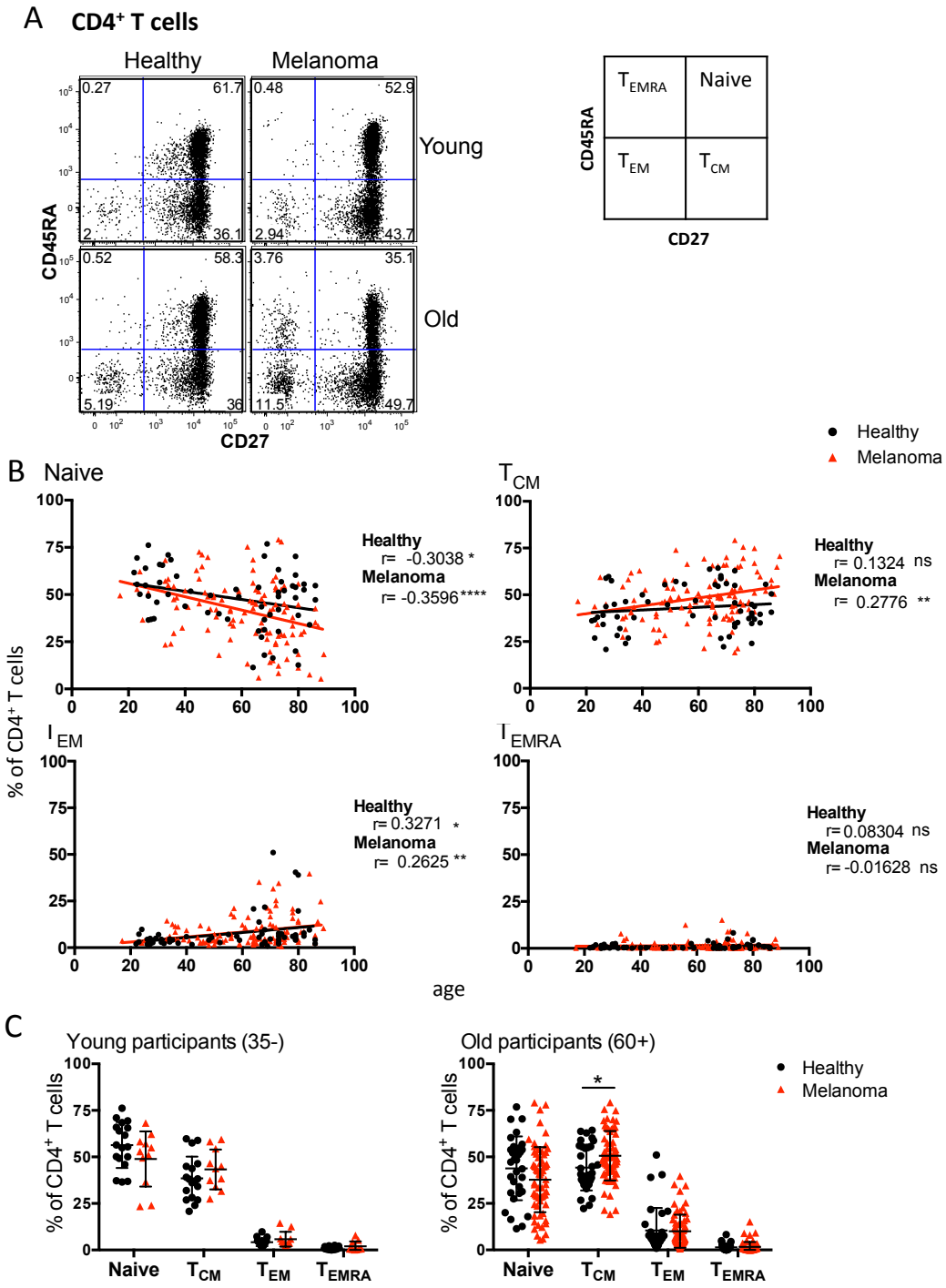


Fig. 3-2: Blood CD4$^+$ T cell subset distribution with age in melanoma patients and healthy controls

A: Representative FACS plots showing CD4$^+$ T cell CD45RA/CD27 expression patterns in young and old melanoma patients and healthy controls. The schematic diagram on the right shows the names given to the subpopulations in each gate.
B: Frequencies of each of the CD45RA/CD27 subsets within total CD4$^+$ T cells are correlated against age in melanoma patients (n=113) and healthy controls (n=59). Lines of best fit were generated by linear regression and the correlation (r-value) and significance were assessed by Pearson and Spearman rank.
C: Direct comparison of percentages of the CD4$^+$ T cell subsets between young melanoma patients (n=11) and young healthy controls (18) and between old melanoma patients (n=67) and old healthy controls (n=33). Populations were compared using the unpaired T-test or the Mann-Whitney test. Horizontal lines depict mean values with standard deviation for all graphs.
*=p<0.05; **=p<0.01; ***=p<0.001; ****p>0.0001

3.3.1.2 **CMV infection is associated with altered $CD4^+$ T cell differentiation in old melanoma patients**

CMV infection is known to heavily influence global T cell differentiation patterns of carriers, especially with advanced age (Chidrawar et al. 2009; Derhovanessian et al. 2011). It was therefore of interest to determine whether CMV played a role in the T cell differentiation patterns observed among the melanoma patients and healthy controls. Analyses were made in the old (60 years and above) age group only, as no differences were found in global $CD4^+$ T cell differentiation between young patients and controls. Old participants were identified as CMV responders and non-responders via $CD4^+$ T cell cytokine production (measured by intracellular flow cytometry, Fig. 3-3A) following overnight incubation with viral lysate. Results obtained by this method have been shown to correlate with screening through serological testing by our group previously (Fletcher et al. 2005). The frequency of CMV responders and the magnitude of the *in vitro* response to the lysate (Fig. 3-3B) were not found to differ significantly between old patients and controls (64% and 58% CMV responders respectively; p= 0.5040).

Melanoma patients were further subdivided according to their disease stage with stages I-II being local disease and III-IV disseminated. No stage IV patients were available for this analysis. Amongst the melanoma patients, only 26% stage I melanoma patients were found to be CMV^+, whilst 72% and 62% of stage II and III were positive respectively. It should be noted that although we attempted to compare age matched groups in this work, old melanoma patients belonging to different disease stages were not age matched**:** Stage I participants were on average younger that stage II and stage III patients (mean ages= 69.7, 74.0 and 72.6 respectively). Any differences detected between patients of different stages might therefore be solely due to discrepancies in age.

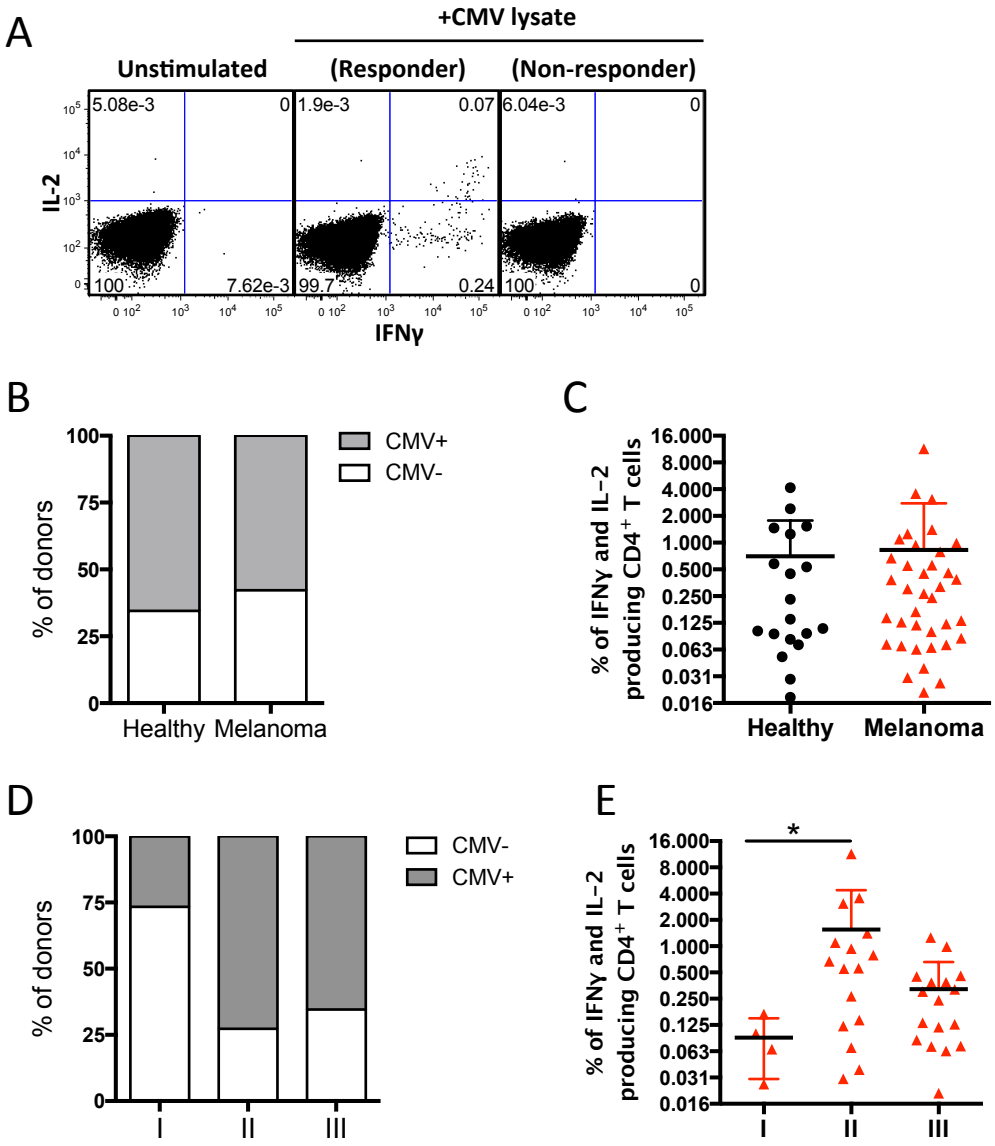


Fig. 3-3: Identification of CMV infected individuals among old (aged 60 or above) patients and controls

A: Representative FACS plot showing identification of CMV positive individuals through detection of IFNγ$^+$ or IL-2$^+$ CD4$^+$ T cells following overnight stimulation of PBMCs with CMV lysate.
B: Percentages of CMV positive and negative donors amongst old melanoma patients (n=64) and healthy controls (n=29)
C: Frequency of CD4$^+$ T cells producing cytokines in response to CMV lysate among old CMV positive melanoma patients (n=37) and healthy controls (n=19). Horizontal lines depict mean values with standard deviation.
C: Percentages of CMV positive and negative donors amongst stage I (n=15), II (n=22) and III (n=26) melanoma patients.
D: Frequency of CD4$^+$ T cells producing cytokines in response to CMV lysate among CMV positive stage I, II and III melanoma patients (n=4, 16 and 17 respectively). Populations were compared using the Kruskal Wallis test. Horizontal lines depict mean values with standard deviation for all graphs.
*p>0.05

Among the melanoma patients, a CMV^+ status was associated with a significant decrease in naïve cell frequencies and increase in T_{EM} and T_{EMRA}, compared to patients that were CMV^- ($p=0.0221$, $p>0.001$ and $p=0.0053$ respectively; Fig. 3-4A). Similarly, healthy old CMV^+ individuals had increased frequencies of T_{EM} and T_{EMRA} compared to healthy old CMV^- donors ($p=0.006$ and $p= 0.0204$, respectively). The increase of T_{CM} detected in the old melanoma patients compared to healthy controls did not persist after excluding all the participants that were CMV^+. Instead, $CD4^+$ T_{CM} was increased compared to the controls in the CMV carrier group only ($p= 0.0448$).

In order to investigate whether the increase in T_{CM} among CMV^+ melanoma patients varied with tumour stage, participants were separated into their respective disease stages and analysed for subset frequencies (Fig. 3-4C). However, as mentioned above, the groups were not age matched and no significant differences in naïve, T_{CM}, T_{EM} and T_{EMRA} $CD4^+$ subset distributions were detected among patients of different stages.

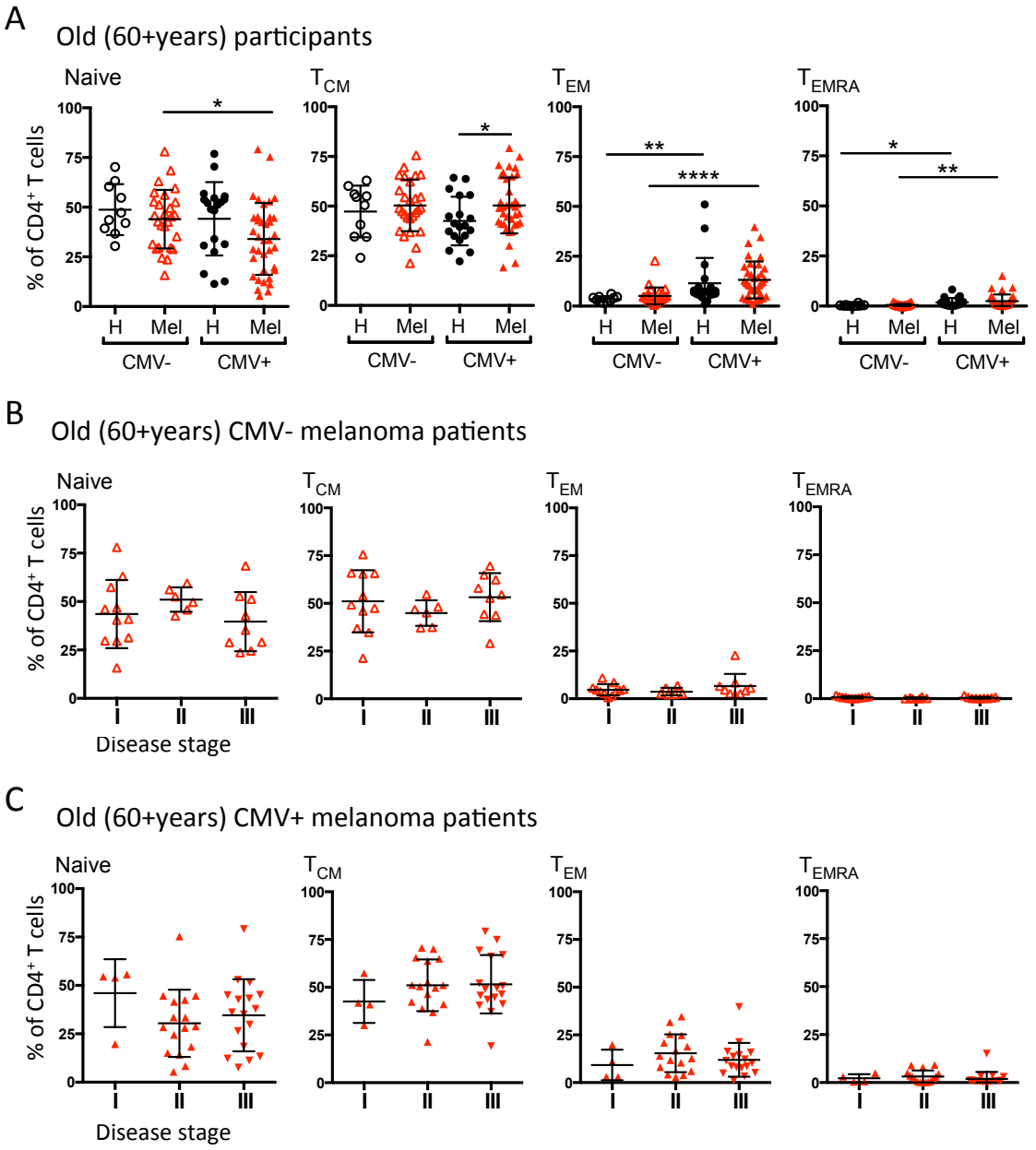


Fig. 3-4: The role of CMV status and disease stage in the subset distribution of circulating CD4$^+$ T cells in old melanoma patients and healthy controls

A: Percentages of CD4$^+$ T cell subpopulations in old (>60 years) CMV positive and CMV negative melanoma patients (n=37 and 27, respectively) and healthy controls (n=19 and 10, respectively).
B: Frequency of CD4$^+$ T cell subsets in old (>60 years) CMV negative melanoma patients according to their disease stage (n=11 for stage I, n=6 for stage II and n=9 for stage III).
C: Frequency of CD4$^+$ T cell subsets in old (>60 years) CMV positive melanoma patients according to their disease stage (n=4 for stage I, n=15 for stage II and n=17 for stage III).
Horizontal lines depict mean values with standard deviation for all graphs and populations were compared using the Student t-test in A and one-way ANOVA in B and C. Note that the old patients of different stages are not age matched.
*=p<0.05; **=p<0.01; ***=p<0.001; ****p>0.0001

3.3.1.3 **$CD4^+FoxP3^+$ Tregs are not increased in old melanoma patients**

$CD4^+$ $FoxP3^+$ Tregs have suppressive activity and are often associated with malignancies (Viguier et al. 2004; Chen et al. 2005). In both, old melanoma patients and healthy controls, the majority of $FoxP3^+$ Tregs were confined to the T_{CM} compartment within the $CD4^+$ T cells (Fig. 3-5A,C). It was therefore investigated if the increase in T_{CM} detected amongst the old CMV positive patients could be linked to an increase in $FoxP3^+$ Tregs in the patients. This was not found to be the case as $FoxP3^+$ Treg frequencies remained similar between circulating $CD4^+$ T cells of old melanoma patients and healthy aged matched controls (Fig. 3-5B).

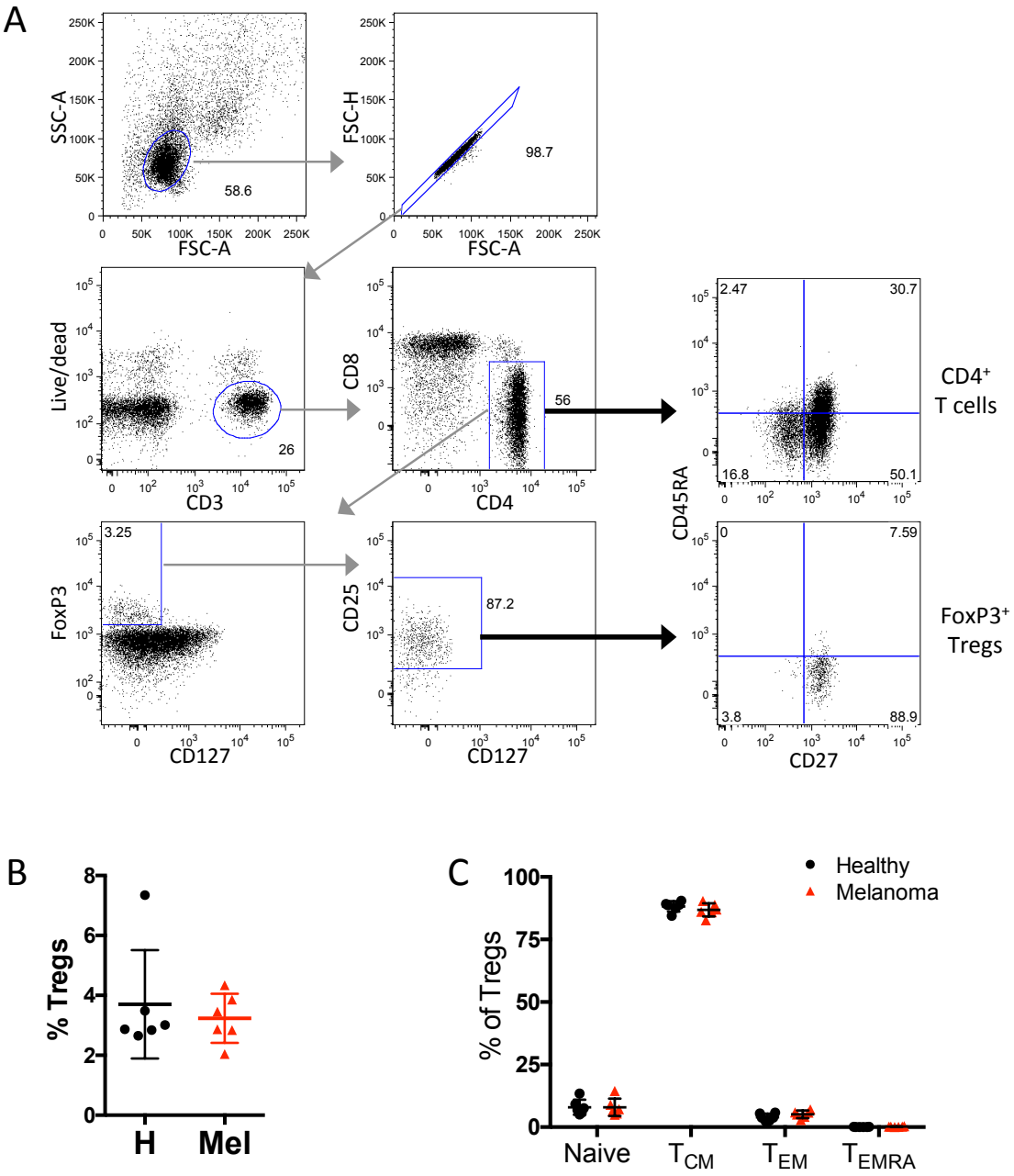


Fig. 3-5: Quantification and phenotyping of circulating CD4$^+$ FoxP3$^+$ Tregs in old melanoma patients and healthy controls

A: Representative gating strategy showing identification of CD4$^+$FoxP3$^+$CD127$^-$CD25$^+$ cells, also known as Tregs, and showing the CD45RA/CD27 phenotype of total CD4$^+$ T cells and Tregs.
B: Percentages of Tregs amongst the CD4$^+$ T cells of old melanoma patients and healthy age matched controls (n=6 each).
C: CD45RA/CD27 subset distribution of Tregs derived from melanoma patients and healthy controls (n=6 each).
Horizontal lines depict mean values with standard deviation for all graphs.

3.3.2 Global circulating $CD8^+$ T cell differentiation in melanoma patients compared to healthy controls

3.3.2.1 Old melanoma patients display an increase in $CD8^+$ T_{EMRA} compared to healthy age matched controls

Each CD45RA/CD27 subset was measured as percentage of the total $CD8^+$ T cell population and plotted against age in healthy individuals and melanoma patients (representative staining and cumulative data are shown in Fig. 3-6A,B). In both healthy and melanoma groups, naïve $CD8^+$ T cells decreased significantly with age (both $p<0.0001$), whilst T_{EM} and T_{EMRA} increased ($p=0.0025$ and $p<0.0001$ for T_{EM} and $p=0.0084$ and $p<0.0001$ for T_{EMRA} respectively). The fraction of T_{CM} also increased with age amongst healthy individuals ($p=0.0057$), but was not found to change with age in the melanoma patients. Young melanoma patients did not show a significant difference in the $CD8^+$ T cell subset distribution compared to age matched healthy controls. Old melanoma patients however showed a significant increase in T_{EMRA} ($p=0.0382$; Fig. 3-6C).

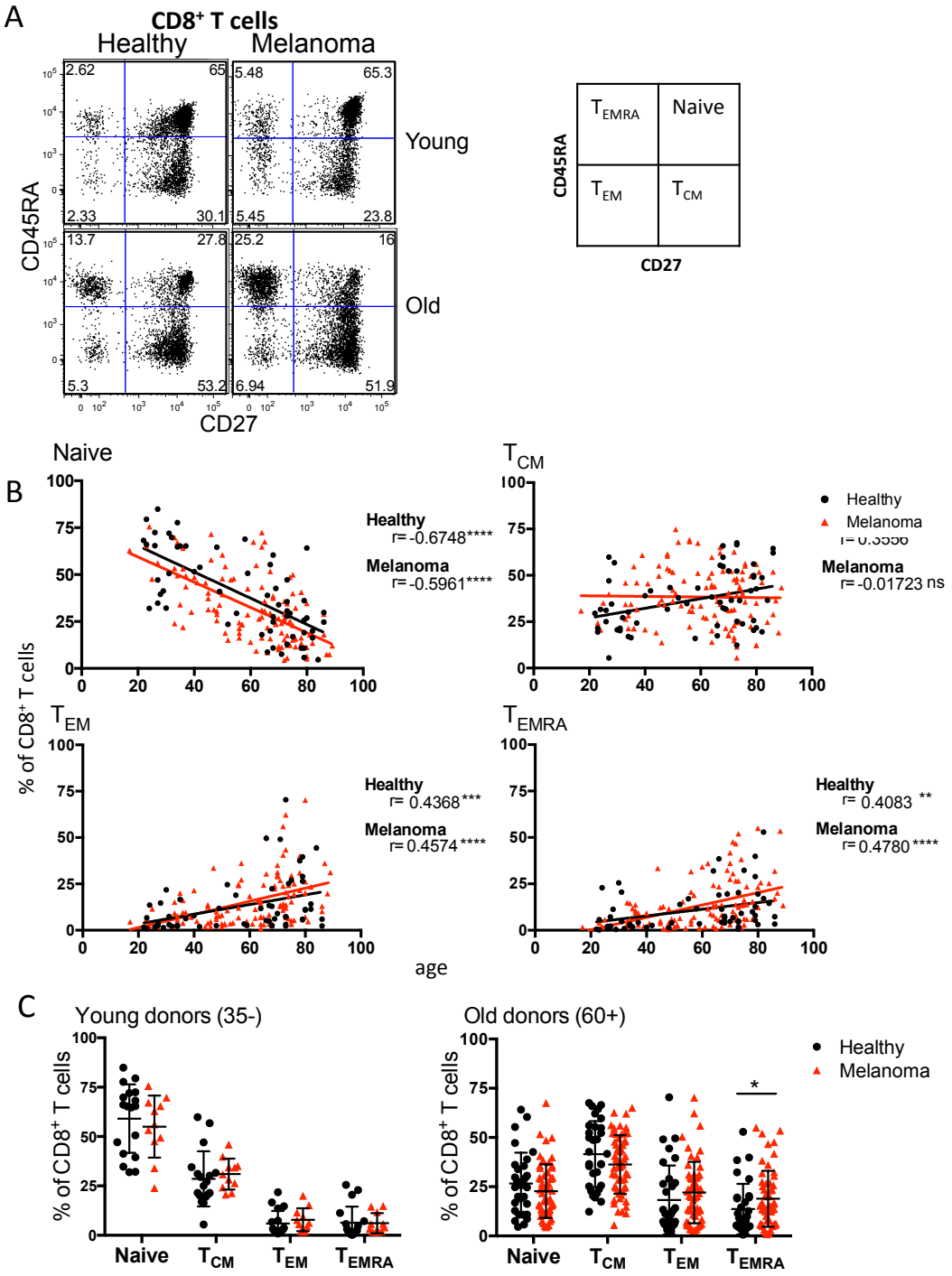


Fig. 3-6: Frequency of CD8$^+$ T cell subsets by age in the blood of melanoma patients and healthy controls

A: Representative FACS plots showing CD45RA/CD27 defined CD8$^+$ T cell subset distribution in young and old melanoma patients and healthy controls. The schematic diagram on the right shows the names given to the subpopulations in each gate.
B: Frequencies of each of the CD45RA/CD27 populations within total CD8$^+$ T cells are correlated against age in melanoma patients (n=113) and healthy controls (n=59). Lines of best fit were generated by linear regression and the correlation assessed by Pearson and Spearman rank.
C: comparison of percentages of the CD8$^+$ T cell subsets between young melanoma patients (n=11) and young healthy controls (18) and between old melanoma patients (n=67) and old healthy controls (n=33). Populations were compared using the unpaired T-test or the Mann-Whitney test. Horizontal lines depict mean values with standard deviation for all graphs.
*=p<0.05; **=p<0.01; ***=p<0.001; ****p>0.0001

3.3.2.2 T_{EMRA} $CD8^+$ T cells are increased in melanoma patients in the absence of CMV

The effect of CMV infection history on $CD8^+$ T cell differentiation in older melanoma patients and healthy controls was also assessed (Fig. 3-7A). Positive CMV status was linked to a decrease in naïve $CD8^+$ T cell frequencies in patients ($p<0.0001$) and controls ($p=0.0156$) and in T_{CM} ($p=0.0088$ in melanoma and $p<0.0001$ in healthy subjects). T_{EM} levels were increased among CMV positive melanoma patients ($p<0.0001$) and controls ($p=0.0024$) compared to individuals from the same groups that were unaffected by the virus and so were T_{EMRA} levels ($p=0.0009$ in melanoma and $p=0.0018$ in healthy subjects). Intriguingly, old melanoma patients who did not have CMV still displayed decreased levels of T_{CM} and increased levels of T_{EM} and T_{EMRA} compared to healthy age matched individuals who were also CMV negative ($p=0.0044$ for differences in T_{CM}, $p=0.0275$ in T_{EM} and $p=0.0470$ in T_{EMRA}; Fig. 3-7A).

In order to determine whether the accumulation of these highly differentiated $CD8^+$ T_{EMRA} cells in melanoma patients was related to disease severity, the CMV negative melanoma patients aged 60 or more were further subdivided into their respective disease stage for comparison of $CD8^+$ T cell subset frequencies (Fig. 3-7B,C). However, no association could be found between increased disease stage and $CD8^+$ T_{EMRA} cell frequency, although it should be noted that the old participants of different stages were not age matched.

A non-CMV associated increase in highly differentiated $CD8^+$ T_{EMRA} cells was therefore detected in old melanoma patients. However, since high T_{EMRA} levels could not be linked to disease stage, no links could be drawn between T_{EMRA} levels and disease severity.

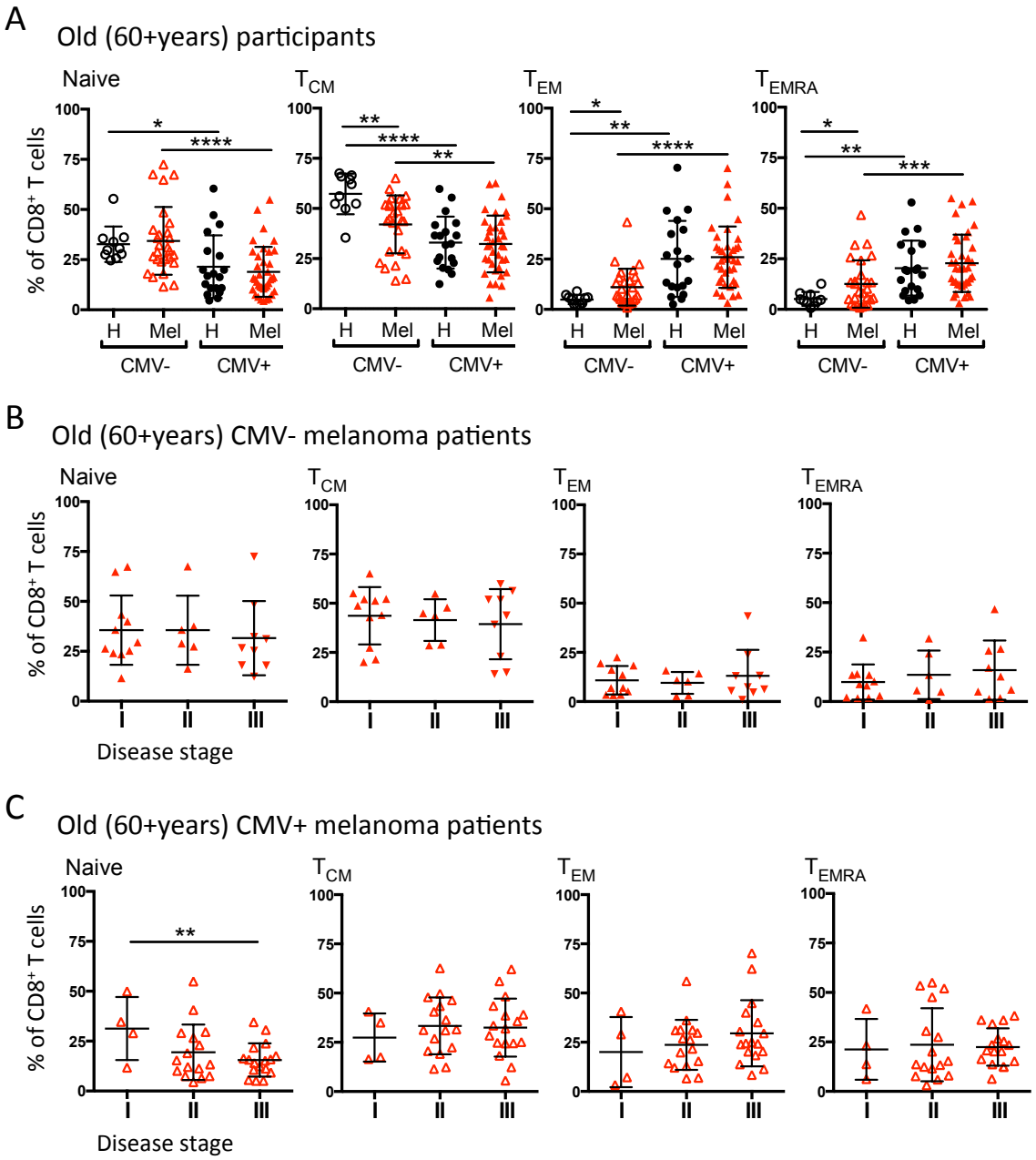


Fig. 3-7: The role of CMV status and disease stage in the CD8$^+$ T cell subset distribution in the circulation of old melanoma patients and healthy controls

A: Percentages of CD8$^+$ T cell subpopulations in old (>60 years) CMV positive and CMV negative melanoma patients (n=37 and 27, respectively) and positive and negative healthy controls (n=19 and 10, respectively).
B: Frequency of CD8$^+$ T cell subsets in old CMV negative melanoma patients according to their disease stage (n=11 for stage I, n=6 for stage II and n=9 for stage III).
C: Frequency of CD8$^+$ T cell subsets in old (>60 years) CMV positive melanoma patients according to their disease stage (n=4 for stage I, n=15 for stage II and n=17 for stage III).
Horizontal lines depict mean values with standard deviation for all graphs and populations were compared using the student t-test or Mann-Whitney test (in A) or one-way ANOVA or Kruskal Wallis test (in B and C). Note that the old patients of different stages are not age matched.
*=p<0.05; **=p<0.001; ***=p<0.001; ****=p<0.0001

3.3.2.3 **EBV infection is not responsible for increased T_{EMRA} levels in CMV negative patients.**

EBV is another common persistent herpes virus that potentially generates $CD45RA^+$ memory cells (Dunne et al. 2002) and might therefore explain the high levels of T_{EMRA} found in some CMV negative melanoma patients. However, cytokine production in response to EBV lysate was only found in one out of six melanoma patients who had previously been shown to display high frequencies of T_{EMRA} in the absence of CMV (Fig. 3-8).

In order to rule out the possibility that the PBMCs from the patients of interest had a defect in their ability to respond to stimuli *in vitro* and might therefore be mis-interpreted as CMV or EBV negative, cells were further treated with Varicella zoster virus (VZV) lysate or the superantigen Staphylococcus Enterotoxin B (SEB). Four out of six melanoma patients responded to VZV lysate and all responded to unspecific stimulation with SEB, confirming that the cells were able to respond to other stimuli (Fig. 3-8).

Additionally to CMV, EBV could therefore be ruled out as potential cause for increased T_{EMRA} in the melanoma patients.

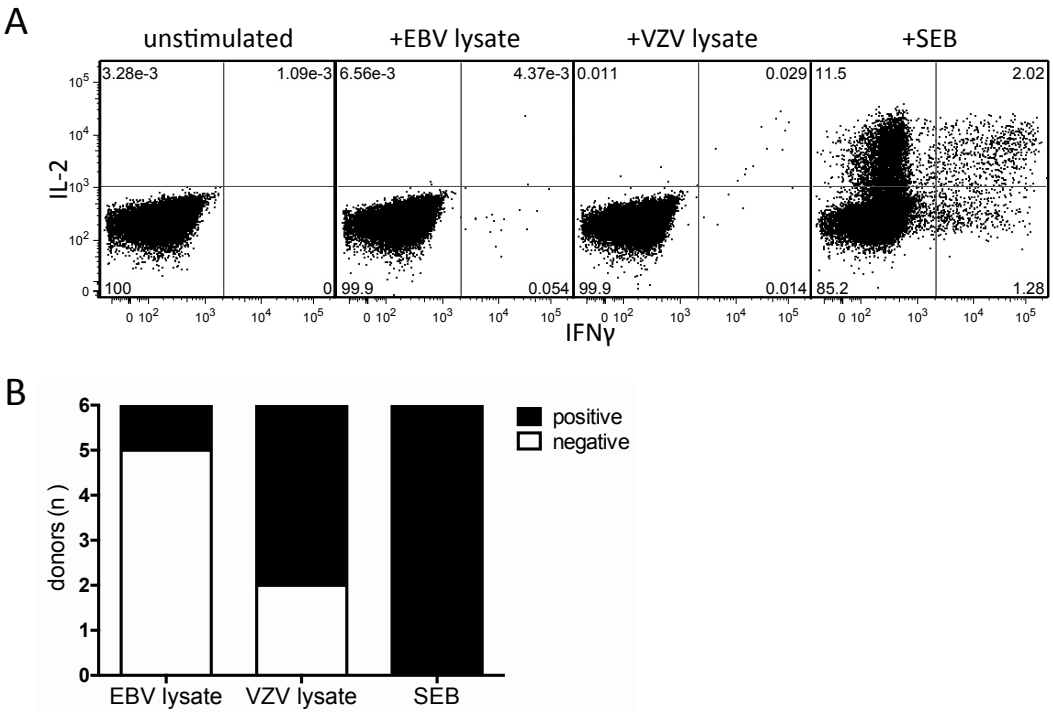


Fig. 3-8: EBV and VZV prevalence in the six old melanoma patients who displayed high frequencies of $CD8^+$ T_{EMRA} in the absence of CMV.

PBMCs were stimulated overnight with Staphylococcus enterotoxin B (SEB), Epstein–Barr virus (EBV) or Varicella zoster virus (VZV) lysate or left unstimulated. IL-2 and IFNγ production were measured intracellularily in the $CD4^+$ T cell subset by flow cytometry and compared to the unstimulated control to determine presence of anti-viral responses. SEB was used as positive control. The donors selected were 6 melanoma patients who had previously been shown to have high $CD8^+$ T_{EMRA} frequencies despite appearing to be CMV negative.
A: representative flow cytometric gating showing cytokine responses of $CD4^+$ T cells under the various stimulation conditions.
B: Stacked histograms showing the prevalence of individuals responding to EBV, VZV and SEB *in vitro*.

3.3.3 Characteristics of $CD8^+$ T cell subsets of old melanoma patients

As an increase in $CD8^+$ T_{EM} and T_{EMRA} was observed in the circulation of patients with melanoma, it was of interest to investigate whether these cells displayed the same functional and phenotypic properties as in healthy individuals. Features of senescence and exhaustion, cytotoxic potential, proinflammatory cytokine production and expression of skin homing markers were therefore assessed in the $CD8^+$ T cell subpopulations of melanoma patients aged 60 and above.

3.3.3.1 $CD8^+$ T_{EMRA} of old melanoma patients display features of senescence

T_{EMRA} are thought to be the most differentiated T cell subset based on their low proliferative capacity and telomere length compared to the other subsets (Di Mitri et al. 2011; Libri et al. 2011; S. M. Henson et al. 2014). Indeed T_{EMRA} of old melanoma patients were confirmed to have the lowest levels of proliferative capacity amongst all the subsets, as shown by ki67 expression following three days of stimulation with plate bound anti-CD3 and irradiated autologous antigen presenting cells (difference between T_{EMRA} and naïve: p=0.0008 and between T_{EMRA} and T_{CM}: p=0.0011; Fig. 3-9). Similarly and as has been described in healthy subsets, T_{EMRA} of melanoma patients showed significantly reduced telomere lengths compared to Naive (p=0.0098) and T_{CM} p=0.0342) subsets (Fig. 3-10) and reduced capacity to produce IL-2 compared to T_{CM} and T_{EM} (p>0.0005; Fig. 3-11).

CD57 and KLRG1 have previously been described as senescence-associated markers. Indeed, the co-expression of these receptors was found to be highest in T_{EMRA} of melanoma patients and healthy controls alike (Fig. 3-12).

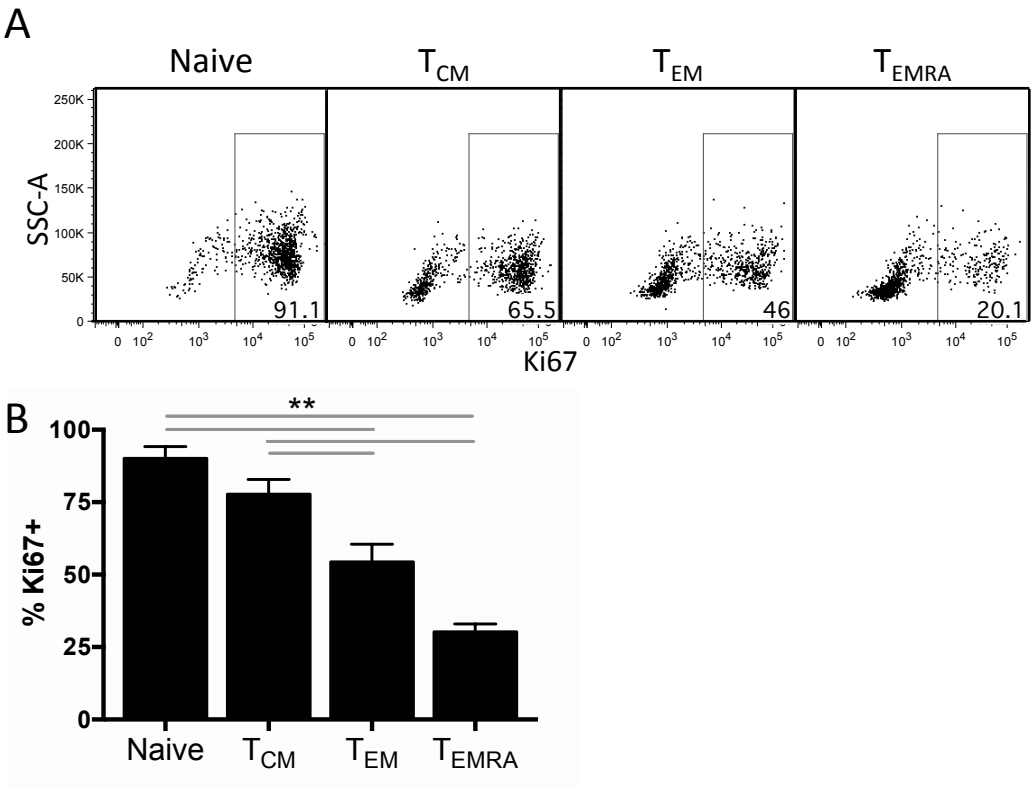


Fig. 3-9: Proliferative capacity of blood-derived $CD8^+$ T cell subpopulations in old melanoma patients measured by Ki67 expression.

$CD8^+$ T cells were FACS sorted according to their CD27 and CD45RA expression and incubated for 3 days in the presence of 0.5µg/ml plate bound anti-CD3 antibody and irradiated autologous antigen presenting cells.
A: Representative flow cytometry dor plots showing Ki67 in the various subsets
B: Cumulative data showing mean Ki67 levels and standard error amongst the various subsets obtained from 5 participants.
Populations were compared using one-way ANOVA.
**p<0.01

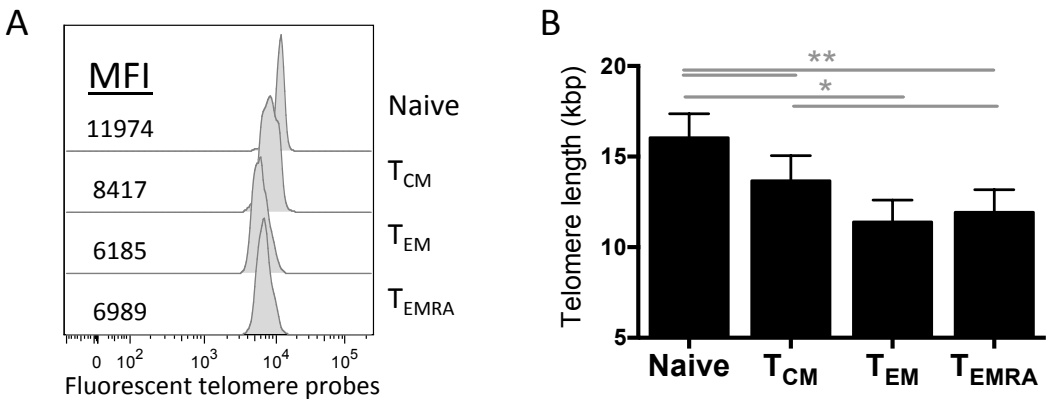


Fig. 3-10: Telomere length of blood-derived CD8$^+$ T cell subsets of old melanoma patients.

Telomere length was measured using the Flow-FISH method (described in detail in the materials and methods chapter).
A: Representative histograms showing mean fluorescence (MFI) of telomere probe stained CD8$^+$ T cells subsets.
B: Bars show mean telomere lengths in kilo base pairs (kbp) in the subsets with standard error in 3 participants. Populations were compared using one-way ANOVA. *=p<0.05; **=p<0.01

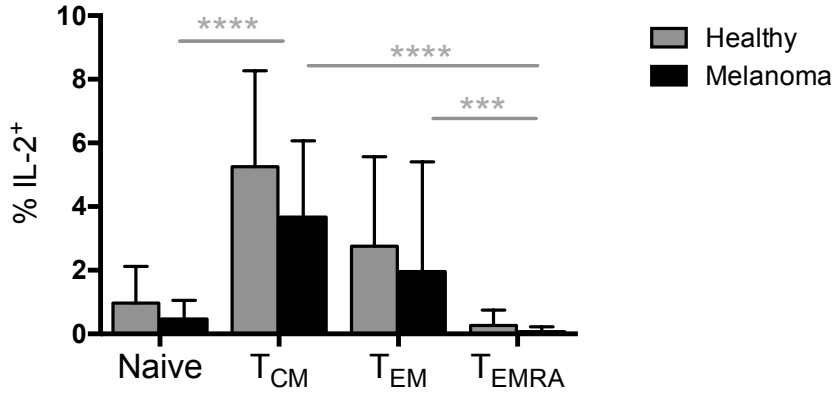


Fig. 3-11 IL-2 production in the blood-derived CD8$^+$ T cell subsets of old melanoma patients and healthy controls

PBMCs from old (60$^+$ years) melanoma (n=17) patients and healthy controls (n=17) were stimulated overnight with the superantigen SEB before being measuring IL-2 production per cell intracellularily via flow cytometry. Horizontal bars and stars highlight significant differences between the melanoma patient subsets, calculated with the Friedman test. Vertical bars indicate standard deviation.
***p<0.001; ****p<0.0001

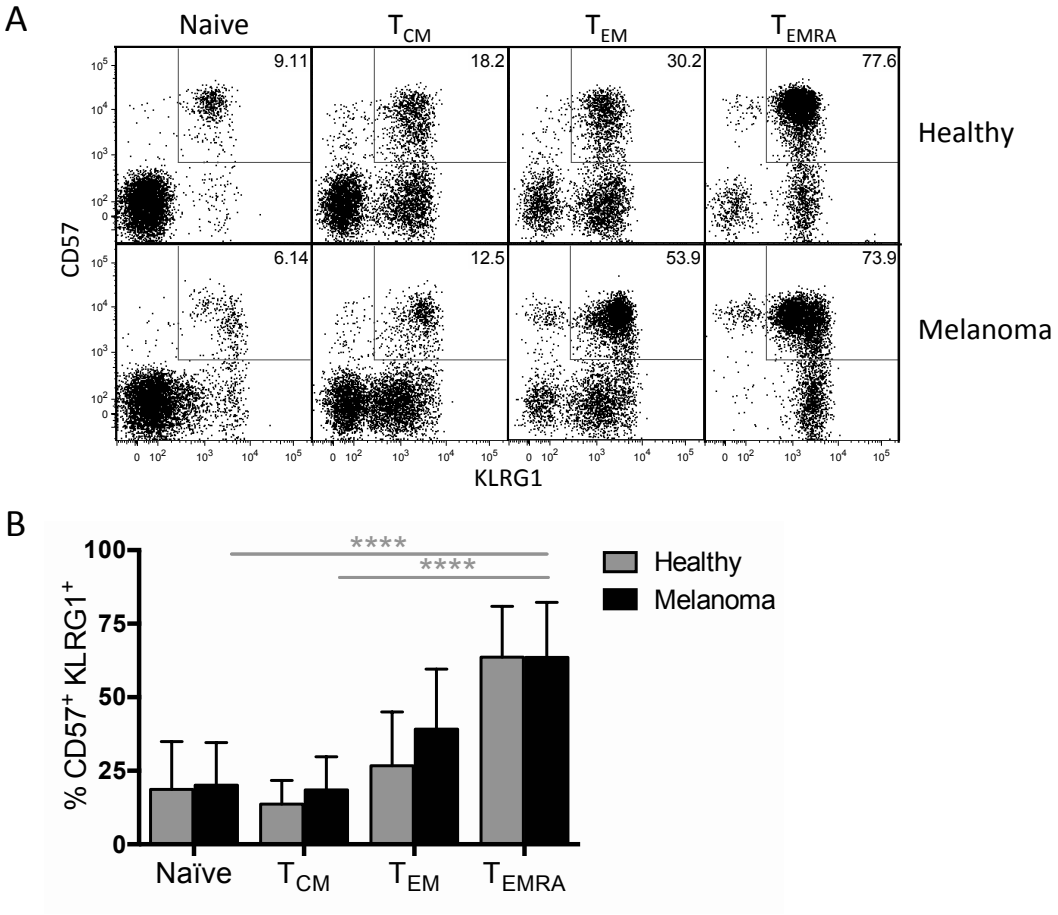


Fig. 3-12: Senescence associated marker expression in blood-derived $CD8^+$ T cell subsets of old melanoma patients compared to healthy controls.

CD57 and KLRG1 expression were measured *ex vivo* in $CD8^+$ T cell subsets using flow cytometry.
A: Representative flow cytometric dot plots showing CD57 and KLRG1 coexpression in the subsets of melanoma patients and healthy controls.
B: Frequency of CD57 and KLRG1 double expressing cells in the $CD8^+$ T cell subsets of old melanoma patients (n=15) and healthy controls (n=19). Vertical bars indicate standard deviations. Stars and horizontal bars highlight significant differences between the melanoma patients' subsets (calculated using the Friedman test). Vertical bars indicate standard deviation.
*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

3.3.3.2 **Cytotoxic potential in the subsets of old melanoma patients**

Despite showing decreased ability to proliferate and an increase in senescent features, T_{EMRA} of healthy individuals display an array of effector functions (Henson et al. 2014). In old melanoma patients and old healthy individuals alike, T_{EMRA} were found to bear the highest levels of perforin and granzyme B coexpression out of all the $CD8^+$ T cell subsets, as 57±23% of T_{EMRA} in healthy and 59±31% of T_{EMRA} in old melanoma patients expressed both granzyme B and perforin (Fig. 3-13).

CD107a, a marker of cytotoxic granule release, was measured after overnight stimulation with anti-CD3 antibody (Fig. 3-14). In old healthy individuals, the highest levels of CD107a were measured in the T_{EM} (14±4%) and T_{EMRA} (15±4%) $CD8^+$ T cell subsets. Melanoma patients were found to have significantly increased levels of CD107a in their T_{EM} compartment (20±6%; p=0.0355), but similar levels the T_{EMRA} compartment (14±6%) compared to healthy controls.

T_{EMRA} of melanoma patients therefore resembled those of healthy individuals in terms of cytotoxic potential.

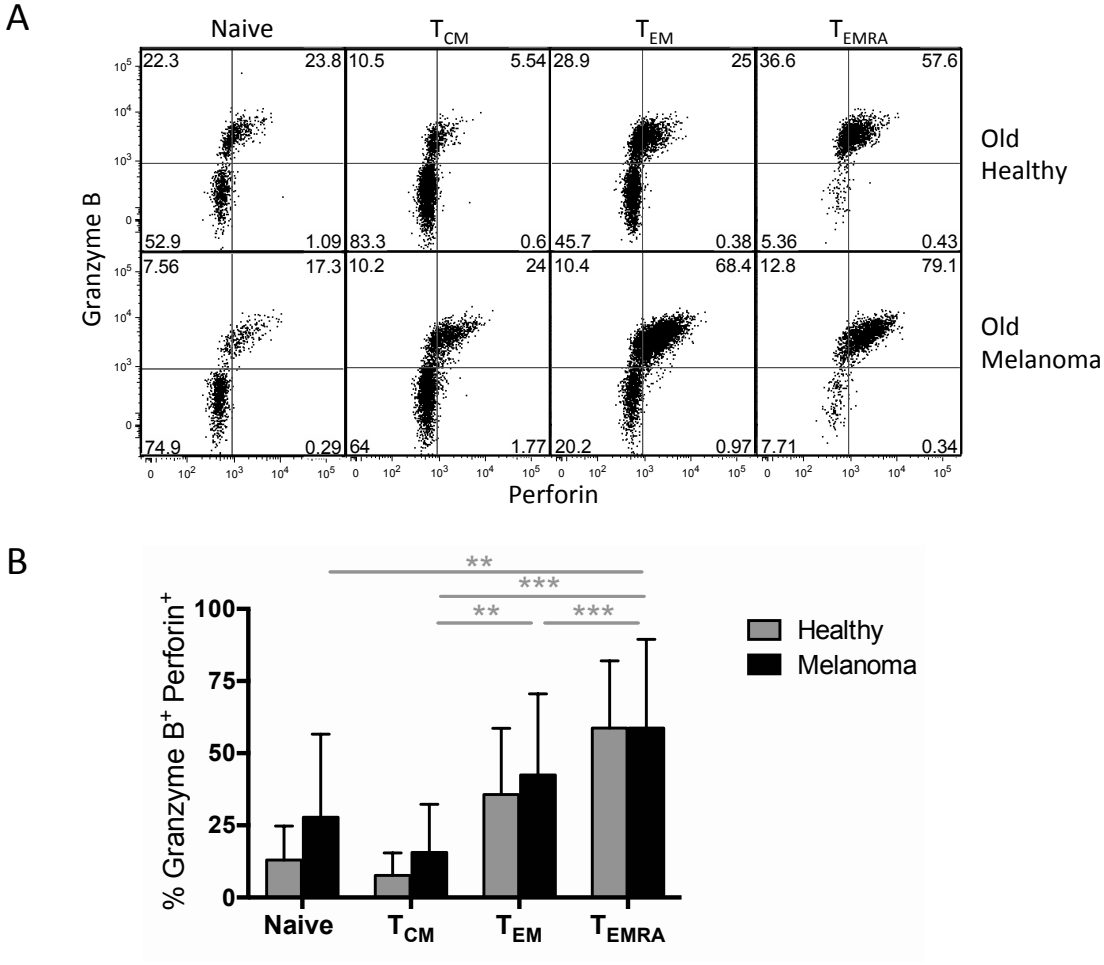


Fig. 3-13: Cytotoxic granule components located in blood-derived CD8$^+$ T cell subsets of old melanoma patients and healthy controls.

A: Representative flow cytometry dot plots showing Granzyme B and Perforin expression patters in the CD8$^+$ T cell subsets of old melanoma patients and healthy controls.
B: Cumulative data showing standard deviations and means of granzyme B and perforin coexpression in the CD8$^+$ T cell subsets of old melanoma patients (n=11) and healthy controls (n=14). Grey horizontal bars and stars mark statistical differences in the frequency of Granzyme B and Perforin coexpressing cells amongst the subsets of the melanoma patients. These were calculated using one-way ANOVA.
**p<0.01; ***p<0.001

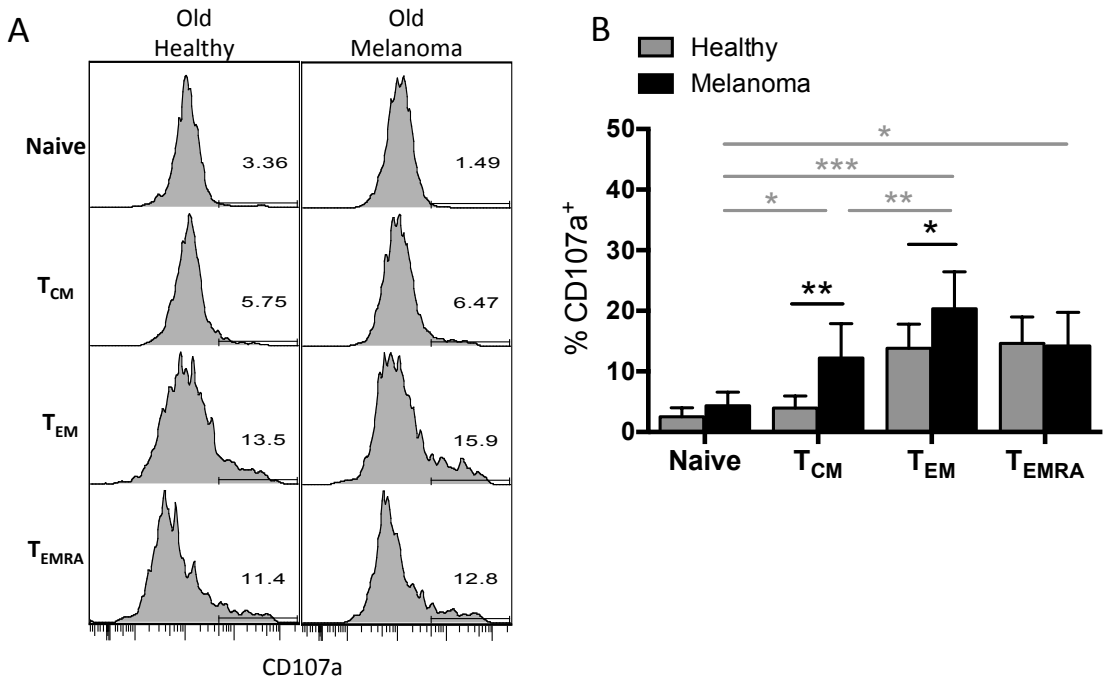


Fig. 3-14: Cytotoxic degranulation in the blood-derived $CD8^+$ T cell subsets of old melanoma patients and healthy controls.

PBMCs were stimulated overnight in the presence of plate-bound anti-CD3 antibody and fluorochrome-conjugated anti-CD107a antibody. Cells were labelled with the appropriate antibodies the following day before being analysed via flow cytometry.
A: Representative flow cytometric histograms showing CD107a expression in the $CD8^+$ T cell subsets of old melanoma patients and healthy controls.
B: Cumulative data showing average percentages and standard deviations of $CD107a^+$ cells in the $CD8^+$ T cell subsets of old melanoma patients (n=8) and healthy controls (n=6). Stars in black denote significant differences in CD107a expression between patient and control subsets (calculated by the unpaired T-test). Stars in grey highlight significant differences between the subsets within the melanoma patients (calculated using one-way ANOVA). Vertical bars indicate standard deviation.
*p<0.05; **p<0.01; ***p<0.001

3.3.3.3 T_{EMRA} of old melanoma patients produce less TNFα than those of healthy controls

Known effector functions associated with T_{EMRA} are not only restricted to cytotoxic granule components but also include increased ability to produce high levels of effector cytokines such as TNFα and IFNγ (Hamann et al. 1997).

In order to test whether T_{EMRA} cells of the old melanoma patients were also able to produce these cytokines, IFNγ and TNFα production were measured in response to overnight stimulation with anti-CD3 antibody via intracellular flow cytometry in the $CD8^+$ T cell subsets of old participants (Fig. 3-15). Whilst T_{EMRA} and T_{EM} were the highest producers of both cytokines among the healthy, T_{EMRA} of old melanoma patients produced significantly less TNFα than their healthy counterparts ($p=0.0111$).

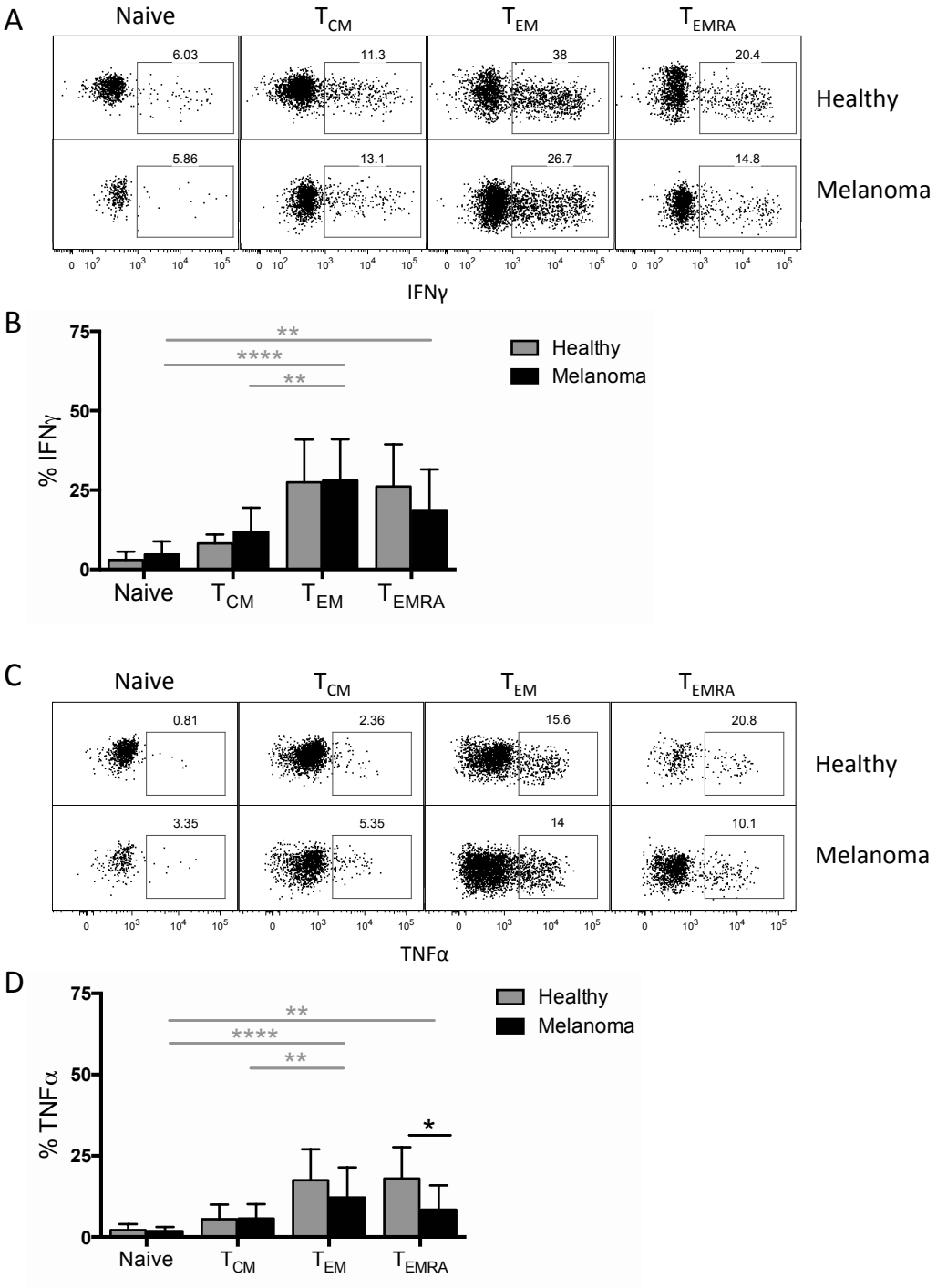


Fig. 3-15: Cytokine production in blood-derived CD8$^+$ T cell subsets in old healthy individuals and melanoma patients (aged 60$^+$)

IFNγ and TNFα cytokine production were measured by intracellular flow cytometry staining in the CD8$^+$ T cell subsets of old melanoma patients (n=13) and healthy controls (n=9) following overnight incubation in the presence of immobilized anti-CD3 antibody.
A: Representative flow cytometric dot plots and
B: Cumulative data showing IFNγ production in the CD8$^+$ T cell subsets.
C: Representative flow cytometric dot plots and
D: Cumulative data showing TNFα production in the CD8$^+$ T cell subsets.
Histograms show mean values with standard deviation. Stars in black denote significant differences in cytokine production between the patients' and control subsets (calculated by the unpaired T-test). Stars in grey highlight significant differences between the subsets within the melanoma patients (calculated using one-way ANOVA).
*=p<0.05; **p<0.01; ****p<0.0001

3.3.3.4 Exhaustion markers PD-1 and CTLA-4 are not increased in the circulating $CD8^+$ T cell subsets of old melanoma patients compared to healthy controls

Exhaustion in T cells is characterized by the simultaneous expression of an array of inhibitory markers on the surface of these cells (Blackburn et al. 2009). Since CTLA-4 and PD-1 expression are associated with immune exhaustion, the expression of these molecules was measured in the T cell subsets of old healthy individuals and melanoma patients. Both PD-1 and CTLA-4 were highest in the T_{CM} and T_{EM} subsets compared to the Naïve and T_{EMRA} cells in both participant groups and not different between patients and controls (Fig. 3-16). Therefore, T_{EMRA} in melanoma patients did not display features of immune exhaustion.

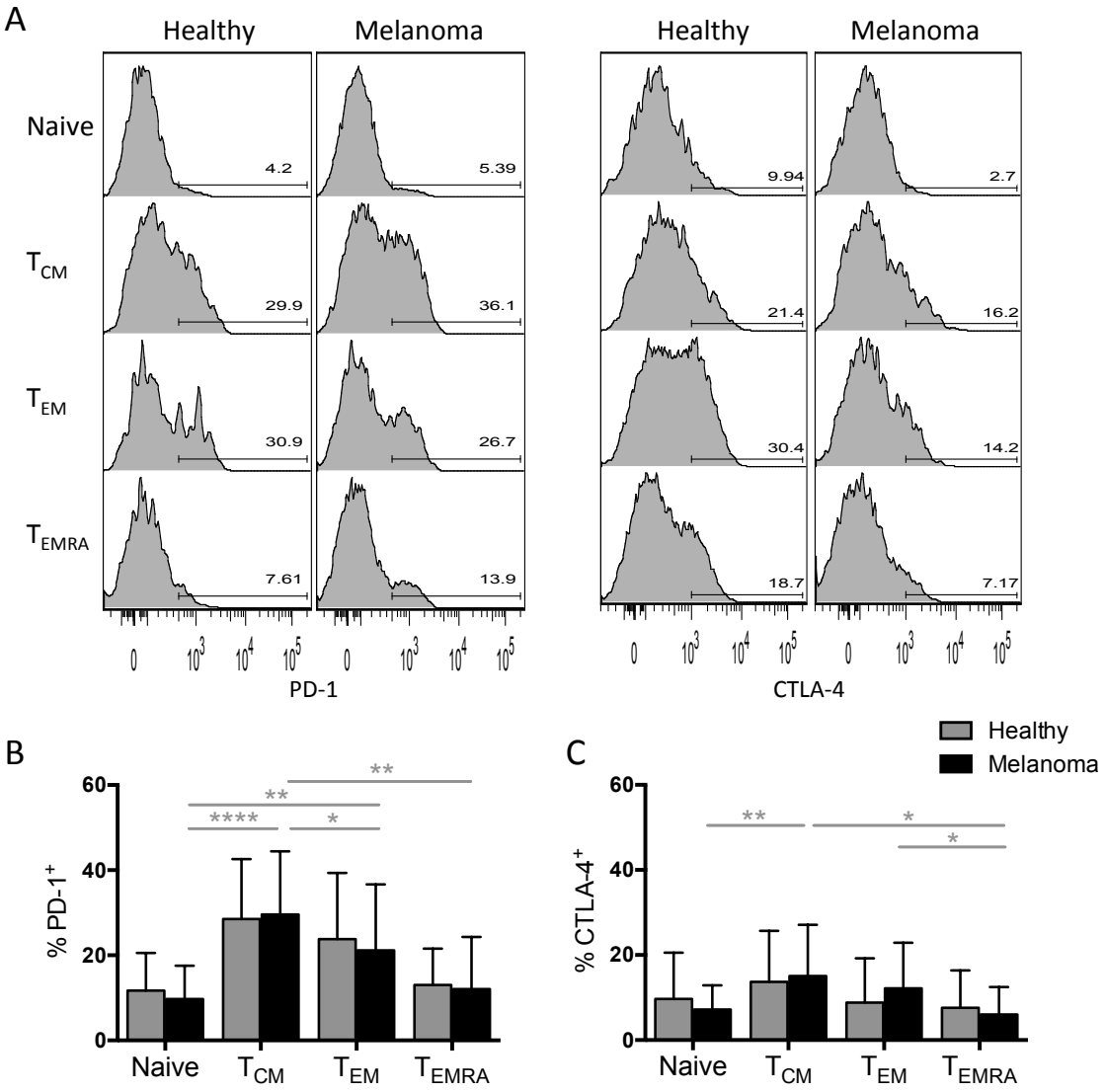


Fig. 3-16: Expression of exhaustion the associated markers PD-1 and CLTLA-4 in the blood-derived CD8$^+$ subsets of old melanoma patients and healthy controls.

PD-1 surface expression was detected ex vivo via flow cytometry on unstimulated cells, whilst intracellular detection of CTLA-4 necessitated stimulation of the PBMCs with immobilized anti-CD3 antibody overnight before intracellular staining.
A: Histograms show representative gating for PD-1 and CTLA-4 in the various CD8$^+$ T cell subsets.
B: Frequencies of PD-1$^+$ cells in the CD8$^+$ T cell subsets of old melanoma patients (n=15) and healthy individuals (n=17).
C: Frequencies of CTLA-4$^+$ cells amongst the CD8$^+$ T cell subsets of old melanoma patients (n=10) and healthy controls (n=10)
Bar graphs depict mean values with standard deviation. Grey horizontal lines and stars highlight statistical differences in PD-1 or CTLA-4 percentage expression between the individual subsets of the melanoma patients and were calculated using one-way ANOVA.
*p<0.05; **p<0.01; ****p<0.0001

3.3.3.5 The skin homing marker CLA is reduced among the T_{CM} and T_{EM} of old melanoma patients

CLA and CCR4 are surface receptors involved in the migration of circulating T cells into the skin. CLA and CCR4 expression were measured on the surface of the $CD8^+$ T cell subsets in old melanoma patients and healthy controls (Fig. 3-17).

In the $CD8^+$ T cells of old melanoma patients, the highest levels of CLA and CCR4 were found on cells of intermediate differentiation stages. CLA was expressed by 11±5% of T_{CM} and 6±6% of T_{EM}. CCR4 was detected on 6±2% of T_{CM} and on 5±3% of T_{EM}. Only 2±1% of naïve or T_{EMRA} subsets of melanoma patients expressed CLA or CCR4. The relative distribution of the homing markers was similar for healthy individuals. Interestingly melanoma patients were found to display significantly decreased levels of CLA on their T_{CM} and T_{EM} subsets ($p=0.0428$ and 0.0077 respectively) compared to healthy controls (Fig. 3-17). Therefore, CLA was reduced in the old melanoma patients compared to healthy controls, particularly in the T_{CM} and T_{EM} subsets.

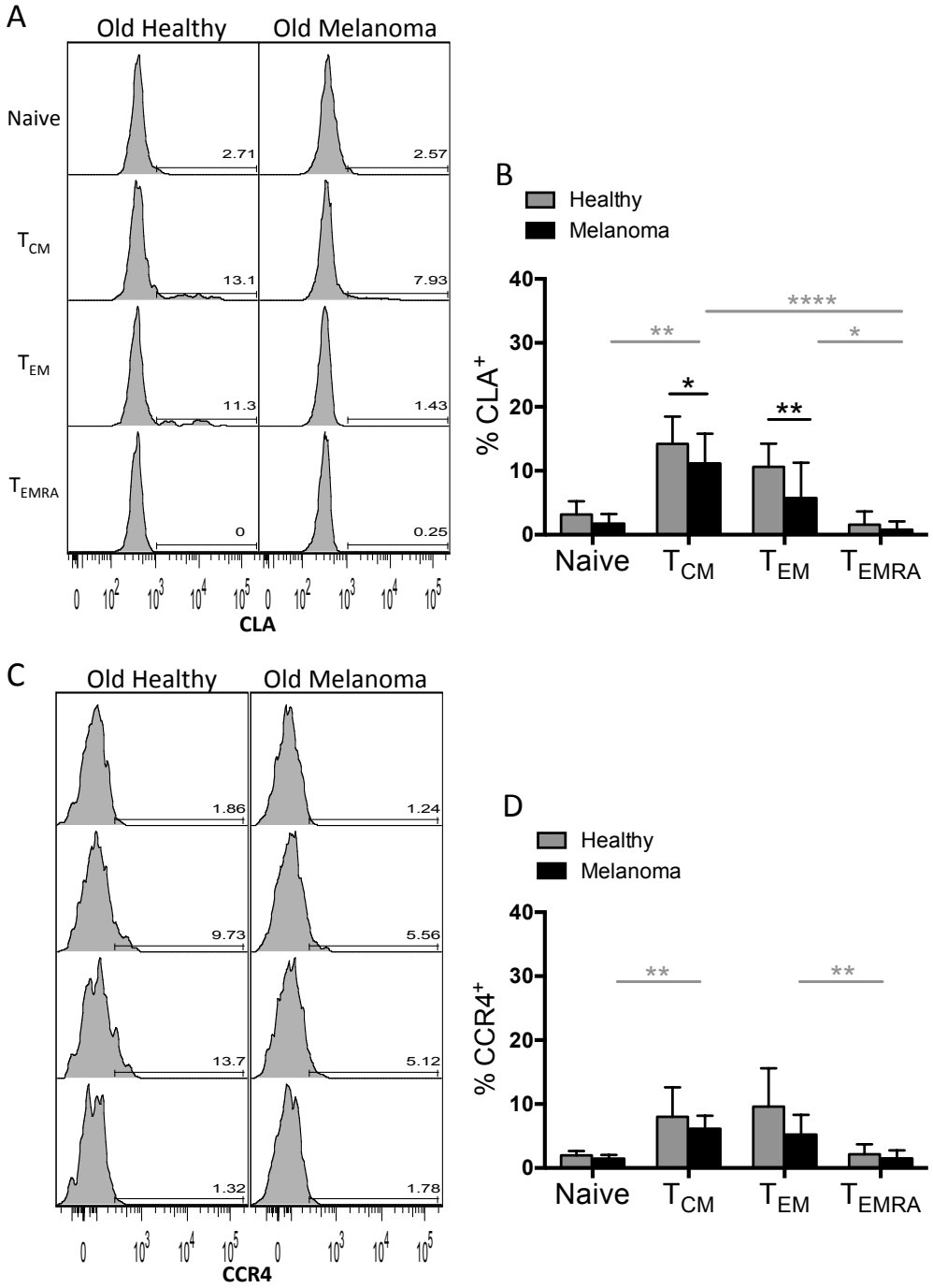


Fig. 3-17: Expression of the skin homing markers CLA and CCR4 in the blood-derived CD8$^+$ T cell subsets of old melanoma patients and healthy controls.

CLA and CCR4 expression were measured ex vivo by flow cytometry in circulating the subsets of CD8$^+$ T cells of old melanoma patients and healthy age matched controls.
A: Representative FACS histograms showing the expression patterns of the skin homing surface receptor CLA in the CD8$^+$ T cell subsets of old melanoma patients and healthy controls
B: Cumulative data showing the percentage CLA surface expression in the CD8$^+$ T cell subsets of old melanoma patients (n=15) and healthy controls (n=11)
C: Representative FACS histograms showing the expression patterns of the skin homing surface receptor CCR4 in the CD8$^+$ T cell subsets of old melanoma patients and healthy controls
D: Cumulative data showing the percentage CCR4 surface expression in the CD8$^+$ T cell subsets of old melanoma patients (n=6) and healthy controls (n=6)
Mean and standard deviation are shown; Participants were compared using the unpaired T-test or Mann-Whitney test, subsets from melanoma patients by one-way ANOVA.
*p<0.05; **p<0.01; ****p<0.0001

3.3.4 **Differentiation status of tumour specific $CD8^+$ T cells in old melanoma patients**

3.3.4.1 **Optimization and gating for the detection of melanoma-specific T cells**

We next investigated whether the highly differentiated T_{EMRA} cells found to be expanded in old melanoma patients were specific to melanoma antigens. Fluorochrome labelled peptide loaded MHC class I multimers are routinely used to identify peptide specific $CD8^+$ T cells in patients with the appropriate matched HLA haplotype. A Melan-A peptide loaded HLA-A2 pentamer was initially used, but produced unreliable and non-specific staining. When a Melan-A specific MHC class I Dextramer was used, it generated reproducible data that allowed labelling of $CD8^+$ T cell populations without staining positively in any HLA-A2 negative participants. Every experiment included a negative control dextramer stain by using a MHC dextramer loaded with an irrelevant peptide (see Fig. 3-18).

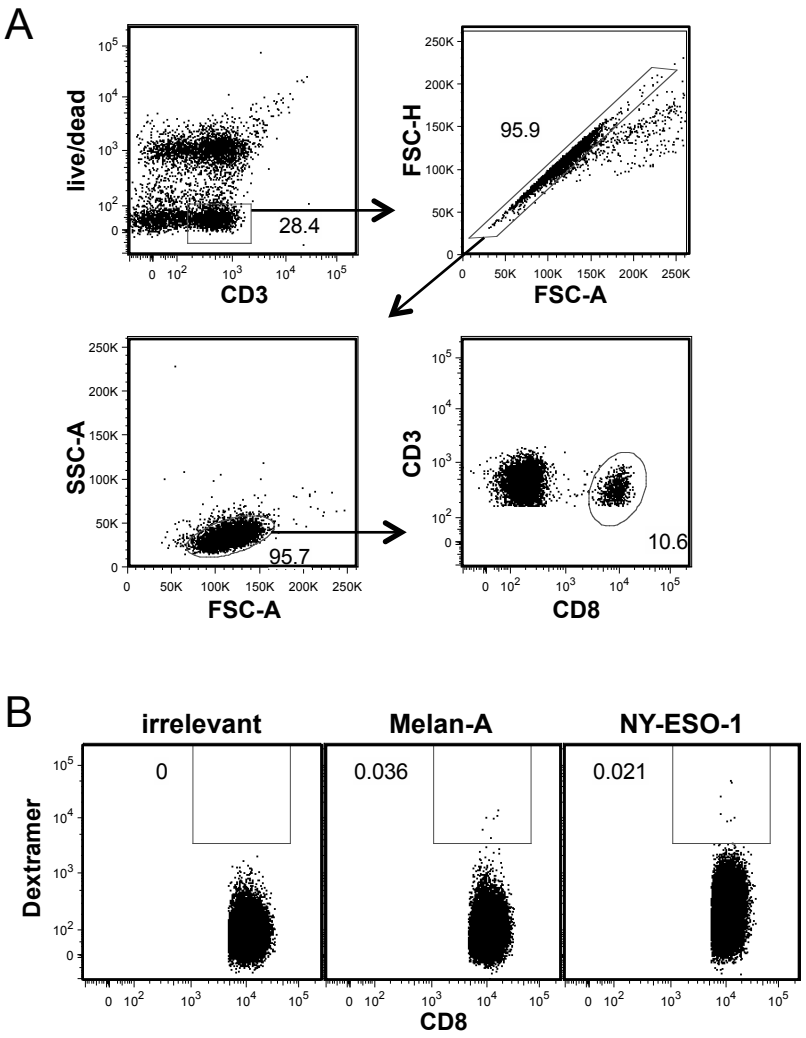


Fig. 3-18: Detection of Melan-A and NY-ESO-1 specific cells among blood-derived CD8+ T cells using fluorochrome conjugated MHC class I dextramers.

Representative flow cytometry gating strategy used to identify melanoma specific $CD8^+$ T cells in a melanoma patient.
A: Gating of true live $CD8^+$ T cells, excluding doublets.
B: Gates for Melan-A and NY-ESO-1 peptide containing Dextramer stains were drawn according to a control sample stained with a dextramer containing an irrelevant peptide as shown in the flow cytometry plots.

3.3.4.2 **Melan-A specific $CD8^+$ T cells in old melanoma patients belong to naïve or T_{CM} subsets.**

Melan-A peptide loaded dextramers were used to detect Melan-A specific cells among the PBMCs of HLA-A2 positive participants. Four out of seven stage I, seven out of eight stage II and ten out of 12 stage III old melanoma patients displayed detectable levels of Melan-A specific $CD8^+$ T cells (Fig. 3-19A). The frequency of these cells ranged between 0.01 to 0.09% of the total $CD8^+$ T cells and was not found to be significantly altered among the old melanoma patients of different stages ($p>0.05$).

Based on their CD27/CD45RA expression profiles, Melan-A cells detected among the patients were found to belong either to the naïve or the T_{CM} subset (Fig. 3-19C). None of the old melanoma patients screened displayed Melan-A specific cells with a T_{EM} or T_{EMRA} phenotype. In order to see if the phenotype of Melan-A specific cells changed with disease stage, we compared the frequency of naïve-like cells among the Melan-A specific cells between patients of stages I, II and III (Fig. 3-19D). The fraction of Melan-A specific cells averaged 84±18% in stage I, 73±33% in stage II and 75±27% in stage III patients. Therefore, frequency and differentiation state of Melan-A specific cells were unchanged between patients of different disease stages.

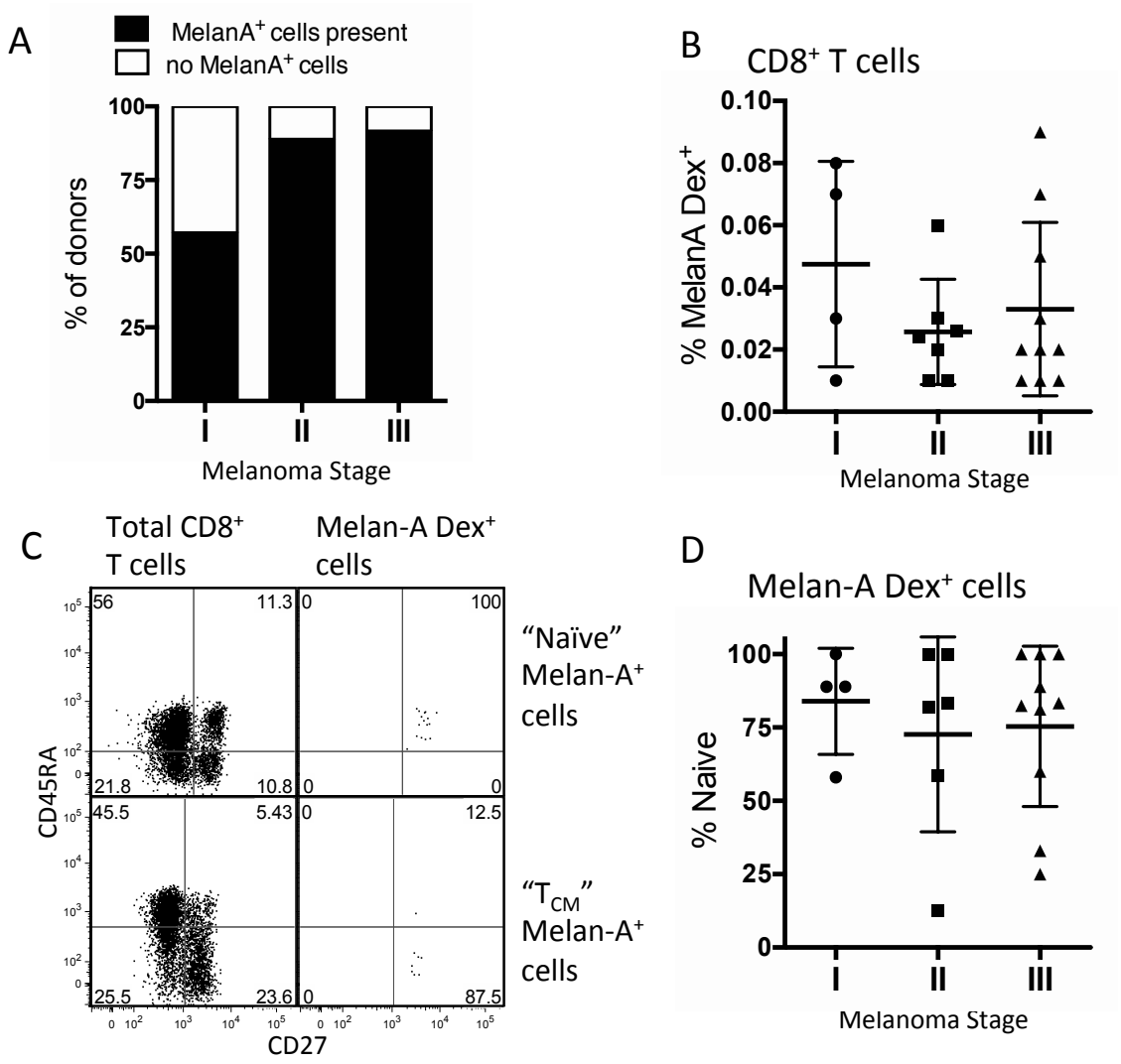


Fig. 3-19: Frequency and differentiation profiles of Melan-A specific CD8$^+$ T cells in the circulation of old melanoma patients.

Melan-A specific CD8$^+$ T cells were detected by flow cytometry using fluorochrome conjugated Melan-A peptide loaded MHC class I HLA-A2 Dextramers.
A: Frequency of stage I, II and III donors presenting with detectable levels of Melan-A specific cells amongst their CD8$^+$ T cell compartment.
B: Percentages of Melan-A specific cells amongst CD8$^+$ T cells of stage I (n=7), II (n=8) and III (n=12) melanoma patients. Means and standard deviation are shown.
C: Representative flow cytometry dot plots showing CD45RA and CD27 expression patterns in total CD8$^+$ T cells and Melan-A specific cells detected in old melanoma patients.
D: Percentages of Melan-A specific cells that display a naïve-like phenotype in stage I (n=4), II (n=6) and III (n=10) melanoma patients. Means and standard deviation are shown.
Populations were compared using the one-way ANOVA.

3.3.4.3 **Low frequency of NY-ESO-1 specific CD8$^+$ T cells amongst melanoma patients**

A second HLA-A2 dextramer containing a peptide of NY-ESO-1, another widely described melanoma antigen, was used to detect and characterize melanoma specific CD8$^+$ T cells amongst the old melanoma patients (Fig. 3-20). Amongst 22 HLA-A2 positive donors tested, only two were found to have detectable levels of NY-ESO-1 specific CD8$^+$ T cells and both were confined to stage III disease. Further, in both donors, the NY-ESO-1 specific CD8$^+$ T cells were confined to the T_{CM} subset.

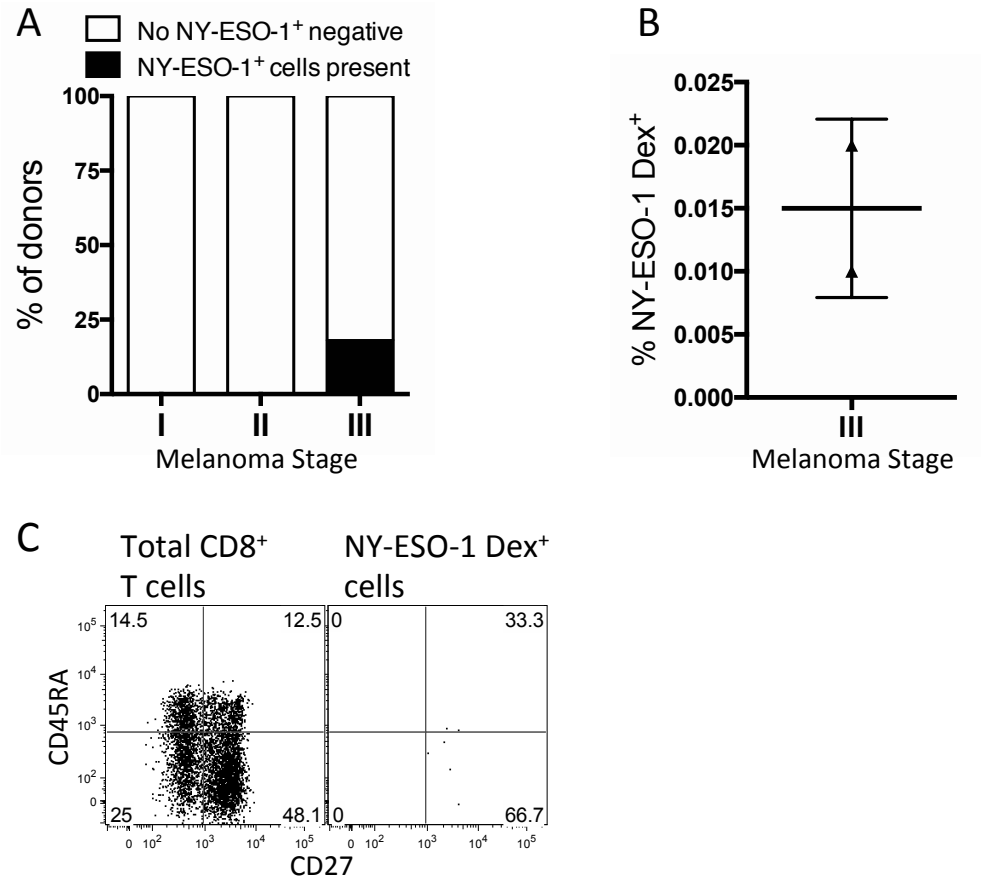


Fig. 3-20 Frequency and differentiation profiles of NY-ESO-1 specific CD8$^+$ T cells in the circulation of old melanoma patients

Melan-A specific CD8$^+$ T cells were detected by flow cytometry using fluorochrome conjugated Melan-A peptide loaded MHC class I HLA-A2 Dextramers.
A: Frequency of patients with detectable levels of NY-ESO-1-specific CD8$^+$ T cells amongst stage I (n=6), II (n=5) and III (n=11) melanoma patients. Mean and standard deviation are shown.
B: Frequency of NY-ESO-1 specific cells detected amongst old stage III melanoma patients.
C: CD45RA/CD27 profile of total and NY-ESO-1 specific CD8$^+$ T cells. NY-ESO-1 specific cells in both donors were >60% T_{CM}.

3.3.5 The role of IL-15 in CD45RA expression

3.3.5.1 IL-15 causes upregulation of CD45RA on $CD8^+$ T cells of old melanoma patients

CD45RA, which is used as a marker to identify naïve and T_{EMRA} cells, can be upregulated in response to IL-15 (Griffiths et al. 2013). By inducing CD45RA expression, IL-15 may therefore generate T_{EMRA}- or naïve-appearing memory cells. This may occur in melanoma patients and cause the increased occurrence of T_{EMRA} cells and occurrence of naïve-like Melan-A specific cells in an antigen independent manner. To address this, melanoma patients' $CD8^+$ naïve, T_{CM}, T_{EM} and T_{EMRA} were therefore FACS sorted and individually cultured in the presence of IL-15. CD45RA and CD27 surface expression were measured on the subsets by flow cytometry directly after sorting and after 14 days in culture. Indeed, CD45RA was significantly increased on T_{CM} and T_{EM} cells after 14 days in culture with IL-15 compared to pre-culture levels at day 0 ($p= 0.0021$ for T_{CM} and $p= 0.0373$ for T_{EM}; see Fig. 3-21A,B). Whilst T_{CM} and T_{EM} cells upregulated CD45RA in response to IL-15, they also retained expression of CD45RO (Fig. 3-21C).

Therefore, IL-15 might explain the high levels of T_{EMRA} cells in some CMV negative old melanoma patients. IL-15 might also lead to the development of naïve-appearing ($CD45RA^+CD27^+$) memory cells and could explain the high frequencies of naïve-like Melan-A specific cells.

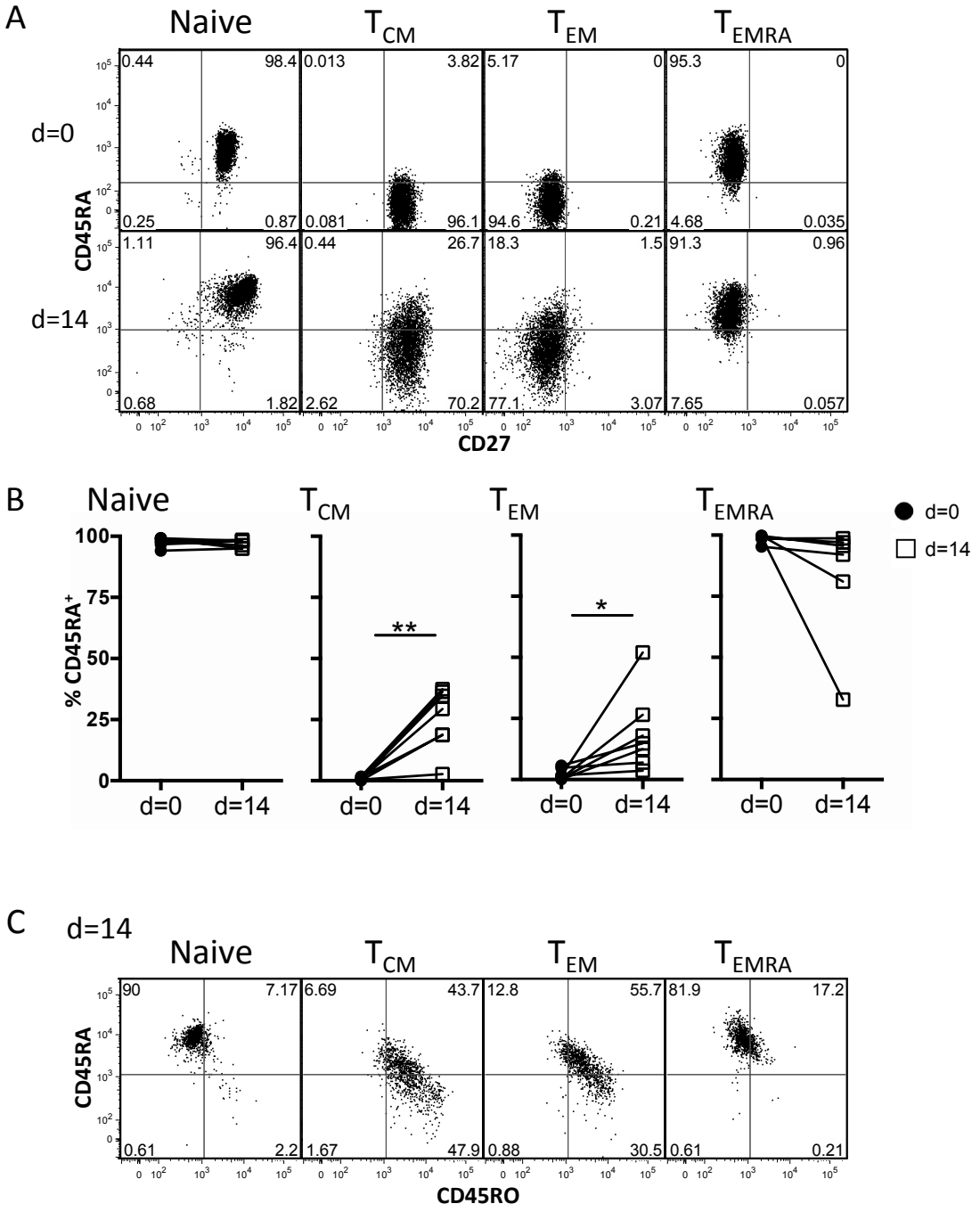


Fig. 3-21: Change in CD45RA surface expression in blood-derived CD8$^+$ T cells from melanoma patients after *in vitro* incubation with IL-15.

CD8$^+$ T cells subsets (based on CD27/CD45RA expression) taken from melanoma patients were FACS sorted and subsequently incubated with IL-15 for 14 days. Surface marker expression was measured via flow cytometry at day 0 (pre-incubation) and day 14 following incubation with IL-15.

A: Representative flow cytometry dot plots showing CD45RA and CD27 surface marker expression at day 0 and day 14 following incubation with IL-15 in T cell subsets derived from an old melanoma patient.

B: Change in percentages of cells expressing CD45RA in sorted CD8$^+$ T cells subsets of 7 melanoma patients after incubation with 10ng/ml IL-15 for 14 days (note: one patient did not yield enough naïve and T_{EMRA} cells for measurement at d=12).

C: Representative flow cytometry dot plots showing CD45RA and CD45RO surface marker expression at day 14 following incubation with IL-15 in T cell subsets derived from an old melanoma patient.

*=p<0.05; **=p<0.01

3.4 Discussion

This chapter explored whether differentiation of circulating T cells is increased in the context of melanoma. Differentiation was measured by assessing CD45RA/CD27 subset frequencies in both $CD4^+$ and $CD8^+$ T cells of participants across all ages. Differences in patients compared to healthy controls were restricted to the old age group and only the changes in the $CD8^+$ T cell compartment were found to be CMV independent. This discussion therefore focuses on results generated in the old cohort and on the $CD8^+$ T cells in particular.

Global T cell differentiation patterns were strongly affected by CMV positivity in both old melanoma patients and healthy controls. However, CMV^+ melanoma patients showed particularly high levels of $CD4^+$ T_{CM} levels compared to age matched CMV^+ controls, suggesting that the melanoma patients were affected more profoundly by their CMV burden than healthy controls. Literature discussing the role of CMV infections in the context of melanoma is scarce, with only one report suggesting an increase in CMV viral loads in patients with malignancies (Dolgikh and Bychkova 2004). CMV is known to reactivate under conditions of psychological stress and immunosuppression (Prosch et al. 1999; Mehta et al. 2000; Torres et al. 2006). The increase in the $CD4^+$ T_{CM} in old CMV^+ melanoma patients compared to old CMV^+ healthy individuals might therefore be due to increased $CD4^+$ T cell stimulation after increased CMV reactivation. This could be caused directly by the immunosuppressive effects of the tumour or indirectly by the psychological stress patients might undergo after becoming aware of their disease. Published follow up studies in healthy elderly participants have linked large expansions of CMV specific T cells to increased mortality (Wikby et al. 2005). In the present study, no correlation between CMV burden and the melanoma patients' health status could be made due to a lack of follow up data.

Within the $CD8^+$ T cell compartment, a significant increase in T_{EMRA} cell percentages could be detected in old melanoma patients compared to healthy age matched controls. High frequencies of $CD8^+$ T_{EMRA} in the melanoma patients were partly CMV independent, as a significant number of the patients with high T_{EMRA} tested negative for the virus. Further analyses confirmed that

these cells matched the functional and phenotypic properties of T_{EMRA} cells in healthy individuals in terms of decreased proliferative capacity, IL-2 production and telomere length and increased cytotoxic capacity and senescence associated marker expression. Increased T cell exhaustion (as measured by PD-1 and CTLA-4 expression) was not detected among circulating $CD8^+$ T cells of old melanoma patients compared to healthy controls.

The question that arose next was whether the $CD8^+$ T_{EMRA} found among the melanoma patients were melanoma specific. Using peptide loaded Dextramers, $CD8^+$ T cells specific to the tumour antigens Melan-A and NY-ESO-1 were detected in a number of the old melanoma patients. However, specific cells in these patients did not have a T_{EMRA} phenotype. It should be noted that MHC multimer technology is restricted to detecting T cell populations specific to a single peptide:MHC combination and might therefore not pick up on other possible melanoma-specific clones. It is consequently still possible that the global T cell changes observed are related to T cells of specificity to other melanoma associated antigens or different epitopes presented on alternative HLA molecules. For example, another widely described tumour-specific T cell population can recognize tyrosinase epitopes. Cells of this specificity were not investigated in this study but have been reported by others to show a senescent-like, unresponsive phenotype in patients with advanced stage disease (Lee et al. 1999; Maczek et al. 2005).

Recent evidence involving CMV specific T_{EMRA} indicates that these cells have lower avidity compared to CMV-specific $CD45RO^+$ cells (Griffiths et al. 2013). Since most anti-tumour specific T cells are thought to be of low affinity (due to deletion of highly self-reactive clones in the thymus) (Zhong et al. 2013), the T_{EMRA} compartment might indeed contain weakly melanoma reactive T cells, which would be difficult to detect *in vitro*. In the absence of evidence for a link between tumour specific immunity and increased global T cell differentiation, non-antigen specific T cell stimuli such as cytokines might also play a role in the generation of T_{EMRA} in melanoma. IL-15 can contribute directly to T_{EMRA} generation and maintenance in an antigen independent manner (Griffiths et al. 2013) and was shown to induce CD45RA in this work in T_{EM} and T_{CM} of melanoma patients. IL-15 improved *in vivo* $CD8^+$ T cell reactivity to murine melanoma and, at high concentrations, T cell responses to human melanoma *in*

vitro (Gamero et al. 1995). Although there are currently no reports suggesting increased IL-15 levels in the circulation of patients, T_{EMRA} in melanoma patients might therefore have been expanded in an antigen-independent manner through cytokines.

Accumulation of highly differentiated CMV-associated T cells in old healthy individuals has been linked to decreased survival (Wikby et al. 2005). Although high T_{EMRA} levels in melanoma patients might similarly be associated with poorer disease outcome, no link was found between the frequency of these cells and disease stage. Current literature suggests no clinical benefit for melanoma patients in having expanded populations of highly differentiated $CD8^+$ cells. Indeed, high levels of $CD8^+CD57^+$ cells were linked to worse prognosis amongst melanoma patients treated with IFN-α (Characiejus et al. 2008). Further, in melanoma treatments involving adoptive transfer of autologous T cells expanded *in vitro* from tumour infiltrating lymphocytes, clinical responses were associated with the level of persistence of the reintroduced T cells. Increased persistence and proliferative capacity were enhanced in cells that had longer telomeres and higher expression of CD28 compared to cells with shorter telomeres, indicating a beneficial role of cells having undergone less differentiation *in vitro* and *in vivo* in mice and humans (Zhou et al. 2005; Huang et al. 2005; Klebanoff et al. 2005; Li et al. 2010).

Interestingly, a decrease in CLA positive $CD8^+$ T_{CM} and T_{EM} could be found in the melanoma patients compared to healthy controls. T_{EMRA} generally only express low levels of this skin homing receptor and expressed similar levels of CLA in the patients compared to controls. The reduction in skin homing cells in the melanoma patients could be due to the cells being recruited to the skin. Indeed, it is published that vitiligo patients, who suffer from autoimmune destruction of cutaneous melanocytes, have reduced frequencies of $CD8^+CLA^+$ cells in the circulation compared to healthy controls, whilst showing increased T cell infiltration in the skin (Antelo et al. 2011).

Using Melan-A and NY-ESO-1 peptide loaded Dextramers, melanoma specific cells were detected in the blood of old melanoma patients. Large expansions of circulating melanoma specific T cells of over 1% of the total $CD8^+$ T cells were not observed, which was surprising given the abundance of literature describing the phenotypes of such cells in the patients. However these are often found in

stage IV melanoma patients that were not available to us (Lee et al. 1999; Fourcade et al. 2009).

The Melan-A specific cells detected among the melanoma specific cells were found to have either a naïve-like phenotype or a T_{CM}-like phenotype. Healthy HLA-A2 positive individuals often present with naïve like Melan-A $CD8^+$ T cells of frequencies around 0.1% of the total $CD8^+$T cells (Pittet et al. 1999). Such an abundance of naïve appearing cells specific for one antigen is unusual as frequencies of naïve precursor T cells against other known epitopes are in average 100 fold lower (Alanio et al. 2010). Data have been published on Melan-A specific T cells being generated at high frequencies in the thymus and having a low proliferative history (Zippelius et al. 2002), supporting the notion that these cells are indeed naïve. However, recent publications have described memory populations expressing markers usually associated with naïve T cells and the upregulation of naïve cell markers in memory cells in response to cytokines (Gattinoni et al. 2011; Griffiths et al. 2013), indicating that naïve appearing cells such as Melan-A cells might not necessarily be truly naïve.

It is thought that the shift in the melanoma-specific T cells from naïve to an activated or memory phenotype requires tumour metastasizing into of the lymph node and beyond in order to activate the cells sufficiently (Dunbar et al. 2000; van Oijen et al. 2004). However, Melan-A specific T cells that had a T_{CM} phenotype could also be detected in the circulation of patients who had localized melanoma only (stages I and II), indicating that melanoma-specific T cells can become activated before melanoma has spread beyond the skin. NY-ESO-1 specific $CD8^+$ T cells, which were detected in two out of 22 HLA-A2 positive patients tested, also showed a T_{CM}-like phenotype. T_{CM} express low levels of effector molecules such as perforin and granzyme B and high levels of costimulatory molecules CD27 and CD28, suggesting that they have a low proliferative history and are not very differentiated. This implies that the memory T cells specific to Melan-A and NY-ESO-1 have only encountered low levels of antigen but have the capacity to differentiate to effector cells if the antigen is re-encountered.

In summary, the results presented in this chapter revealed a CMV-independent expansion of highly differentiated T_{EMRA} cells in a number of old melanoma patients. However, we were not able to show whether these T_{EMRA} comprise

melanoma specific cells. Instead, the Melan-A and NY-ESO-1 specific cells detected in the old melanoma patients displayed less differentiated naïve or T_{CM}-like phenotypes.

It should be noted that T cell subset frequencies were measured in melanoma patients and healthy controls as percentage within the $CD4^+$ or $CD8^+$ T cell compartments and not as absolute cell numbers per volume of blood. Although lymphocyte counts were reportedly unchanged in melanoma patients compared to healthy controls in previous studies (Andres et al. 2006), this might not have been true for the participants of this study. Increased levels of T_{EMRA} cells observed in some melanoma patients might therefore be due to an overall increase in distribution of these cells, or alternatively to the depletion of all other subsets because of an overall decrease in $CD8^+$ T cells. To conclusively determine which scenario is true, absolute cell numbers should have been measured in whole blood via TruCount or similar techniques.

Recent advances in melanoma therapies have focussed on antibody therapies against inhibitory receptors such as PD-1 to boost anti-tumour T cell function. However, these treatments only work on a fraction of patients and beneficial effects are often found to be only transient (Brahmer et al. 2012). Assuming that the increased T_{EMRA} cells detected in the old melanoma patients are melanoma-reactive but hindered in tumour killing by their senescent nature, these patients might benefit from additional treatment by targeting pathways or receptors intrinsic to these senescent T cells, such as p38 signalling which is thought to actively maintain highly differentiated T cells in their senescent-like state (Di Mitri et al. 2011; Henson et al. 2014). This has been attempted during this project and is discussed in the final results chapter (chapter 6).

A point of consideration in this work is the use of CD45RA in conjunction with costimulatory receptor CD27, rather than the lymph node homing receptors CCR7 or CD62L to define T cell memory subsets. The latter were originally used to distinguish the lymph node homing subset T_{CM} from T_{EM} and T_{EMRA} which by definition do not home to the lymph nodes (Sallusto et al. 1999). Despite a broad correlation between CD27 and CCR7/CD62L expression, co-expression of these receptors is not absolute, as T cells can be highly heterogeneous and populations displaying intermediate $CCR7^-CD27^+$ and $CCR7^+CD27^-$ profiles have been described (Appay et al. 2002; Appay et al.

2008). Since T cells irreversibly lose CD27 during differentiation (Hintzen et al. 1993), the use of this marker allows distinction of cells at different stages of differentiation, rather than lymph node homing capability. Although the terms T_{CM}, T_{EM} and T_{EMRA} are used here, CCR7 or CD62L were not measured and the ability of these cells to home to lymphoid organs was therefore not addressed. The use of the $T_{CM}/T_{EM}/T_{EMRA}$ terminology is therefore restricted in the context of this work and comparisons with other studies using these terms should be made with care as different subset markers might have been used.

The frequency and differentiation status of melanoma-specific T cells in the circulation is often thought to be a late event and to have no predictive value to disease outcome (van Oijen et al. 2004). Conversely, the presence of tumour infiltrating lymphocytes has been shown to be a positive prognostic factor by a number of studies (Ladányi et al. 2004; Haanen et al. 2005). The following results chapters will therefore investigate differentiation patterns of T cells in the skin of melanoma patients.

4 Establishing patterns of T cell differentiation in healthy skin

4.1 Introduction

In order to assess T cell differentiation in the skin of patients with melanoma, it is important to understand T cell phenotypes and functions in healthy skin first. The characteristics of T cells in the skin have only been poorly studied compared to the circulation, as blood samples are easier to obtain and technically less challenging to process. However, the last decade has seen a surge in research on tissue resident T cells as their importance in tissue immunity is being recognized.

As described in the previous chapters, T cells in the blood can be divided into subsets, namely naïve, T_{CM}, T_{EM} and T_{EMRA}, with the former being the least and the latter being the most differentiated. These subsets can be distinguished on the basis of surface receptors involved in various physiological processes such as costimulation or migration. Whilst T_{CM} are thought to migrate between secondary lymphoid organs, T_{EM} and T_{EMRA} have often been associated with migration to peripheral tissues. As it was discovered that the skin is populated with a vast number of resident T cells, it was originally proposed that these cells are of the T_{EM} subset because they lack the lymph node homing markers CCR7 and CD62L (Clark et al. 2006; Clark et al. 2012). Although the paradigm has now shifted toward the notion that these cells are not true effector memory T cells (T_{EM}), but instead a separate subset of tissue resident memory T cells (T_{RM}), the idea persists that skin resident T cells share many characteristics with blood borne T_{EM} and are readily poised to respond to antigenic challenges (Schenkel and Masopust 2014; Natsuaki et al. 2014).

The differentiation patterns of skin resident T cells under steady state remain poorly understood, particularly in humans. The following chapter will therefore try to assess the differentiation of skin resident T cells in healthy human skin based on the expression of surface markers commonly associated with differentiation in T cells derived from the blood.

4.2 **Aims**

The aim of this chapter was to establish rules and patterns of T cell differentiation in the skin of healthy individuals to enable the interpretation of changes found in the skin of patients with melanoma (described in the following chapter).

4.3 Results

4.3.1 T cell extraction from skin specimens

4.3.1.1 Optimizing a protocol to isolate T cells from the skin

A skin digestion protocol involving overnight incubation of samples with collagenase type IV in complete medium was adopted in order to disaggregate skin specimen and to obtain a single cell suspension suitable for flow cytometry (outlined schematically in Fig. 4-1A and in full detail in the methods section). This technique is a laborious and time consuming process which requires manual cutting of skin specimen and filtration using a syringe plunger and repeated additions of PBS to pass the sample through a nylon mesh.

In order to reduce processing time and increase cell recovery, other methods of skin disaggregation were explored. The use of mechanical disaggregation methods was tested, initially by using the Medimachine tissue dissociator (by Becton Dickinson Biosciences). However, even small pieces of skin were found to not disaggregate efficiently and to obstruct the rotating blades of the device. This resulted in low cell yields compared to the enzymatic method (Fig. 4-1B). Combining overnight enzymatic digestion with the mechanical disaggregation allowed the blades to rotate unobstructed but did not result in increased yields (Fig. 4-1B). Likewise, no advantage in cell recovery was obtained by a similar device called GentleMACS (by Miltenyi; Fig. 4-1C). Mechanical skin disaggregation methods were therefore abandoned in favour of the combined manual/enzymatic method.

Initial analyses of lymphocytes recovered using collagenase digestion showed decreased surface expression of certain markers such as CD4 but not of others, including CD8 (Fig. 4-2A). Using a different type of collagenase (Type Is, Fig. 4-2A) also affected cell surface markers. Allowing the isolated cells to rest overnight in collagenase-free medium did also not recover the lost epitopes (data not shown). Finally, increasing the serum content in the digestion medium from 10% to 20% or above, as suggested by Mulder and colleagues, allowed prevention of surface marker degradation without significant reduction in collagenase-mediated tissue breakdown (Mulder et al. 1994) (Fig. 4-2B).

The skin samples available from healthy volunteers were generally obtained from redundant plastic surgery skin, whilst melanoma skin samples were obtained from wide local excisions. Cell yields were estimated from counts of leukocytes using a haemocytometer. Cell recovery from skin samples was highly variable, averaging 49,000±35,0000 cells per cm^2 in healthy skin and 116,000±180,000 cells per cm^2 in melanoma patient skin samples (difference in recovery between patients and controls was not significant).

Skin samples were obtained on an irregular basis, at unpredictable times and were subject to availability. Due to these difficulties in sample collection, data obtained on skin T cell differentiation is spread across participants of all ages and adequate age matching between participants was not always possible, particularly in the melanoma cohort.

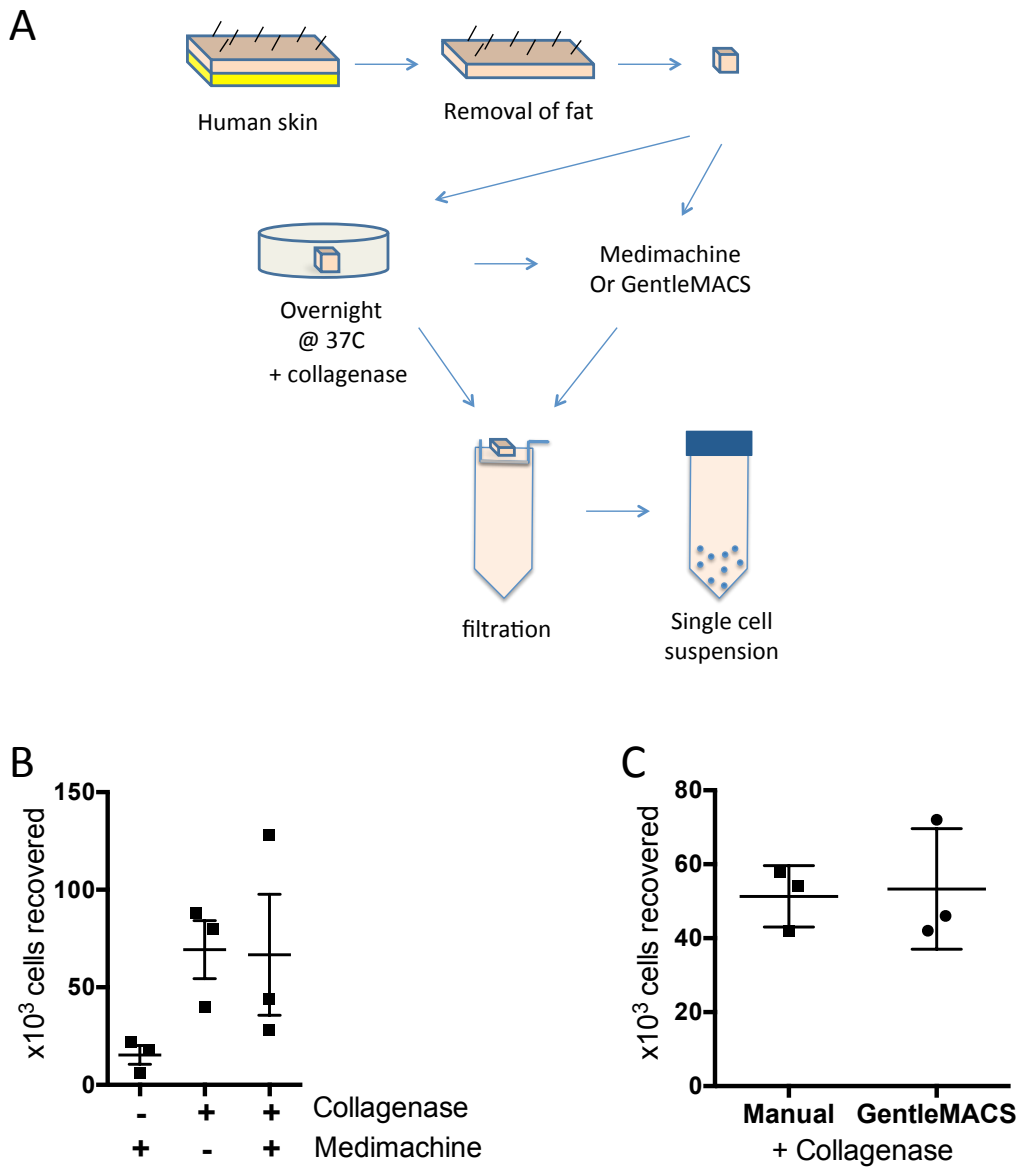
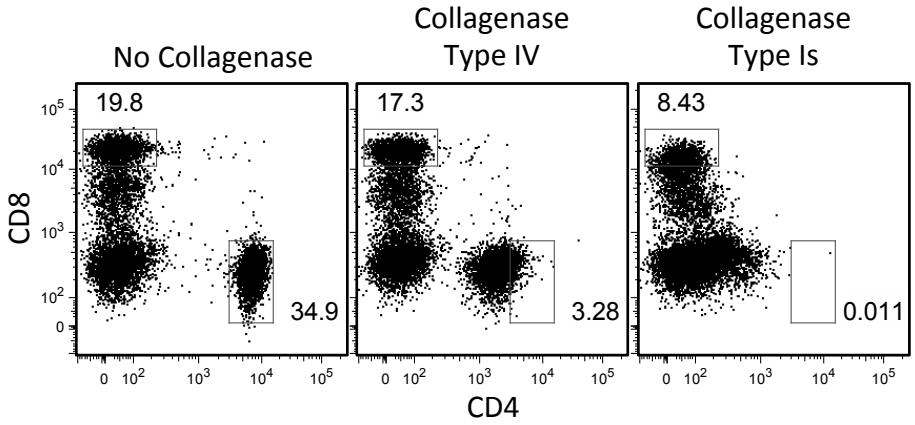


Fig. 4-1: Comparison of cell yields using various methods of skin disaggregation.

A: Schematic diagram outlining the method of skin disaggregation for the extraction of single cell suspensions. Manual disaggregation involved filtration directly after overnight digestion. Mechanical disaggregation using the Medimachine or GentleMACS was tested on undigested or overnight digested skin before filtration.
B: Cell yields were compared using enzymatic (overnight collagenase treatment) and mechanical (Medimachine) skin disaggregation methods, alone or in combination on 8mm punch biopsies. Horizontal lines depict mean values with standard deviation between three biopsies.
C: Cell yields of skin T cells from punch biopsies obtained following collagenase digestion via manual/enzymatic disaggregation or mechanical/enzymatic disaggregation using the GentleMACS tissue dissociator. Horizontal lines depict mean values with standard deviation between three 8mm punch biopsies.

A



B

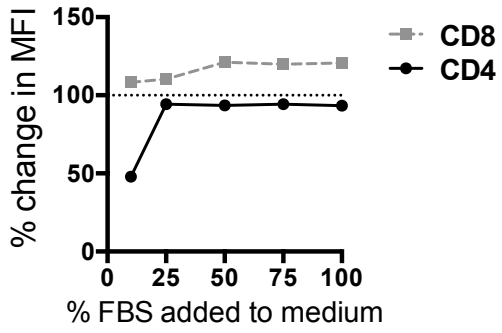


Fig. 4-2 Loss of T cell surface markers after incubation of PBMCs from a healthy donor with collagenase and prevention of this phenomenon through increased FBS concentration

A: Comparison of cell surface marker expression following collagenase digestion. CD4 and CD8 surface expression were assessed by flow cytometry following overnight incubation of PBMCs with 0.8mg of collagenase type IV and type Is and 10% FBS in RPMI medium. Flow cytometry gates were drawn on the dot plots according the untreated sample and applied to collagenase treated samples.
B: Cell surface marker detection after digestion in the presence of varying levels of FBS. Cells were incubated overnight with medium containing 0.8mg of collagenase type IV and varying concentrations of FBS. CD4 and CD8 surface expression were measured via flow cytometry the following day and percentage change in MFI for both markers was calculated compared to untreated cells.

4.3.2 T cell differentiation in healthy skin

To date little is still known about the T cell differentiation in the skin of healthy humans. It was therefore necessary to first investigate T cell differentiation patterns in the skin of healthy individuals, before being able to assess the state of T cell differentiation in the skin of patients with melanoma. The characterization of T cell differentiation in healthy skin, based on T cell differentiation markers commonly used in the blood, was therefore attempted.

4.3.2.1 Skin derived T cells in healthy individuals express high levels of the tissue retention marker CD69

First, expression of CD69 was measured in order to verify if cells extracted from the skin samples were true skin resident T cells. CD69 is not normally expressed on resting T cells in the circulation, but is stably expressed on tissue resident T cells, as it is thought to be involved in tissue retention (Ledgerwood et al. 2008; Sathaliyawala et al. 2013). Blood derived T cells from healthy individuals did not express CD69, whilst 80±8% of $CD4^+$ T cells and 91±5% of $CD8^+$ T cells expressed CD69 in the skin (Fig. 4-3A,B).

To see which of the differentiation subsets in the skin were the most likely to express CD69, expression of the receptor was measured in the CD45RA/CD27 defined differentiation subsets of skin derived $CD4^+$ and $CD8^+$ T cells. In the skin $CD4^+$ T cells the CD27 positive subsets (Naïve and T_{CM}) expressed significantly lower levels of CD69 than the CD27 negative subsets (T_{EM} and T_{EMRA}), as 88±6% of T_{EM} and 92±5% of T_{EMRA} expressed CD69, whilst only 63±25% of naïve and 60±13% of T_{CM} did so. All $CD8^+$ T cell subsets expressed CD69 equally at averages of 87-92%.

Therefore, the majority of skin $CD8^+$ T cells extracted had characteristics of resident T cells, whilst 20% of $CD4^+$ T cells appeared to be transient as they were CD69 negative. The majority of these transient $CD4^+$ T cells belonged to the naïve and T_{CM} subsets.

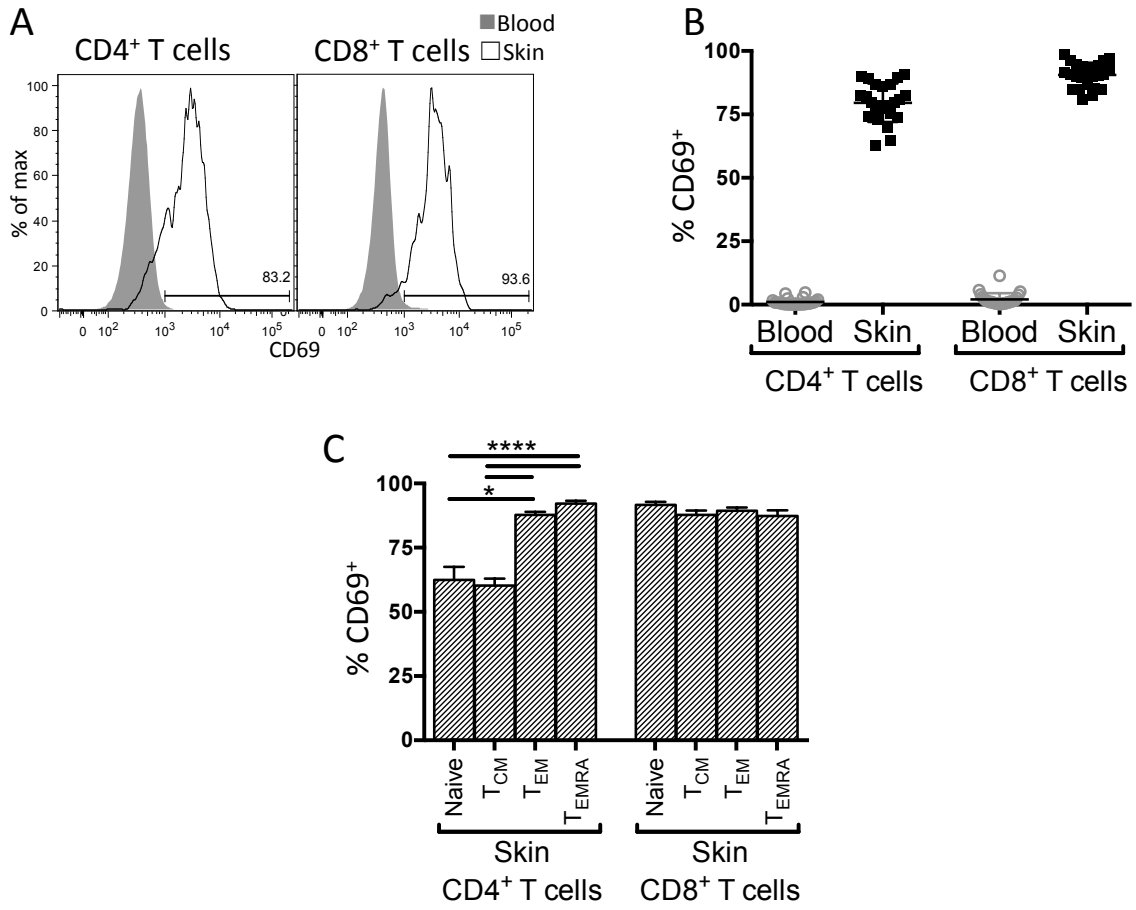


Fig. 4-3: *Ex vivo* expression of the tissue retention marker CD69 in skin and blood derived T cells in healthy individuals.

CD69 expression was measured by flow cytometry in blood and skin derived $CD4^+$ and $CD8^+$ T cells derived from healthy individuals.
A: representative flow cytometry histogram showing CD69 expression in matched blood and skin derived $CD4^+$ and $CD8^+$ T cells.
B: Cumulative data showing CD69 expression in $CD4^+$ and $CD8^+$ T cells from the blood (n=26) and skin (n=24) of healthy individuals. Mean and standard deviation are shown.
C: Cumulative data showing CD69 expression in the CD45/CD27 defined subsets of skin derived $CD4^+$ and $CD8^+$ T cells from 24 healthy individuals. Mean and standard error are shown. Subsets were compared using the Friedman test.
*=p<0.05; ****=p<0.0001

4.3.2.2 **The majority of healthy skin resident CD4$^+$ T cells have a T_{EM} phenotype, irrespective of age**

As described previously, CD27 and CD45RA are commonly used to identify T cell differentiation subsets in the blood. CD27 and CD45RA based subset distribution was therefore assessed by flow cytometry in T cells extracted from the skin and blood of healthy individuals (Fig. 4-4A).

No significant age-related changes in subset distribution were observed among the skin derived CD4$^+$ T cells, although this might be due to the low number of skin donors and high variation between the samples (Fig. 4-4B). The dominant skin subset in donors of all ages was found to be the T_{EM} subset, which in average constituted 60±15% of CD4$^+$ T cells. A smaller population of T_{CM} cells was also present at 32±13%, whilst the CD45RA expressing naïve and T_{EMRA} subsets were only detected at minor frequencies (2±3% and 6±5% respectively).

When comparing paired samples in young and old individuals respectively, skin derived naïve T cells (p= 0.0023 and 0.0101 for young and old respectively) and T_{CM} cells (p=0.0255 and 0.0624) were reduced and T_{EM} (p=0.0007 and 0.0006) and T_{EMRA} (p= 0.2889 and 0.0408) increased when compared to matched blood samples.

Therefore, CD4$^+$ T cell differentiation profiles are profoundly altered in the healthy skin compared to the blood and are dominated by T_{EM}- like cells in both young and old donors.

CD4⁺ T cells

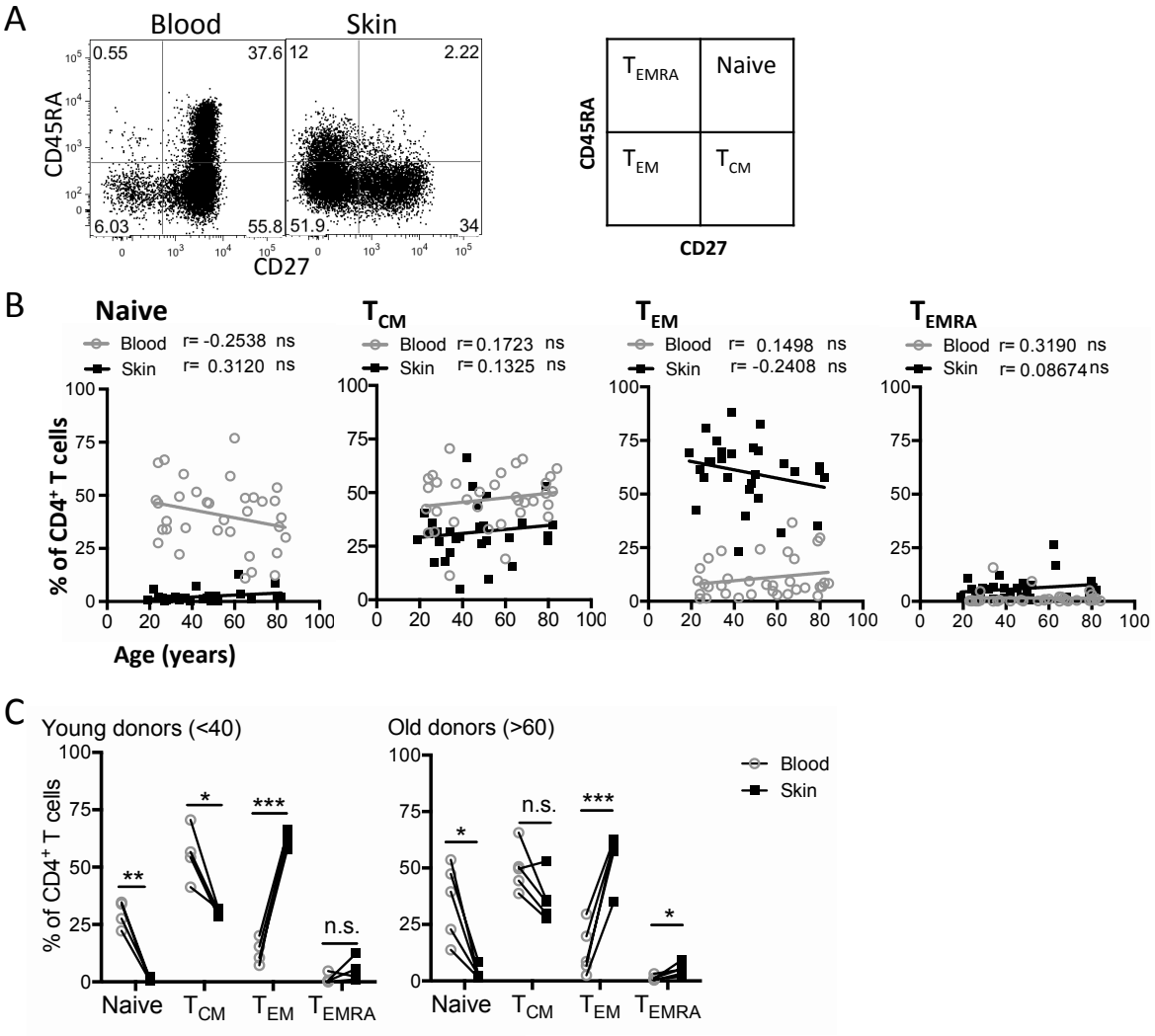


Fig. 4-4: CD4⁺ T cell subset distribution with age in skin compared to blood in healthy donors.

CD45RA and CD27 expression were measured via flow cytometry in CD4⁺ T cells of healthy blood and skin donors.

A: Representative flow cytometry plots show CD45RA and CD27 expression in blood and skin derived CD4⁺ T cells obtained from the same healthy donor. The diagram on the right highlights the names given to the four subsets.

B: Relationship with age of subset frequencies in the skin (n=29) and blood (n=35) of healthy individuals. Lines of best fit were generated by linear regression and the correlation (r-value) and significance were assessed by Pearson and Spearman rank.

C: Comparison of paired blood and skin T cell subset frequencies in young (n=4; aged 40 and younger) and old (n=5; aged 60 and older) healthy individuals. Percentages were compared using the paired T test or the Wilcoxon test.

*=p<0.05; **=p<0.01; ***=p<0.001

4.3.2.3 **$CD8^+$ T cell differentiation patterns are altered in the skin compared to the blood but show all four differentiation subsets**

CD45RA/CD27 subset gates were applied to $CD8^+$ T cells derived from the blood and skin of healthy individuals (Fig. 4-5A). As was the case with skin derived $CD4^+$ T cells, $CD8^+$ T cell CD45RA/CD27 subset frequency did not change significantly with age, although there seemed to be a trend towards increased abundance of T_{CM} and decreased T_{EM} in the older age groups (Fig. 4-5B). T_{CM} and T_{EM} cells comprised on average 34±21% and 33±16% respectively, whilst naïve (17±12%) and T_{EMRA} (16±15%) populations were also present. Paired blood and skin samples were compared in the young and old (Fig. 4-5C). In the young, skin derived $CD8^+$ T cells were found to contain significantly less naïve and more T_{EM} cells compared to the blood of the same donors ($p= 0.0489$ and 0.0432, respectively). Similar patterns were observed in the old, where they were not significant ($p= 0.0724$ and 0.0552).

Therefore, based on their CD45RA/CD27 expression, $CD8^+$ T cells in the healthy skin are comprised mainly of T_{EM} and T_{CM}, as well as lower levels of T_{EMRA} and naïve-live cells.

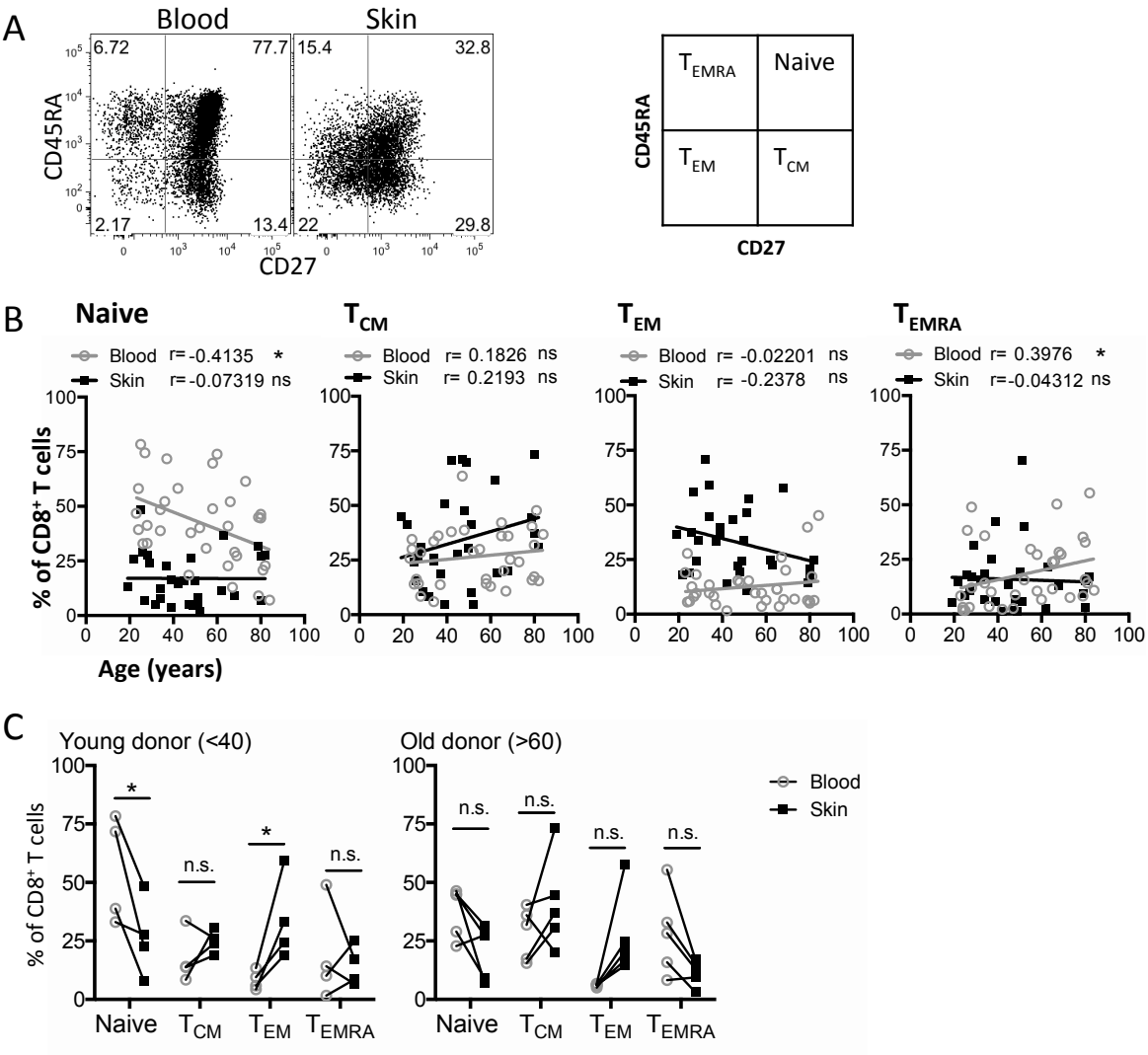


Fig. 4-5: Change in CD8$^+$ T cell subset distribution with age in skin compared to blood in healthy donors.

CD45RA and CD27 expression were measured via flow cytometry in CD4$^+$ T cells of healthy blood and skin donors.
A: Representative flow cytometry plots show CD45RA and CD27 expression in blood derived and skin derived CD8$^+$ T cells obtained from the same healthy donor. The diagram on the right highlights the names given to the four subsets.
B: Relationship with age of subset frequencies in the skin (n=29) and blood (n=35) of healthy individuals. Lines of best fit were generated by linear regression and the correlation (r-value) and significance were assessed by Pearson and Spearman rank.
C: Comparison of paired blood and skin T cell subset frequencies in young (n=4; aged 40 and younger) and old (n=5; aged 60 and older) healthy individuals. Percentages were compared using the paired T test or the Wilcoxon test.
*=p<0.05; **=p<0.01; ***=p<0.001

4.3.2.4 **T cells in the skin do not display surface receptor patters commonly associated with highly differentiated cells in the blood**

A number of surface marker combinations other than CD27/CD45RA are often used on blood derived T cells to distinguish cells at different stages of differentiation. This includes combined measurement of CD27 and CD28. T cells that express both CD27 and CD28 are thought to be the least differentiated, whilst double negative cells, which accumulate with age, are the most differentiated. This means that $CD27^+CD28^+$ cells typically comprise naïve and T_{CM} cells, whilst CD27-CD28- cells include T_{EM} and T_{EMRA} cells. Cells that express either CD27 or CD28 are in an intermediate differentiation stage that mostly includes T_{CM} (S. M. Henson et al. 2009; Appay et al. 2008).

CD27 and CD28 coexpression was analyzed in the skin and blood of healthy individuals (Fig. 4-6). The majority of skin resident $CD4^+$ (67±10%) and $CD8^+$ T cells (44±14%) expressed CD28 but no CD27. Only 21±8% of skin resident $CD4^+$ T cells expressed both CD27 and CD28, whilst 11±7% expressed neither. Cells that only expressed CD27 but no CD28 were virtually absent among skin resident $CD4^+$ T cells (1±1%). Similarly, minor populations of $CD27^+CD28^+$ (16±8%) and CD27-CD28- (28±10%) cells could be detected among the skin resident $CD8^+$ T cells. $CD27^+$CD28- cells were also present among the skin $CD8^+$ T cells (16±8%).

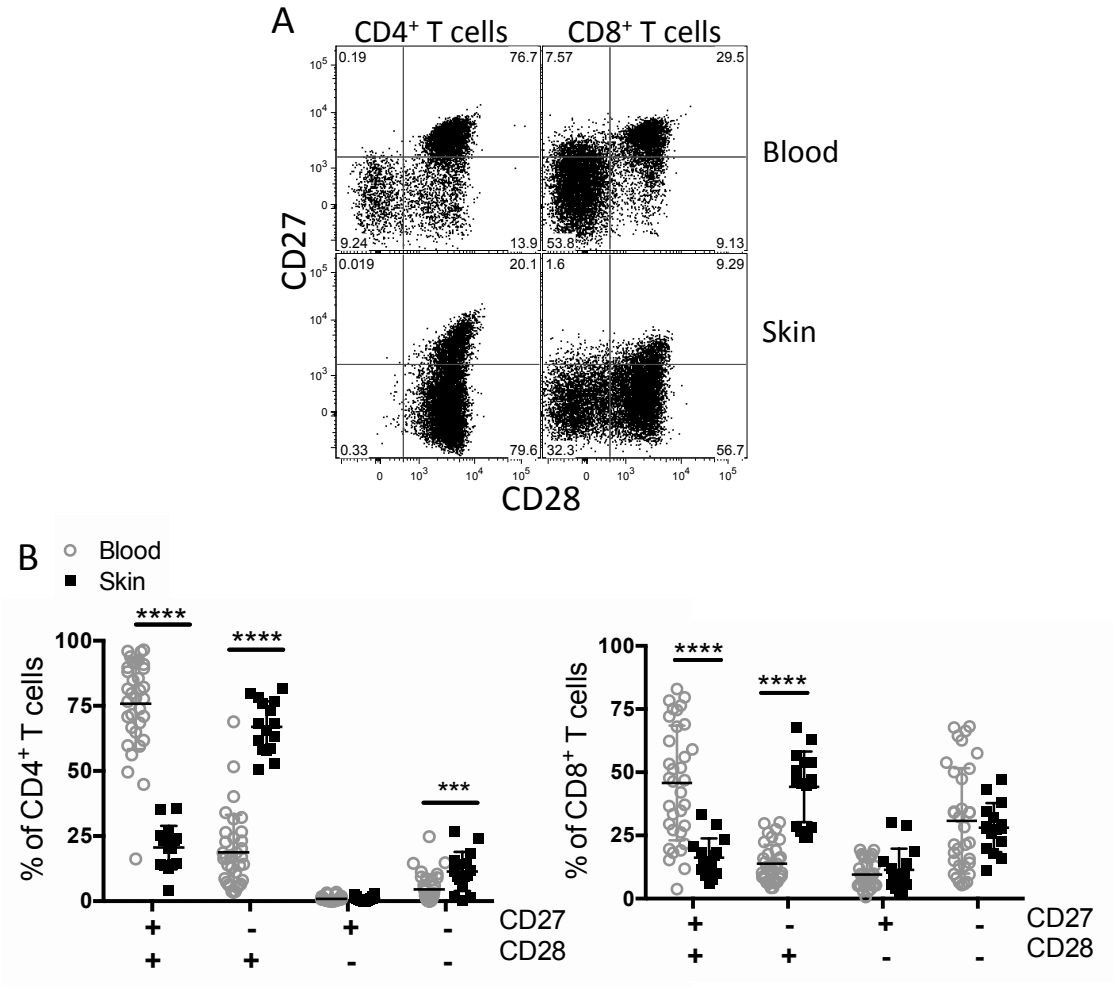


Fig. 4-6: CD27 and CD28 coexpression in skin and blood derived T cells of healthy individuals

CD27 and CD28 were measured ex vivo by flow cytometry in blood and skin derived $CD4^+$ and $CD8^+$ T cells

A: Representative flow cytometric dot plots showing CD27 and CD28 expression in $CD4^+$ and $CD8^+$ T cells derived from the blood and skin of a healthy individual.

B: Cumulative data showing the distribution of subpopulations defined by CD27 and CD28 expression in $CD4^+$ (on the left) and $CD8^+$ T cells (on the right) derived from the blood (n=34) and skin (n=16) of healthy individuals. Comparisons were made using the Student or paired t-test.

*=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001

As previously mentioned, KLRG1 and CD57 can also be used to identify highly differentiated cells and are increased in the blood on cells of the T_{EM} and T_{EMRA} subsets. KLRG1 and CD57 expression were therefore measured in T cells extracted from healthy skin and compared to levels in the blood (Fig. 4-7). The majority of skin resident T cells were negative for both CD57 and KLRG1 (85±9% for $CD4^+$ and 65±15 for $CD8^+$ T cells), whilst double-expressing cells remained rare in the $CD4^+$ T cells (1±2%) and low in the $CD8^+$ T cells (9±12%; Fig. 4-7). Interestingly, a population of $CD57^+KLRG1^-$ cells among the $CD4^+$ (10±6%) and $CD8^+$ T cells (20±10%) was detected in the skin, which was not present in the blood, as T cells in the blood cells were unlikely to express CD57 in the absence of KLRG1.

Therefore, based on commonly used differentiation-associated markers, the majority of skin resident T cells appeared to be of an intermediate (CD27-$CD28^+$CD57-KLRG1-) differentiation stage.

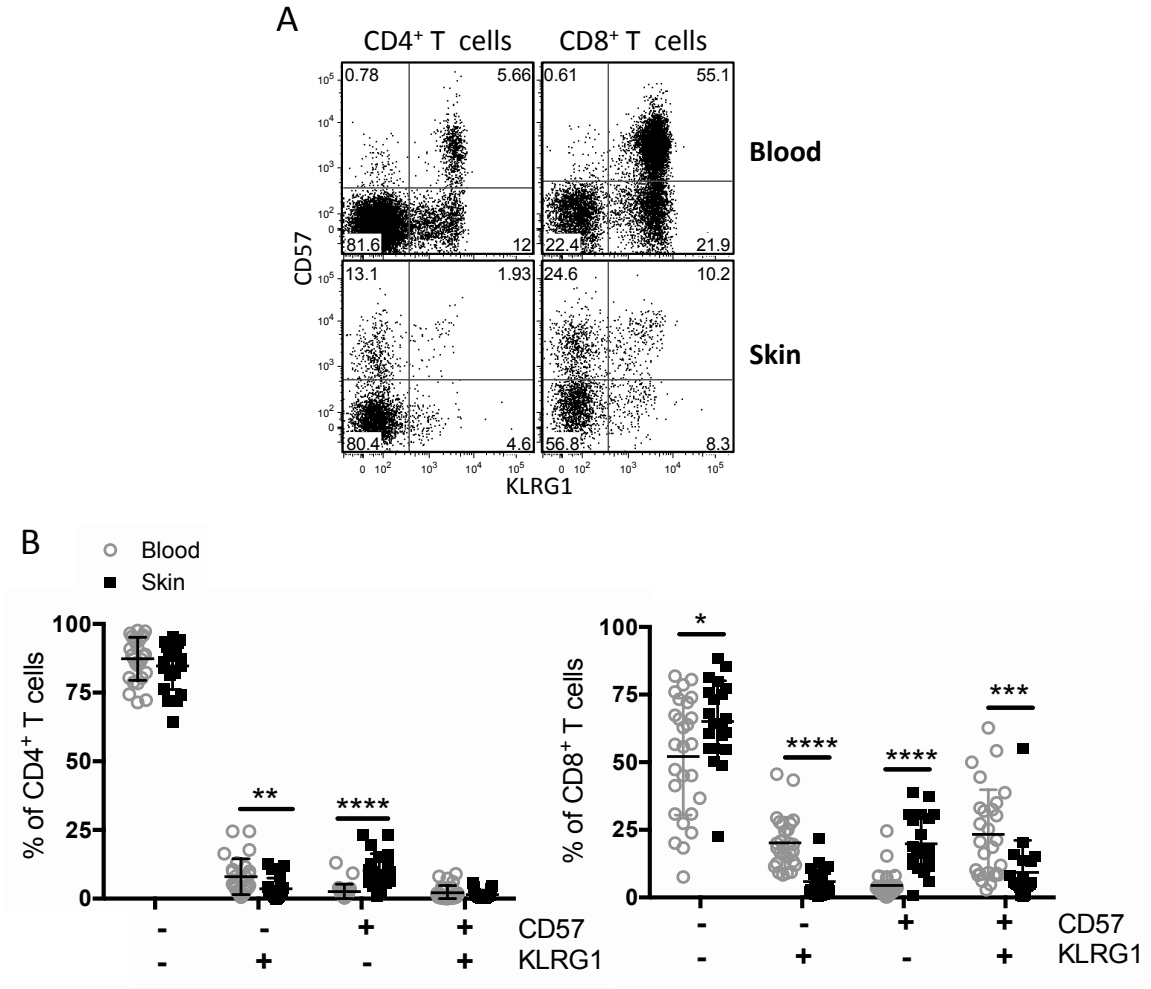


Fig. 4-7: Expression of senescence-associated markers KLRG1 and CD57 in the blood and skin T cells of healthy donors.

KLRG1 and CD57 were measured ex vivo by flow cytometry in blood and skin derived $CD4^+$ and $CD8^+$ T cells of healthy donors.
A: Representative flow cytometric dot plots showing KLRG1 and CD57 expression in $CD4^+$ and $CD8^+$ T cells derived from the blood and skin of the same individual.
B: Cumulative data showing the distribution of subpopulations defined by KLRG1 and CD57 expression in $CD4^+$ (on the left) and $CD8^+$ T cells (on the right) derived from the blood (n=27) and skin (n=21) of healthy individuals. Populations were compared using the Student or paired t-tests.
*=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001

4.3.2.5 **CD45RA/CD27 T cell subsets in the skin have different characteristics to CD45RA/CD27 subsets in the blood**

As described in the previous chapters, CD45RA and CD27 expression patterns in blood T cells reliably predict other phenotypic properties. T_{EMRA} cells for example do not express CD28 but express high levels of the KLRG1 and CD57 (Appay et al. 2008). To determine if this was also true in skin derived T cells, surface expression of CD28, KLRG1 and CD57 was measured in the CD45RA/CD27 subsets of skin derived T cells from healthy individuals and compared to the patterns in the blood subsets (Fig. 4-8).

CD28 expression is known to decrease during differentiation in blood derived T cells and is therefore generally found to be highest in the naïve and T_{CM} subsets, lower in the T_{EM} and lowest in the T_{EMRA}. It was confirmed that this was the case in blood derived $CD4^+$ and $CD8^+$ T cells of healthy individuals (Fig. 4-8). CD28 expression followed a similar pattern in the skin, where CD28 was higher in the T_{CM} cells compared to the T_{EM} and lowest in the T_{EMRA} cells in both $CD4^+$ and $CD8^+$ T cells. Interestingly, the majority of the naïve appearing skin $CD8^+$ T cells did not express CD28.

Expression of senescence associated markers KLRG1 and CD57 increases gradually with differentiation. Consequently, expression of both markers was found to be lowest in naïve and T_{CM}, higher on T_{EM} and highest in T_{EMRA} in blood derived $CD4^+$ and $CD8^+$ T cells. However, skin T cell subsets did not follow this pattern: CD57 and KLRG1 were not markedly increased in the skin T_{EMRA} compared to the other subsets and were particularly low in the T_{EM} among of the $CD8^+$ T cell compartment.

Therefore CD45RA and CD27 surface expression was disconnected from senescence associated marker expression in the skin. Further, $CD45RA^+CD27^+$ naïve appearing $CD8^+$ T cells in the skin are unlikely to be truly naïve as they expressed reduced levels of CD28.

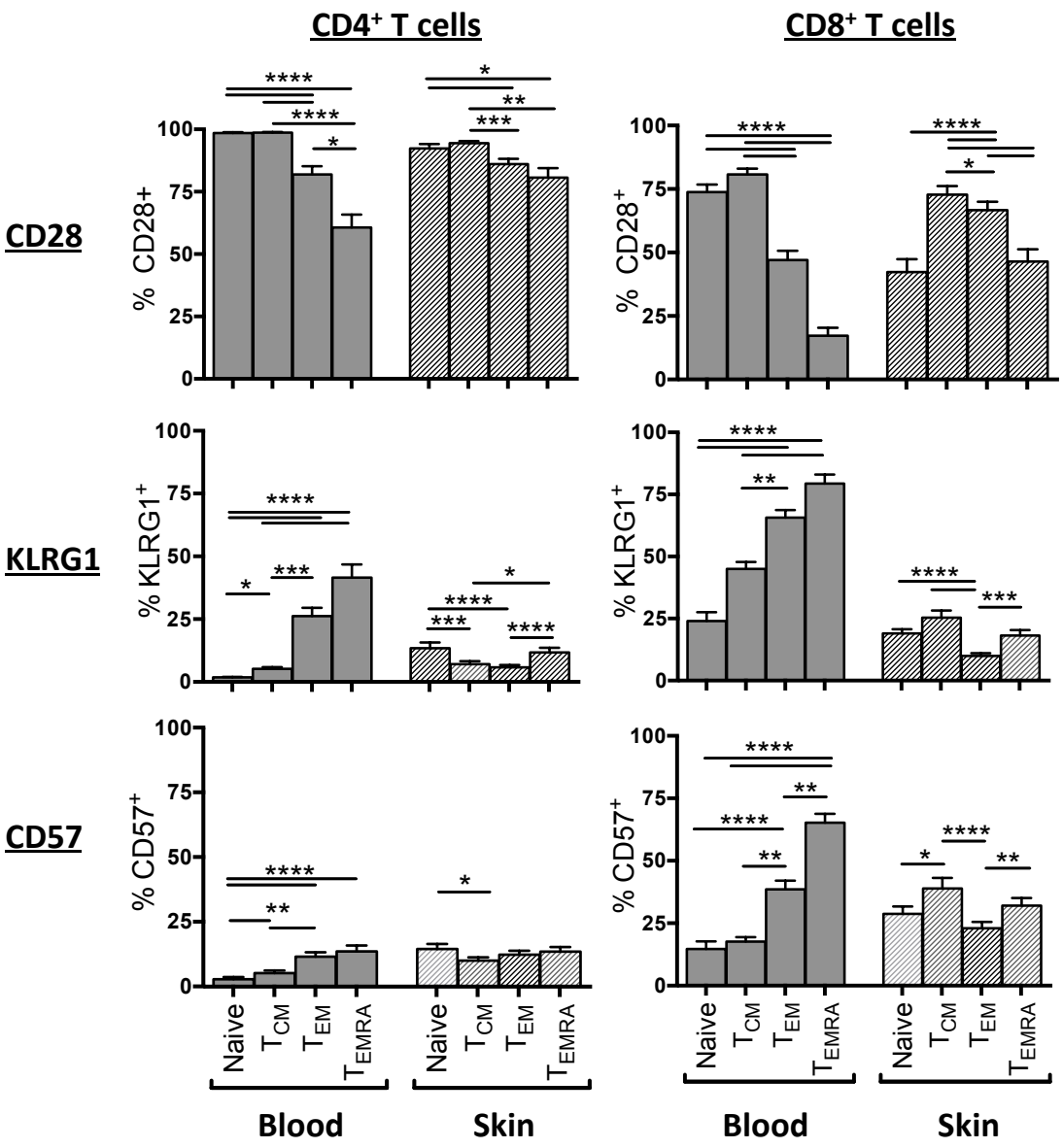


Fig. 4-8: Expression of markers commonly used to define T cell differentiation in the blood in the CD45RA/CD27 subsets in the blood and skin of healthy individuals.

CD28, KLRG1 and CD57 expression were measured ex vivo in blood and skin derived $CD4^+$ (graphs on the left) and $CD8^+$ (graphs on the right) T cell subsets. Naïve, T_{CM}, T_{EM} and T_{EMRA} T cell subsets were based on CD45RA and CD27 expression as previously described. The bar graphs show means and standard errors. Populations were compared using one-way ANOVA or the Friedman test. Participant numbers were as follows for blood and skin donors respectively: 26 and 17 for CD28, 32 and 27 for KLRG1 and finally 27 and 21 for CD57 measurements.
*=p<0.05; **=p<0.005; ***=p<0.0005; ****=p<0.0001

4.3.2.6 **PD-1 but not CTLA-4 expression is increased in skin T cells from healthy individuals compared to the blood.**

CTLA-4 and PD-1 play major roles in maintaining T cell tolerance and have been associated with T cell exhaustion (but not senescence) in various disease settings including cancer (Baitsch et al. 2011; Blackburn et al. 2009; Duraiswamy et al. 2013). In order to investigate expression of these receptors in tissue resident T cells in the absence of melanoma, PD-1 and CTLA-4 expression were measured in skin derived T cells and compared to the blood of healthy individuals (Fig. 4-9). PD-1 expression was significantly increased in skin $CD4^+$ and $CD8^+$ T cells (35±18% and 51±18%, respectively) compared to the blood ($CD4^+$: 10±6%, p= 0.0002; $CD8^+$: 14±8%, p< 0.0001). Conversely, CTLA-4 expression levels were low in the skin and similar to levels in the blood in both $CD4^+$ T cells (16±8% in the skin and 20±12% in the blood) and $CD8^+$ T cells (5±5% in the skin and 8±7% in the blood).

In the blood PD-1 and CTLA-4 expression were highest in the subsets of intermediate differentiation (T_{CM} and T_{EM}) in both $CD4^+$ and $CD8^+$ T cells (Fig. 4-10). In the skin, PD-1 expression was increased in all the subsets, but highest in the T_{CM}-like subsets, where 46±16% of CD4 and 60±19% of $CD8^+$ T_{CM} expressed this marker. CTLA-4 expression in the skin was low in all subsets apart from the T_{CM}, where 38±15% of $CD4^+T_{CM}$ and 10±6% of $CD8^+$ T_{CM} expressed the receptor.

Therefore PD-1 (but not CTLA-4) was significantly increased in skin derived T cells compared to the blood, and the T_{CM}-like subset in the skin expressed the highest levels of both these markers.

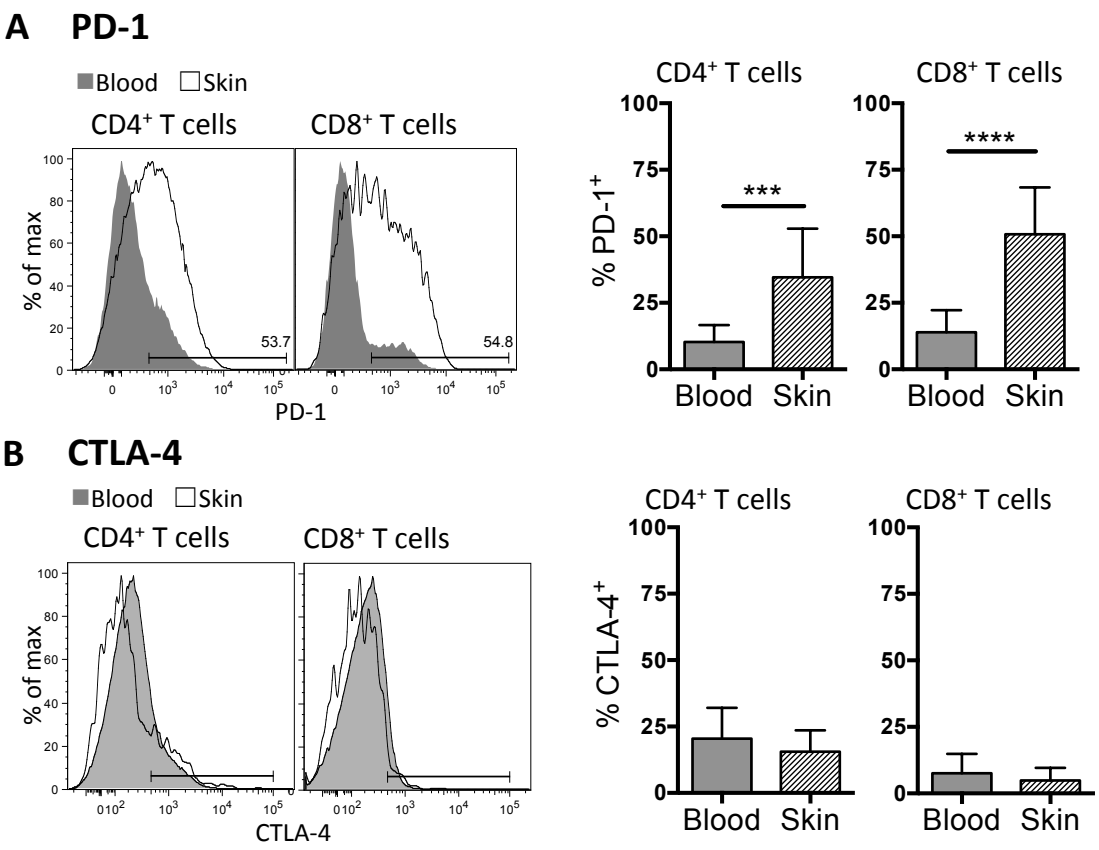


Fig. 4-9 Surface expression of the inhibitory/exhaustion markers PD-1 and CTLA-4 in blood and skin derived total CD4$^+$ and CD8$^+$ T cells derived from healthy individuals.

Extracellular PD-1 expression was measured by flow cytometry *ex vivo*, intracellular CTLA-4 expression following overnight stimulation of the cells with immobilized anti-CD3 antibody.
A: Representative flow cytometry histograms and cumulative data showing PD-1 expression in total CD4$^+$ and CD8$^+$ T cells in the blood (n=12) and matched skin (n=12) of healthy individuals. Populations were compared using the paired t-test.
B: Representative flow cytometry histograms and cumulative data showing CTLA-4 expression in total CD4$^+$ and CD8$^+$ T cells in the blood (n=19) and skin (n=14) of healthy individuals. Populations were compared using the Student t-test.
Mean and standard deviation are shown. ***=p<0.001; ****=p<0.0001

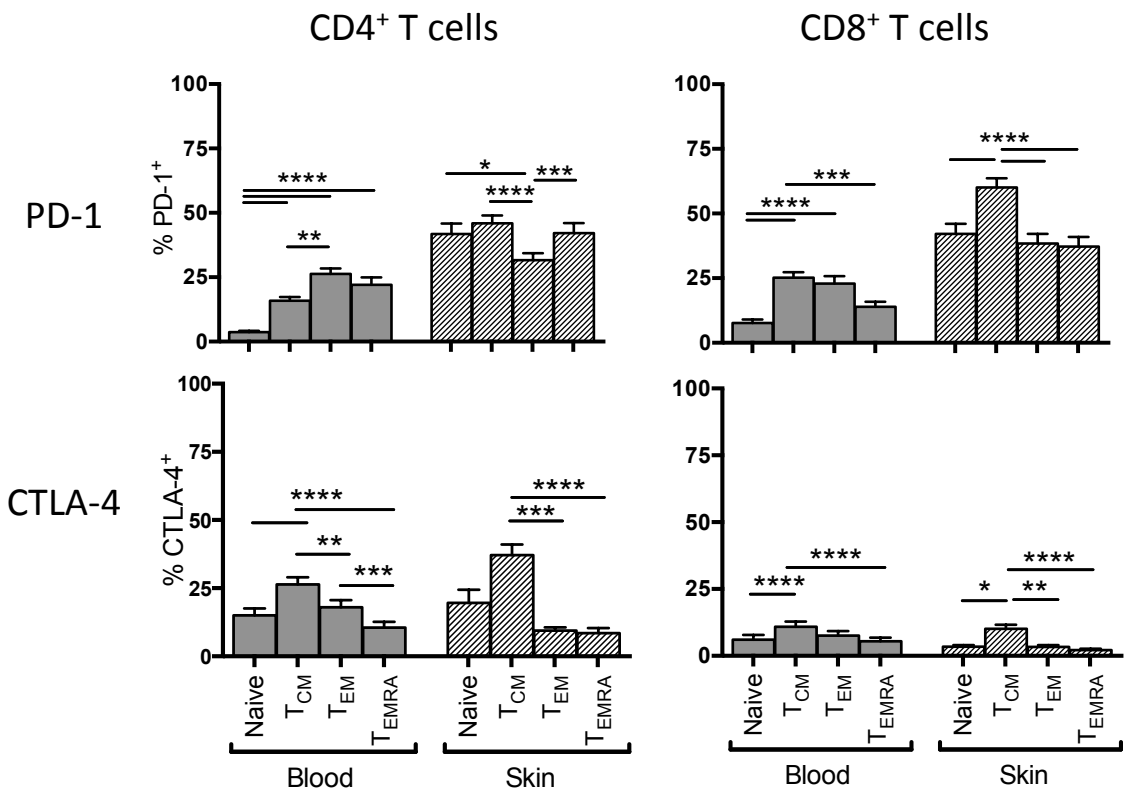


Fig. 4-10: PD-1 and CTLA-4 expression in the CD45RA/CD27 subsets of skin and blood derived T cells in healthy individuals.

PD-1 expression was measured by flow cytometry ex vivo, CTLA-4 expression following overnight stimulation of the cells with immobilized anti-CD3 antibody. Participant numbers were as follows: 35 blood and 29 skin donors for PD-1 measurements and 19 blood and 14 skin donors for CTLA-4 measurements.
Means and standard error are shown, differences in PD-1 and CTLA-4 expression were calculated using one-way ANOVA or the Friedman test.
*=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001

4.3.2.7 **$CD4^+$ $FoxP3^+$ Tregs in the skin are confined to the T_{CM} subset**

Suppressive $CD4^+FoxP3^+$ Treg cells are known to be important for immune tolerance in the skin and it was of interest to understand their phenotypic properties in that tissue. Consistent with previous findings (Sanchez Rodriguez et al. 2014), $FoxP3^+$ Treg frequency in the $CD4^+$ T cell compartment was increased in the skin compared to the blood (9±2% versus 4±2%, p= 0.0019), as measured by flow cytometry (Fig. 4-11A,B). Like circulating $FoxP3^+$ Tregs, Tregs in the skin were found to be confined to the CD45RA-$CD27^+$ T_{CM} compartment but made up only 23±6% of the skin T_{CM} compartment (Fig. 4-11C,D). Compared to the total $CD4^+$ T cells in the skin, skin $FoxP3^+$ Tregs expressed lower levels of CD69 and increased levels of CTLA-4 (Fig. 4-11E).

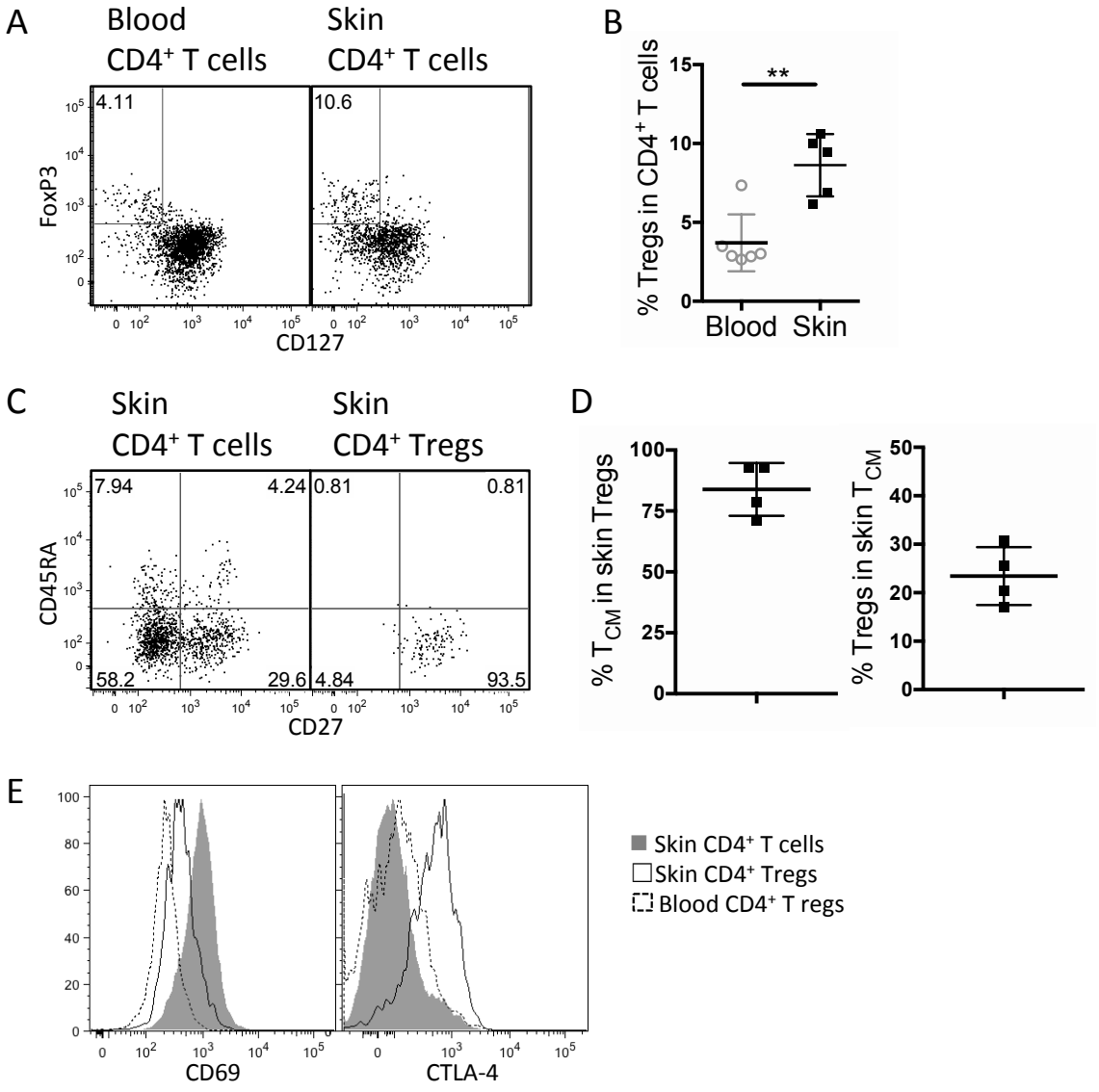


Fig. 4-11 Frequency and phenotypic properties of FoxP3$^+$ Tregs in healthy skin

Skin and blood derived T cells were analysed by flow cytometry and gating was applied to identify total CD4$^+$ T cells and FoxP3$^+$CD25$^+$CD127$^+$ Tregs within the CD4$^+$ compartment.
A: Flow cytometric dot plot showing frequency of FoxP3$^+$CD127- cells in the blood and skin of healthy individuals.
B: Frequency of FoxP3$^+$CD25$^+$CD127$^+$ Tregs in the CD4$^+$ T cell compartment in samples derived from the blood (n=6) and skin (n=5) of healthy individuals. Mean and standard deviation are shown. Populations were compared using the t-test.
C: Flow cytometric dot plot showing CD45RA/CD27 surface expression on skin derived total CD4$^+$ T cells and FoxP3$^+$ CD4$^+$ Tregs.
D: Frequency of T_{CM} among FoxP3$^+$ Treg compartment and of FoxP3$^+$ Treg cells in the T_{CM} compartment of skin derived CD4$^+$ T cells (n=4). Mean and standard deviation are shown.
E: Flow cytometry histogram showing CD69 and CTLA-4 expression in total skin CD4$^+$ T cells and FoxP3$^+$ CD4$^+$ Tregs.
**=p<0.01

4.3.2.8 The majority of skin resident $CD8^+$ T cells do not express granzyme B or perforin

Memory cells expressing high levels of the cytotoxic granule components perforin and granzyme B can be detected in the blood of healthy individuals and are generally associated with a more differentiated phenotype (Henson et al. 2014). Whilst these cells therefore have low proliferative capacity and reduced functional plasticity due to their lineage commitment, they are thought to confer immediate protection against infected and malignant cells compared to other less differentiated cells types that would need more time to synthesize effector proteins upon activation. The expression of cytotoxic granule components and markers associated with cytotoxicity have yet to be fully investigated in skin resident T cells.

Intracellular granzyme B and perforin expression were therefore measured in the blood and skin derived $CD8^+$ T cells of healthy individuals by flow cytometry (Fig. 4-12). Whilst granzyme B and perforin could be detected in blood samples in varying amounts ranging from 5-57% and 3-35% respectively, skin $CD8^+$ T cells consistently expressed low levels of granzyme B (averaging at 6±3%, maximum 11%), and no perforin (2±2%).

In order to verify that the process of skin cell extraction was not the reason for the low levels of perforin and granzyme B measured, immunofluorescent staining of frozen skin sections was performed. Staining for either granzyme B or perforin together with CD8 confirmed that the majority of skin resident T cells did not express granzyme B or perforin *in situ* (Fig. 4-13).

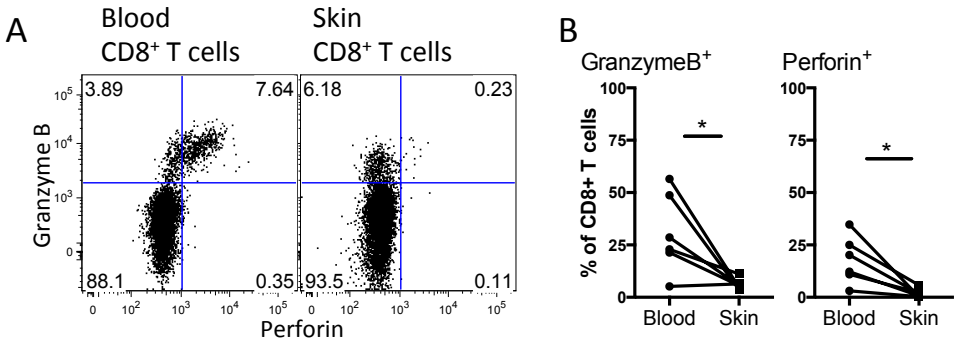


Fig. 4-12: Perforin and Granzyme B expression in CD8$^+$ T cells derived from blood or skin of healthy individuals.

A: Representative dot plots showing typical granzyme B and Perforin expression patterns in blood and skin derived CD8$^+$ T cells.
B: Percentage of CD8$^+$ T cells expressing granzyme B or perforin in the skin compared to the blood of the same donor. Populations were compared using the paired t-test.
*=p<0.05

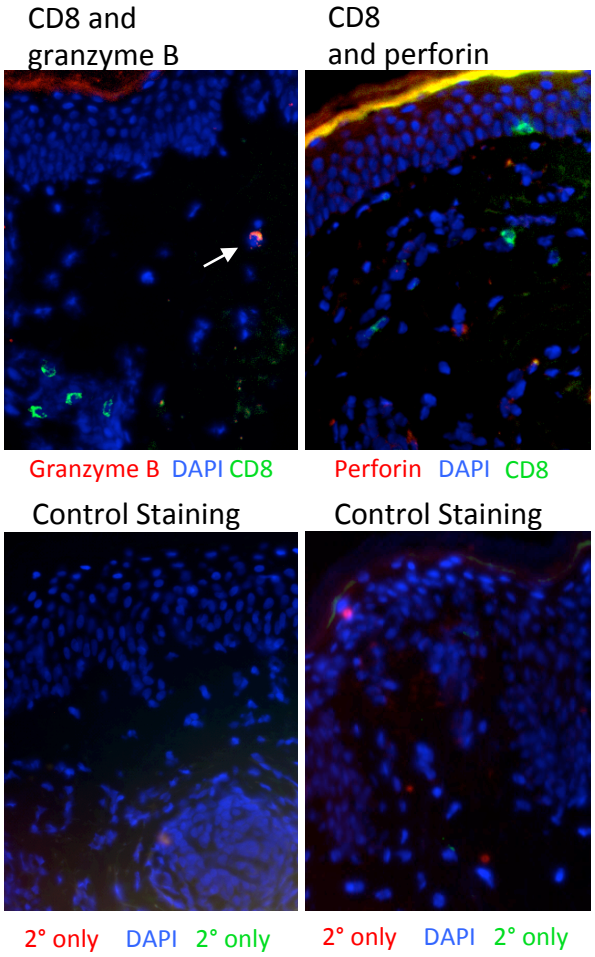


Fig. 4-13: Immunofluorescent staining of healthy skin for granzyme B or perforin and CD8$^+$ T cells.

Immunofluorescence stain performed on frozen sections of healthy skin using CD8 (green), DAPI (blue) and either granzyme B or perforin (both red). The white arrow indicates the presence of a rare CD8 and granzyme B double positive staining cell. The first row shows full staining, whilst the second row shows control stains using only the appropriate 2° antibodies, omitting primary antibodies for granzyme B and CD8 (on the left) and antibodies for perforin and CD8 (on the right).

4.3.2.9 Disconnection between CD45RA/CD27 defined subsets and expression of cytotoxic markers in the skin

Among memory T cells in the blood, perforin and granzyme B expression are associated with a highly differentiated phenotype. Cells carrying these cytotoxic granule components therefore mainly belong to the T_{EM} and T_{EMRA} phenotypes and express low levels of the costimulatory marker CD28 and high levels of the senescence-associated marker KLRG1 (Henson et al. 2014; Appay et al. 2008; Speiser et al. 1999). It was confirmed that in the blood $CD8^+$ T cells, granzyme B expression was highest among the T_{EM} and T_{EMRA} (Fig. 4-14) and was strongly associated with low levels of CD28 and high levels of KLRG1 (Fig. 4-15). Among the skin derived $CD8^+$ T cells, granzyme B and perforin were only expressed in a minority of T_{EMRA} cells (16±16% and 12±15% respectively) and particularly low in the T_{EM}-like subset (8±10% and 2±5% respectively; Fig. 4-14).

Loss of CD28 in skin $CD8^+$ T cells did not correlate with an increase in granzyme B as it did in the blood (Fig. 4-15B). Indeed, cells in the skin that expressed granzyme B and those that did not comprised equal levels of CD28 expressing cells (60±12% and 62±15% respectively; Fig. 4-15C). Interestingly, granzyme B expressing cells in the skin showed increased levels of KLRG1 (22±10%) compared to granzyme B negative $CD8^+$ skin T cells (5±4%; p=0.0002; Fig. 4-15). However, KLRG1 was not a suitable marker for granzyme B expression in the skin, as the majority of Granzyme B expressing skin T cells did in fact not express KLRG1.

$CD8^+$ T cells in the skin therefore appear to have low cytotoxic potential under steady state conditions, as they only express low levels of cytotoxic granule components. Further, the small fraction of cells expressing granzyme B in the skin did not display the surface markers commonly associated with a cytotoxic phenotype in memory T cells in the blood.

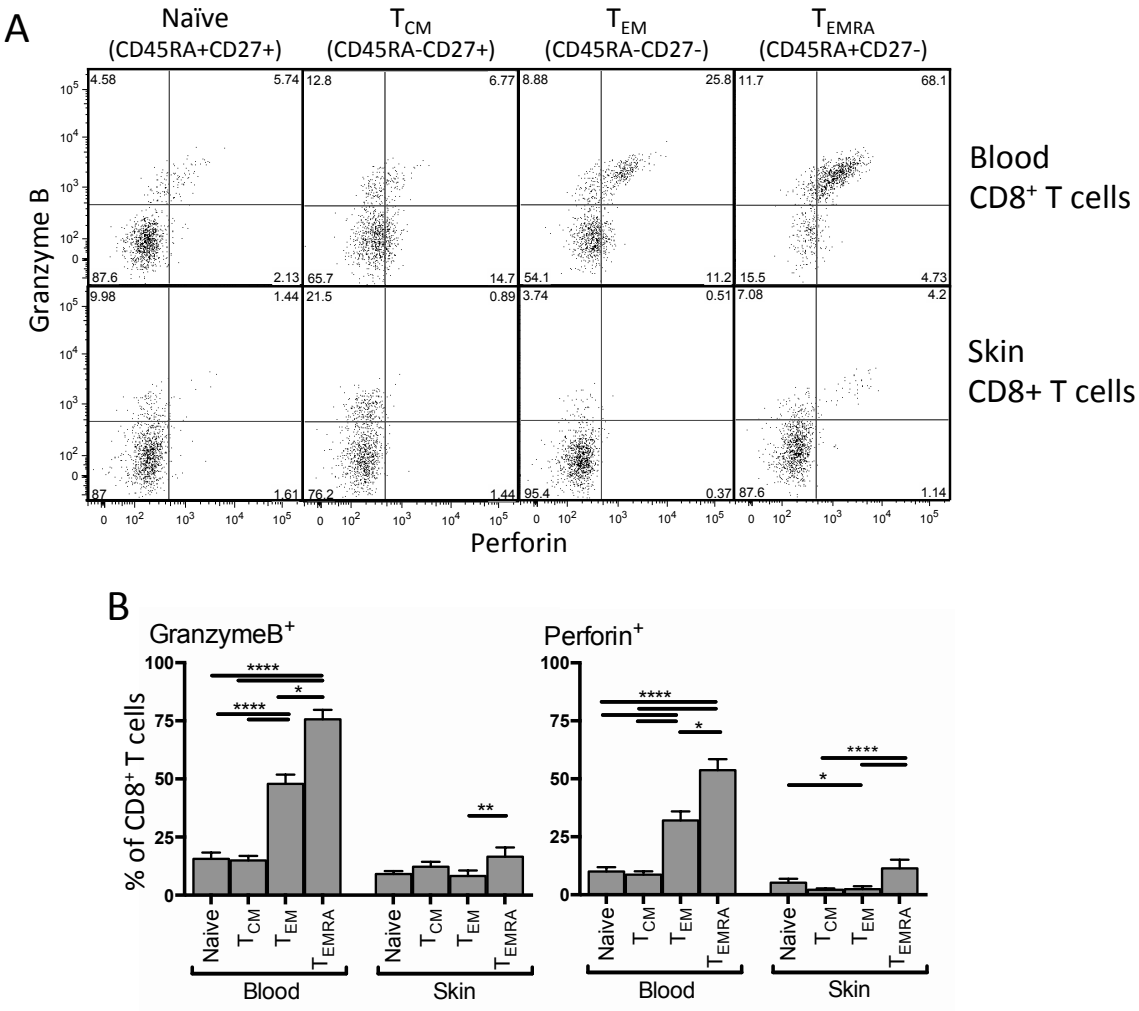


Fig. 4-14: Expression of the cytotoxic granule components granzyme B and perforin in the CD8$^+$ T cell subsets derived from the skin and the blood of healthy donors.

A: Representative flow cytometry dot plots showing the expression of Perforin and Granzyme B in the subsets of skin and blood derived T cells.
B: Histograms showing the frequency of granzyme B and perforin expressing cells in the CD8$^+$ T cell subsets in the blood (n= 34) and in the skin (n=17) of healthy volunteers. Mean and standard error are shown, populations were compared using one-way ANOVA.
*=p<0.05; ****=p<0.0001

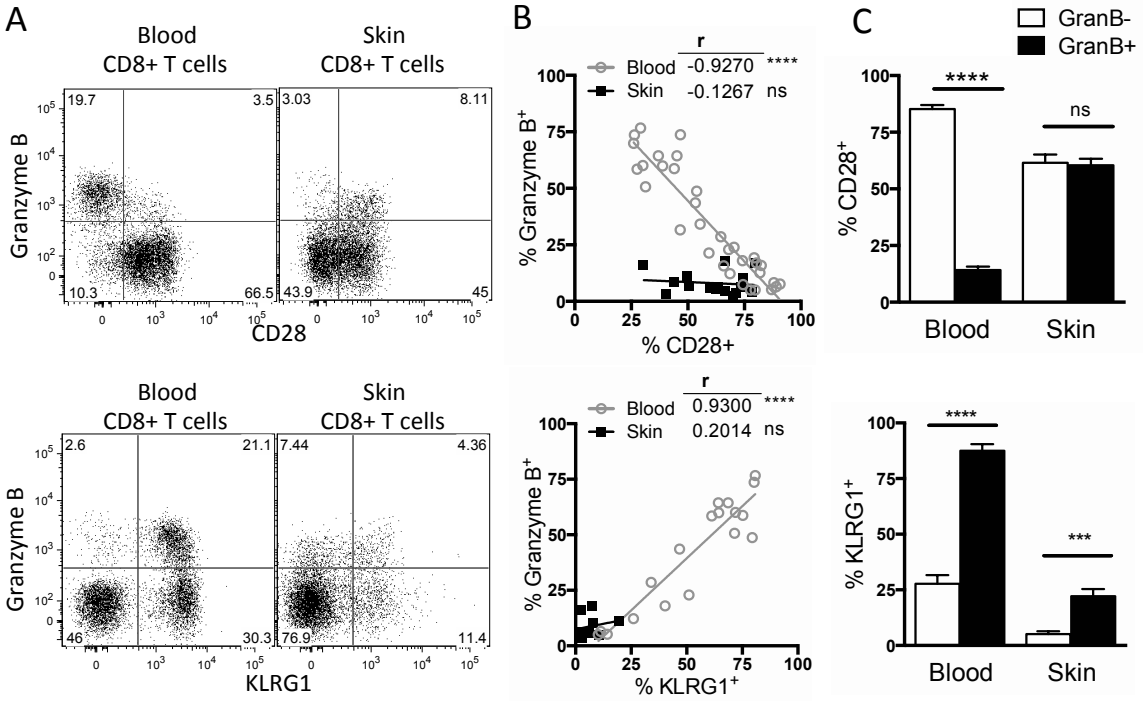


Fig. 4-15: Correlation of surface CD28 and KLRG1 to intracellular granzyme B expression in CD8$^+$ T cells derived from skin and blood of healthy individuals.

A: Representative flow cytometry dot plots showing CD28 and KLRG1 expression in relation to Granzyme B expression in blood and skin derived CD8$^+$ T cells.
B: Correlations between percentage CD28 or KLRG1 expressions with percentage granzyme B expression in blood and skin derived CD8$^+$ T cells. Lines of best fit, r values and significance are shown.
C: Cumulative data showing frequencies of CD28 or KLRG1 expressing cells in granzyme B positive (GranB$^+$) and granzyme B negative (GranB-) blood and skin derived CD8$^+$ T cells. Averages and standard errors are shown. Populations were compared using the paired t-test. ***p<0.001, ****p<0.0001.

4.4 Discussion

4.4.1 Optimizing a protocol to isolate T cells from the skin

The fact that isolation of skin resident T cells is technically challenging has long hampered advances in skin immunology research. However, a number of protocols have been published, most relying on enzymatic digestion or crawl-out methods. In 2006 Clark and colleagues described a technique yielding in average about 250,000 T cells per cm^2 of skin, by using a method involving culture of skin explants for up to 21 days and harvesting cells spilling out from the tissue (Clarket al. 2006a; Clark et al. 2006b). The authors argued that this technique yielded unaltered cells and was therefore superior to collagenase digestion, which caused degradation of surface markers such as CCR4 and yielded on average only around 2 000 T cells per cm^2.

However, culture conditions and relying on a cell's ability to migrate out of the skin sample might affect compositions and phenotypes of cell populations harvested. An enzymatic digestion based method was therefore adopted for this work in order to reduce the time samples spent in culture. Methods for mechanical skin disaggregation were tested to further reduce processing time but were found to be unsatisfactory for efficient skin cell recovery. Using an optimized method of overnight collagenase-based skin digestion, an average of 50,000 cells were recovered per cm^2 of skin, although this count likely included cell types other than T cells due to morphological similarities when viewed under the light microscope. Surface marker degradation was prevented reliably by increasing the concentration of FBS in the digestion medium from 10% to 20% as recommended by Mulder and colleagues, who proposed that this would block contaminating proteases in the commercial collagenase preparations (Mulder et al. 1994).

It is estimated that a million cells reside in each cm^2 of healthy skin under steady state conditions (Clark et al. 2006) and the technique used here only allowed for the recovery of a fraction of these cells for analysis. However, we are confident that the cells recovered (and therefore the data presented here) are a reliable representation of skin cells *in vivo*, as a majority of results

matched previously published results and/or were matched by histological evidence.

4.4.2 **Characterizing skin derived T cells**

It is often reported that skin resident T cells are T_{EM}-like (Schenkel and Masopust 2014; Natsuaki et al. 2014). The findings described here on healthy skin T cells suggest that the majority of skin resident $CD4^+$ and $CD8^+$ T cells may appear indeed T_{EM}-like on the basis that most expressed neither CD45RA nor CD27. However, further phenotyping revealed high levels of CD28 expression (commonly found on less differentiated cells) and low levels of CD57, KLRG1, granzyme B and perforin, which are commonly associated with the T_{EM} subset (Appay et al. 2008) . Therefore, based on the associations made in the blood (by us and others) between surface marker expression patterns and T cell differentiation, one might deduce that skin resident T cells have an intermediate differentiation phenotype. However such projection of rules established in the blood onto cells in the skin should be made with care: Cell surface markers might have different physiological functions in the tissue and correlations made between these markers and cellular differentiation might be circumstantial rather than casual, as their role in T cell differentiation is still poorly understood. The fact that phenotypic markers established in the blood might not hold true in the context of skin resident T cells is supported by our observation that the CD45RA/CD27 defined subsets in the skin predicted poorly the expression of CD28, KLRG1 and CD57 compared the blood. Similarly, markers commonly associated with cytotoxic potential in the blood were not applicable to granzyme B expressing cells in the skin.

The question on how differentiated resting skin resident T cells are therefore remains enigmatic. A recent study on mice showed that skin T cells were predominantly $KLRG1^+$ during the effector phase shortly after activation, whilst the long-lived memory cells remaining after disease resolution were KLRG1-. Interestingly, these memory cells were not derived from the dominant $KLRG1^+$ population, but instead from a minor KLRG1- memory precursor population generated early during infection (Mackay et al. 2013). The predominantly KLRG1- memory T cells observed in the healthy human skin might therefore similarly be derived from such precursor cells with low differentiation rather than more differentiated cells with effector properties. Future experiments should include measurements of the telomere length and proliferative capacity of the skin resident T cells in order to confirm their relative state of differentiation.

PD-1 and CTLA-4 are inhibitory markers involved in immune regulation and maintenance of tolerance (Waterhouse et al. 1995; Nishimura et al. 1999). In the context of persistent disease, they have also been associated with immune exhaustion (Nakamoto et al. 2009; Ahmadzadeh et al. 2009; Pentcheva-Hoang et al. 2014). Skin derived T cells displayed significantly increased levels of PD-1 compared to blood derived cells, whilst CTLA-4 levels were similar between the two organs. Previous studies have shown that PD-1 levels are also increased (compared to the blood) on T cells in other healthy non-lymphoid tissues such as the brain and gastric mucosae (Sadagopal et al. 2010; Saito et al. 2013). This suggests that PD-1 may be important in regulating tissue resident T cells in the skin and other peripheral organs.

Despite the often-stated assumption that skin resident T cells are readily poised effector cells, granzyme B and perforin expression were low in healthy skin. This observation has been made previously by others who confirmed low expression of both enzymes at protein and mRNA levels *in situ*. Interestingly, the same authors noted an increase in these molecules in the skin during vitiligo, Lichen sclerosus, psoriasis and atopic dermatitis, confirming that T cells can express granzyme B and perforin in the skin during inflammatory conditions (van den Wijngaard et al. 2000; N. Yawalkar et al. 2001; Hunger et al. 2007). Granzyme B is not only important for target cell killing, but can also, when secreted, promote inflammation and extracellular matrix degradation and threaten skin matrix integrity (Hiebert and Granville 2012). Perforin and granzyme B expression might therefore be maintained at low levels in healthy skin in order to limit immune-mediated tissue damage.

Finally, it was noted that the skin is not formed of phenotypically uniform $CD4^+$ and $CD8^+$ T cells. The $CD4^+$ T cell compartment for example included a T_{CM}-like population, which contained $FoxP3^+$ Treg cells expressing high levels of CTLA-4 but no CD69. Confirming previous reports, Treg frequency was higher in the skin compared to blood (Clark and Kupper 2007; Booth et al. 2010; Vukmanovic-Stejic et al. 2013; Sanchez Rodriguez et al. 2014). $FoxP3^+$ Tregs are known to have strong immunosuppressive functions and their role in maintaining immune tolerance in the skin has been highlighted in knockout models in mice (Dudda et al. 2008). Other smaller populations of skin resident T

cells that might deserve special attention are the KLRG1-CD57^{+} cells not present in the blood.

In summary, the results in this chapter suggest that the majority of skin resident T cells are at an imbalance with T cells from the blood. They appear to have an intermediate differentiation phenotype with low cytotoxic potential and may be regulated through surface expression of PD-1. The signals involved in licensing skin resident T cells to become potent effector cells will be explored in the final results chapter. First, T cell differentiation of melanoma patients' skin T cells will be examined in the following chapter.

5 T cell differentiation in the skin of patients with melanoma

5.1 Introduction

Much research has been devoted to phenotyping melanoma specific cells in the circulation, whilst little is known about the T cells in the skin of the patients. This is despite the fact that frequency and phenotypes of circulating melanoma specific cells do not correlate with disease outcome, whilst immune processes in the uninvolved skin as well as lesions of the patients have prognostic value. For example, the presence of tumour infiltrating lymphocytes has been shown to be a positive prognostic factor in a number of studies (Ladányi et al. 2004; Haanen et al. 2005). Similarly, delayed type hypersensitivity responses in melanoma vaccination studies were very good indicators of treatment efficacy (A. Baars et al. 2000).

After establishing differentiation marker expression patterns in the skin of healthy donors, we therefore investigated if and how differentiation patterns might be altered in the skin T cells of melanoma patients. In this study, the majority of melanoma skin samples were derived from wide local excision skin, as described in Fig. 5-1. Skin specimens available for research were removed from the outermost margins of the excision, which was deemed unlikely to contain tumour cells. Primary melanoma tissue itself was only available for research purposes on very rare occasions (when the lesion had grown very large), as these samples were needed for clinical histological grading.

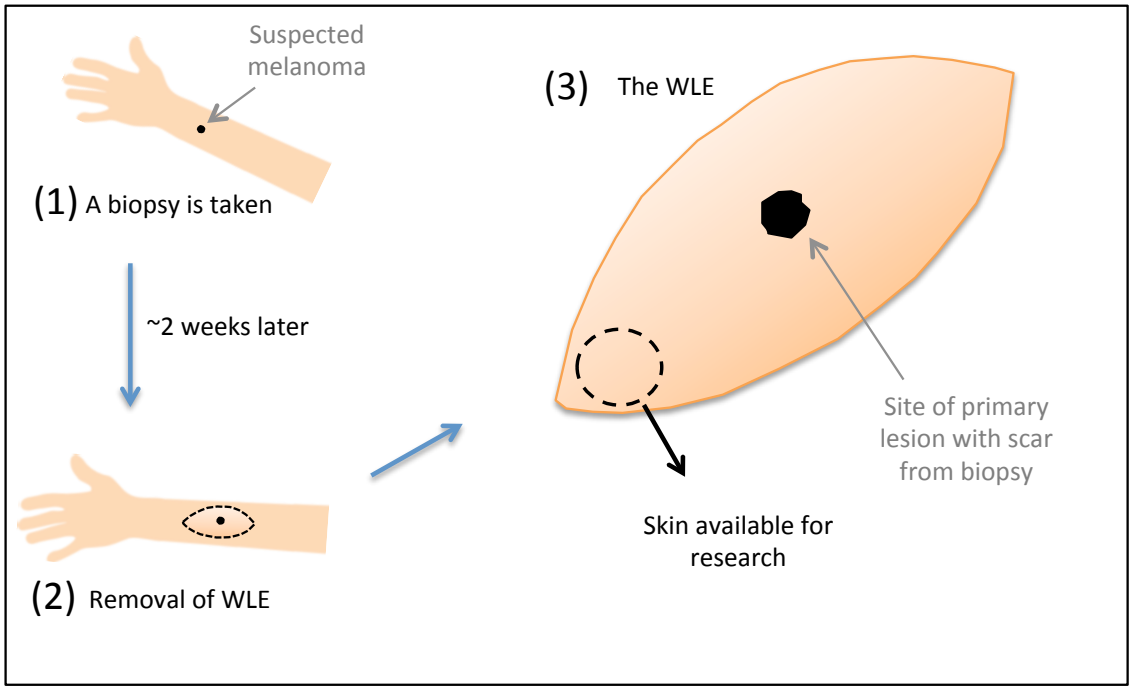


Fig. 5-1: Schematic diagram outlining the procedure for obtaining skin from melanoma patients for research purposes.

Step (1): The patient presents a suspicious mole and a biopsy is taken from the site. The biopsy is analysed by histology to confirm malignancy.
Step (2): After confirmation of malignancy (typically 2 weeks after the first visit), the patient undergoes surgical removal of a wide area around the melanoma lesion. This wide local excision is performed by removing the skin surrounding the primary melanoma in a margin of at least 2cm in order to ensure total removal of tumour cells from the skin of the patients.
Step (3): Skin from the outermost areas of the wide local excision is made available for research purposes.
WLE= wide local excision

5.2 Aims

The aim of this chapter was to investigate T cell differentiation in uninvolved skin and sites of tumour invasion in melanoma patients and compare the phenotypes where possible to T cells derived from the skin of healthy controls.

5.3 Results

5.3.1 Assessing T cell differentiation in the wide local excision skin of patients with melanoma

5.3.1.1 Imbalance of phenotypic properties between melanoma patient skin and blood T cells

First, phenotypic properties were compared between the blood and skin of patients with melanoma, as skin derived T cells were found to differ markedly from their circulating counterparts in healthy individuals (see chapter 4).

Frequencies of CD45RA and CD27 defined subsets were measured by flow cytometry and compared between the blood and the skin of patients with melanoma (Fig. 5-2). Compared to the cells in the circulation, skin T cells of melanoma patients displayed a significant increase in $CD4^+$ and $CD8^+$ T_{CM} ($p= 0.0002$ and $p< 0.0001$, respectively). This was accompanied by a decrease in naïve $CD4^+$ T cells ($p< 0.0001$) and $CD8^+$ T cells ($p= 0.0273$), an increase in $CD4^+$ T_{CM} ($p= 0.0002$) and a decrease in $CD8^+$ T_{EMRA} ($p= 0.0100$).

In circulating T cells, CD28 expression is lost and KLRG1 expression is gained with differentiation. Naïve and T_{CM} in the blood therefore have high CD28 and low KLRG1, whilst T_{EM} and T_{EMRA} have low levels of CD28 and high levels of KLRG1 (Appay et al. 2008). To determine whether this was also true in skin derived T cells from patients with melanoma, matched blood and skin samples of patients were analysed via flow cytometry for these markers (Fig. 5-3). Whilst blood derived $CD4^+$ and $CD8^+$ T cell subsets of the patients followed the expected pattern, T_{EM} and T_{EMRA} cells in the skin expressed high CD28 and decreased KLRG1 compared to their circulating counterparts.

Similarly to previous observations in healthy individuals, melanoma patients therefore presented with altered T cell subset compositions in the skin compared to the blood and a disconnection was found between the skin T cell subsets and markers of differentiation.

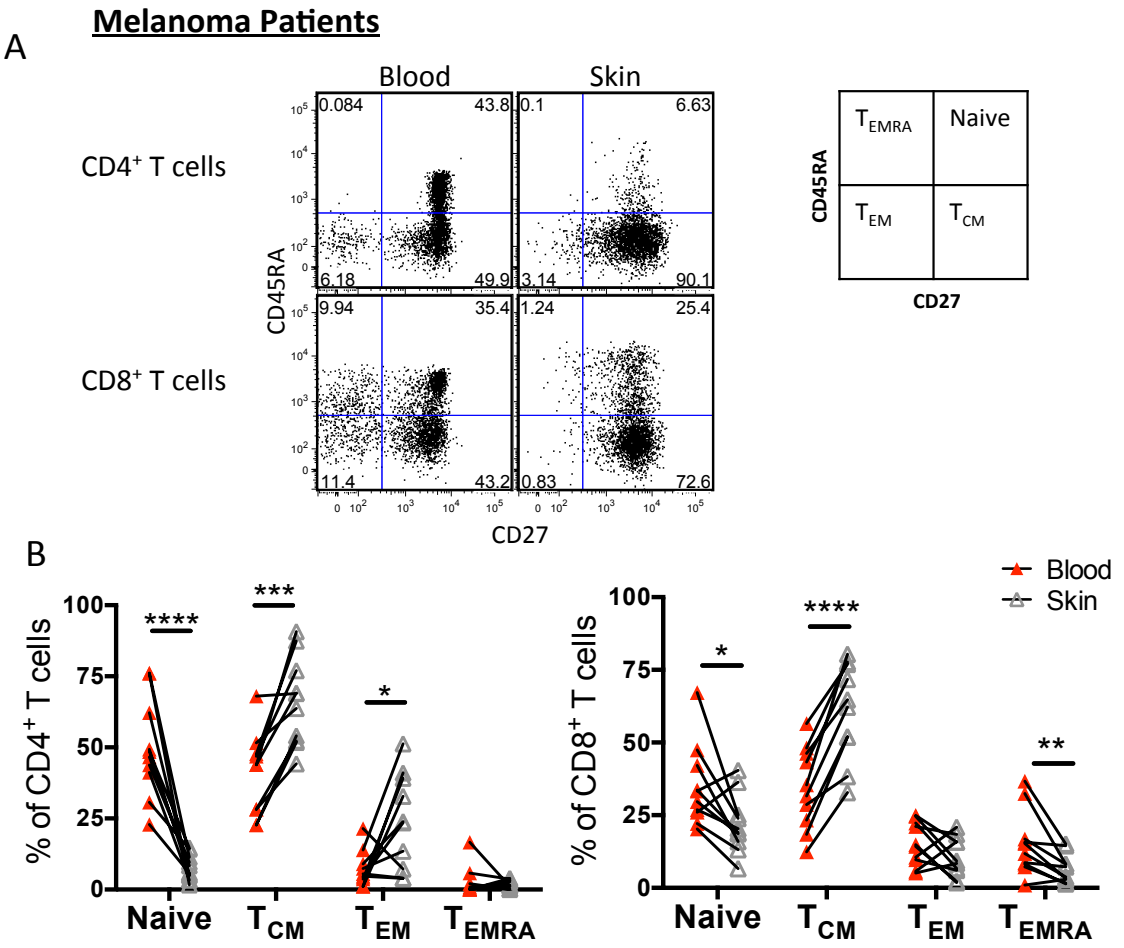


Fig. 5-2: CD45RA/CD27 subset distribution patterns of $CD4^+$ and $CD8^+$ T cells derived from melanoma wide local excision skin compared to the blood of the same donor.

T cell CD45RA/CD27 subset distributions were measured by flow cytometry in cells derived from paired skin and blood samples from patients with melanoma (n=10).
A: Representative flow cytometric dot plot showing CD45RA/CD27 expression in the blood and skin derived $CD4^+$ and $CD8^+$ T cell compartments of the same donor. The diagram on the right highlights the names given to the four subsets.
B: Direct intraperson comparison of skin and blood T cell subset distribution. Populations were compared using the paired t-test.
*=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001

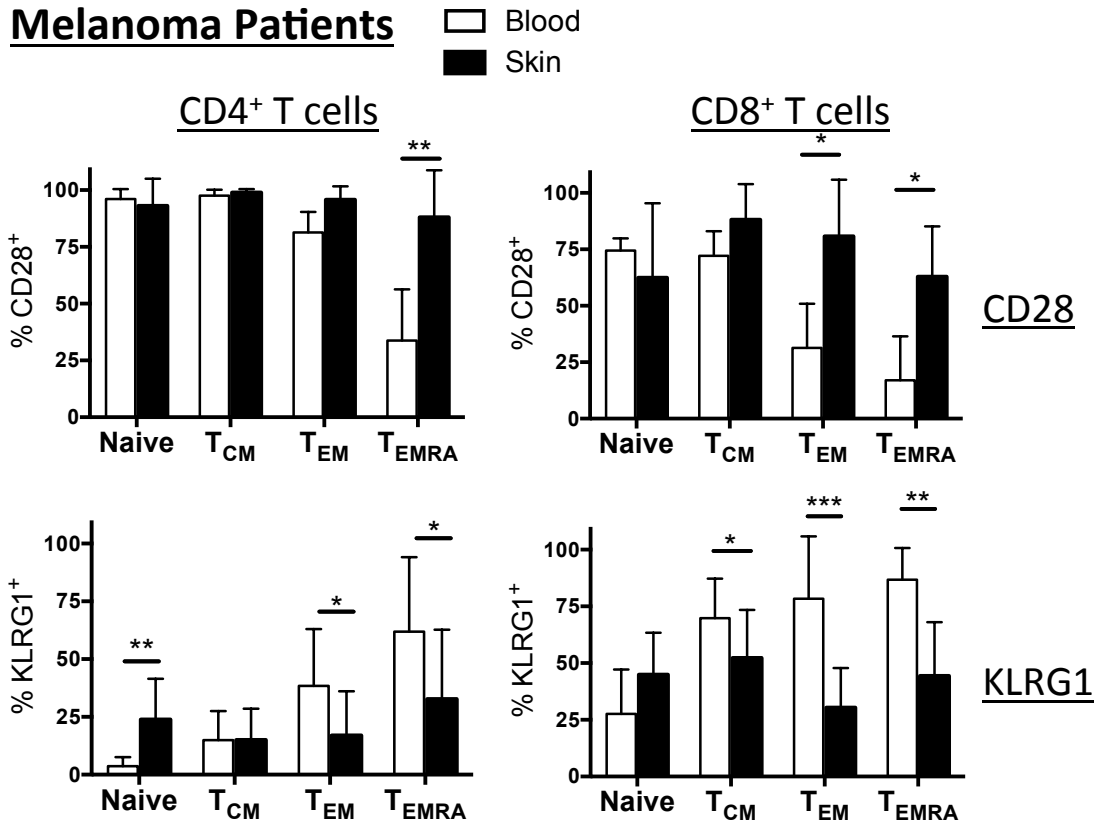


Fig. 5-3: CD28 and KLRG1 expression in $CD4^+$ and $CD8^+$ T cell subsets in the skin compared to the blood in melanoma patients.

CD28 (n=3) and KLRG1 (n=9) were measured by flow cytometry in the CD27/CD45RA subsets of $CD4^+$ and $CD8^+$ T cells derived from paired blood and skin samples from patients with melanoma. Mean and standard deviation are shown.
Frequencies of positive cells in each subset were compared between blood and skin using the paired t-test.
*=p<0.05; **=p<0.01; ***=p>0.001

5.3.2 **CD45RA/CD27 subset distribution is altered in the skin derived T cells of melanoma patients compared to healthy controls**

Next, CD45RA/CD27 $CD4^+$ T subset distribution was assessed in the skin of melanoma patients and compared to healthy controls (Fig. 5-4A). In healthy individuals, the skin $CD4^+$ T cells were mostly T_{EM} with a smaller proportion of T_{CM}-like cells and this did not change with age. Melanoma patients on the other hand displayed an age dependent change in subset distribution by displaying a significant decrease of T_{CM}-like cells ($p=0.0082$) and an increase of T_{EM}-like cells ($p=0.0069$) with age (Fig. 5-4B). Young (aged under 40) melanoma patients' $CD4^+$ T cells showed high levels of T_{CM}-like cells that were significantly increased compared to age matched healthy controls (79±11% compared to 27±9% in healthy; $p<0.0001$) and this was accompanied by a significant reduction in T_{EM} (16±11% compared to 67±11%; $p<0.0001$) and T_{EMRA}-like cells in the patients (0.6±0.6% compared to 5±4%; $p=0.0494$; Fig. 5-4C). Old melanoma patients (aged 60 and above) showed lower levels of T_{CM} compared to young patients, but these levels were still increased compared to healthy age matched controls (49±18% in old melanoma compared to 32±11% in old healthy skin, $p=0.0266$; Fig. 5-4C). Old melanoma patients also displayed a significant decrease in T_{EMRA}-like cells compared to healthy controls (2±2% versus 10±9% in healthy; $p=0.0182$), whilst T_{EM} levels were at similar levels (46±19% versus 53±14% in healthy; $p=0.2731$).

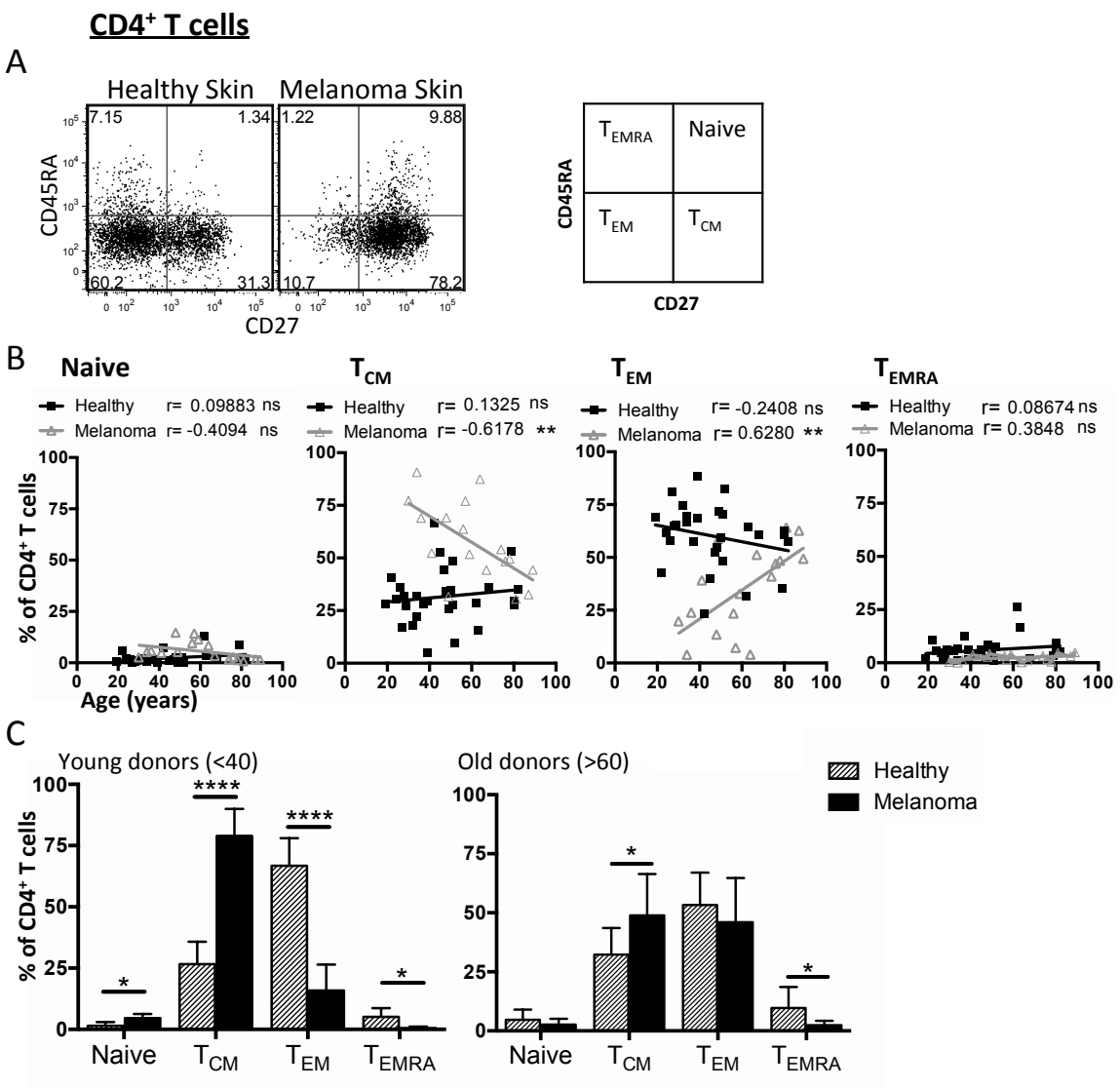


Fig. 5-4: CD4$^+$ T cell CD45RA/CD27 subset distribution patterns in the wide local excision skin of melanoma patients compared to skin derived cells of healthy controls.

CD4$^+$ T cell CD45RA/CD27 subset distributions were measured by flow cytometry in cells derived from the skin of melanoma patients and healthy controls of all ages.

A: Representative flow cytometric dot plot showing CD45RA/CD27 expression in the CD4$^+$ T cell compartment in skin derived cells of a melanoma patient and a healthy individual. The diagram on the right highlights the names given to the four subsets.

B: Cumulative data showing CD4$^+$ T cell subset distribution with age in skin derived cells from melanoma patients (n=17) and healthy controls (n=29). Lines of best fit were generated by linear regression and the correlation (r-value) and significance were assessed by Pearson or Spearman rank.

C: Direct comparison of CD4$^+$ T cell subset distribution in melanoma patients and healthy controls in the young (aged under 40 years; n=3 for melanoma and n=13 for healthy) or old (aged 60 and above; n=8 for melanoma and n=7 for healthy) cohorts. Populations were compared using the unpaired T test.

*=p<0.05; ****=p<0.0001

Changes in the $CD8^+$ T cell compartment in the skin of melanoma patients resembled those observed in the $CD4^+$ T cells and again, age-dependent changes is subset frequencies were found in melanoma patients but not healthy controls. In melanoma patients, T_{CM} and naïve-like frequencies decreased (not-significant and p=0.0181 respectively) and T_{EM} levels increased (p=0.0007) with age (Fig. 5-5B). When comparing subset frequencies in patients and controls, young melanoma patients' $CD8^+$ T cells displayed an increase in T_{CM}-like cells compared to age matched healthy controls (76±5% compared to 25±14% in healthy; p< 0.0001), and this was accompanied by a significant decrease in T_{EM} (5±2% vs 39±15%; p=0.0021) and T_{EMRA}-like cells (2±1% vs 17±11; p=0.0384), whilst naïve-like cells remained at similar levels (17±4% for melanoma and 19±11% for healthy young donors). Conversely, old melanoma patients exhibited similar levels of subset frequencies as healthy controls, apart from displaying a significant decrease of naïve-like cells (11±5% in melanoma patients compared to 21±12% in healthy controls; p=0.0495).

Based on CD45RA/CD27 expression, melanoma patients' skin derived $CD4^+$ and $CD8^+$ T cells therefore appeared to display an increase in T_{CM}-like cells and decrease in T_{EM}-like cells compared to healthy controls, particularly at younger age.

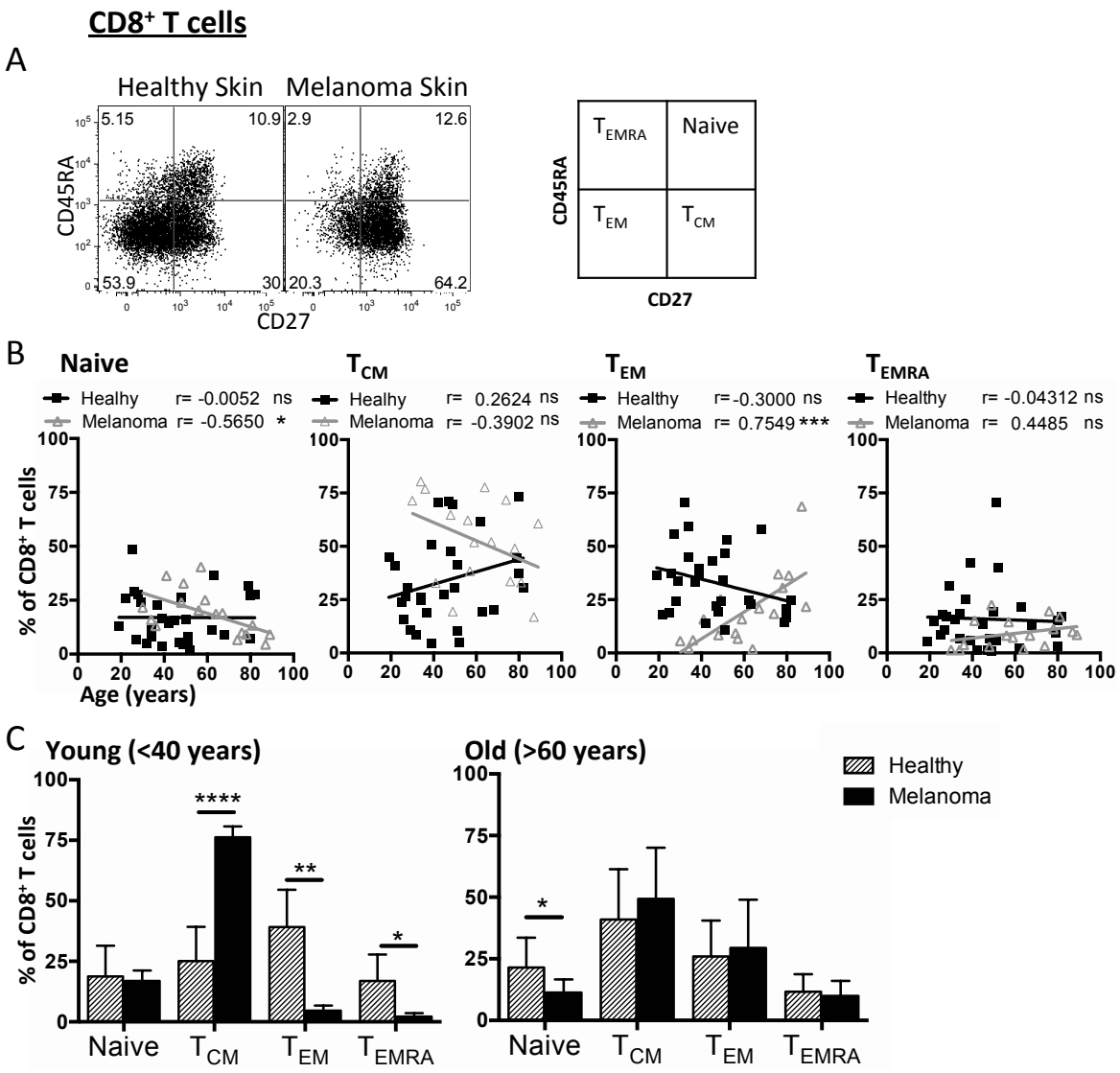


Fig. 5-5: Changes in CD8$^+$ T cell subset distribution patterns in the skin of melanoma patients compared to healthy controls

CD45RA/CD27 subset distributions were measured by flow cytometry in the skin derived CD8$^+$ T cells of melanoma patients and healthy controls of all ages.

A: Representative flow cytometric dot plot showing CD45RA/CD27 expression in the CD8$^+$ T cell compartment in skin derived cells of a melanoma patient and a healthy individual. The diagram on the right highlights the names given to the four subsets.

B: Cumulative data showing CD8$^+$ T cell subset distribution with age in skin derived cells from melanoma patients (n=17) and healthy controls (n=29). Lines of best fit were generated by linear regression and the correlation (r-value) and significance were assessed by Pearson or Spearman rank.

C: Direct comparison of CD8$^+$ T cell subset distribution in melanoma patients and healthy controls in the young (aged under 40 years; n=3 for melanoma and n=13 for healthy) or old (aged 60 and above; n=8 for melanoma and n=7 for healthy) cohorts. Populations were compared using the unpaired T test.

*=p<0.05; **=p<0.005; ****=p<0.0001

5.3.2.1 **CD45RA/CD27 subset distribution in primary melanoma lesions resembles that of melanoma skin**

Our observations made on T cell phenotypes in the skin of melanoma patients were based on wide local excision skin, as this was the tissue most readily available. We were also able to obtain tissue from primary melanoma lesions from old donors on 3 occasions. We extracted resident T cells from these primary lesions via collagenase digestion and measured CD45RA and CD27 surface expression via flow cytometry. T cell subset distribution of primary melanoma derived cells was compared to cells from wide local excision skin and healthy skin of age matched individuals (Fig. 5-6). T cells from primary melanoma lesions from old donors displayed similar CD45RA/CD27 expression patterns to those from old melanoma wide local excisions and increased T_{CM} levels compared to T cells from the skin of old healthy individuals, particularly among the $CD4^+$ T cells ($p=0.0269$). T cell subset distribution in primary melanoma lesions therefore appears to follow similar patterns to wide local excision skin by displaying an increase in T_{CM}-like cells. However, more primary melanoma lesions should be collected in the future to confirm this.

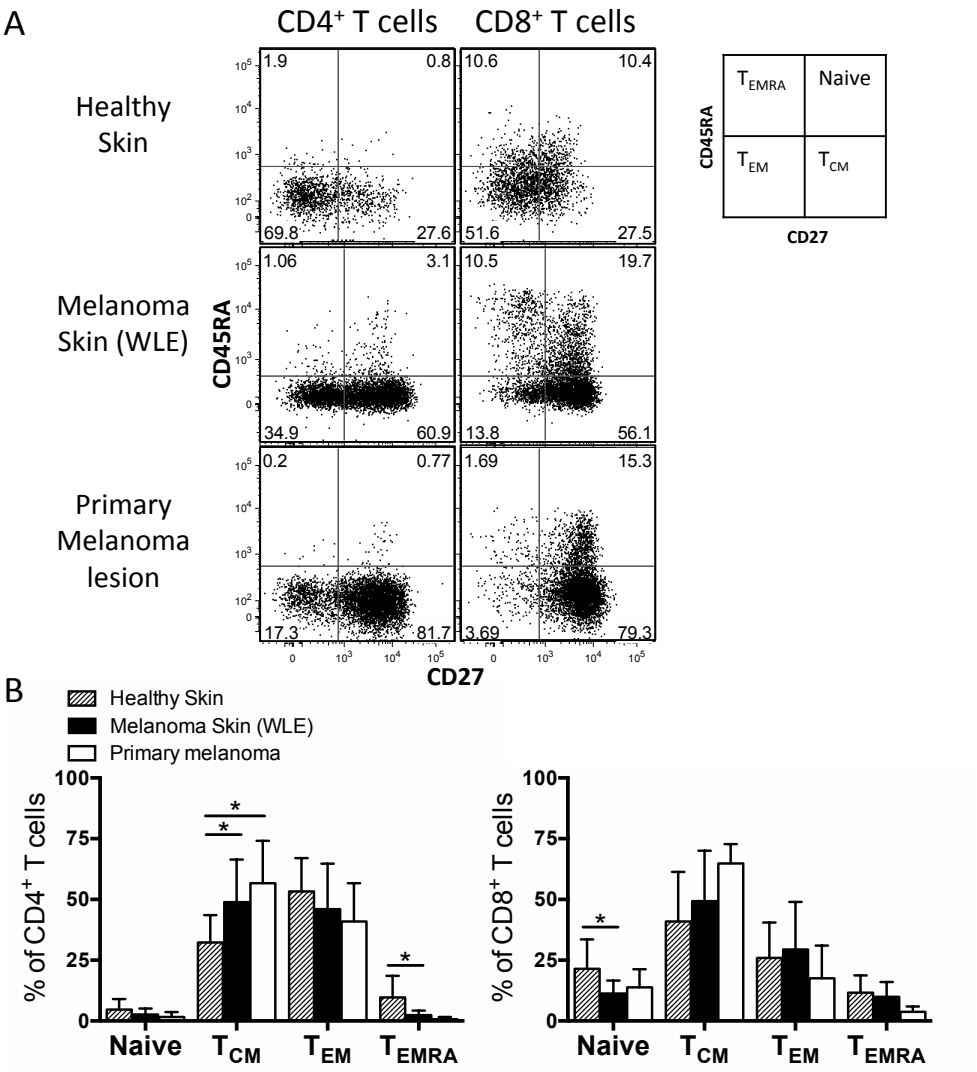


Fig. 5-6: CD27 and CD45RA subset distribution in T cells derived from primary melanoma lesions compared to melanoma patient skin from wide local excisions and skin from healthy controls.

Cells were extracted from tissues using collagenase digestion and analysed via flow cytometry. All participants were aged 60 or above. CD45RA and CD27 expression was compared between $CD4^+$ and $CD8^+$ T cells from primary melanoma lesions (n=3) , melanoma skin from wide local excisions (WLE; n=8) and healthy skin (n=7).

A: Representative flow cytometric dot plots showing CD45RA and CD27 expression in $CD4^+$ T cells and $CD8^+$ T cells in the three different conditions. The diagram on the right highlights the names given to the four subsets.

B: Cumulative data showing average percentages and standard deviation of each CD45RA/CD27 subset in healthy skin, wide local excision skin and primary melanoma skin.

Percentages in different conditions were compared using the standard T-test. *=$p<0.05$

5.3.2.2 **CD28 expression is increased on T cells in the skin of patients with melanoma compared to healthy controls**

CD27 and CD28 are costimulatory markers which are lost on highly differentiated cells (Appay et al. 2008). Because we found an increase in CD27 expressing cells in the skin of patients with melanoma compared to healthy controls, we investigated whether this was also true for CD28. We measured CD28 expression on the T cells extracted from the skin of patients with melanoma and compared levels to the T cells extracted from the skin of healthy individuals (Fig. 5-7). Because expression of CD28 did not change with age in both patients and controls, participants were compared across all age groups. A significant increase of CD28 could be detected among the $CD4^+$ T cells of melanoma patients as 96±4% expressed the receptor compared to 89±8% in healthy individuals (p=0.0157). CD28 was also increased on the $CD8^+$ T cells of some melanoma patients compared to healthy controls but this difference was not significant (75±21% compared to 61±14% in healthy individuals; p=0.0693). Therefore, like CD27, CD28 was increased on the T cells of patients with melanoma.

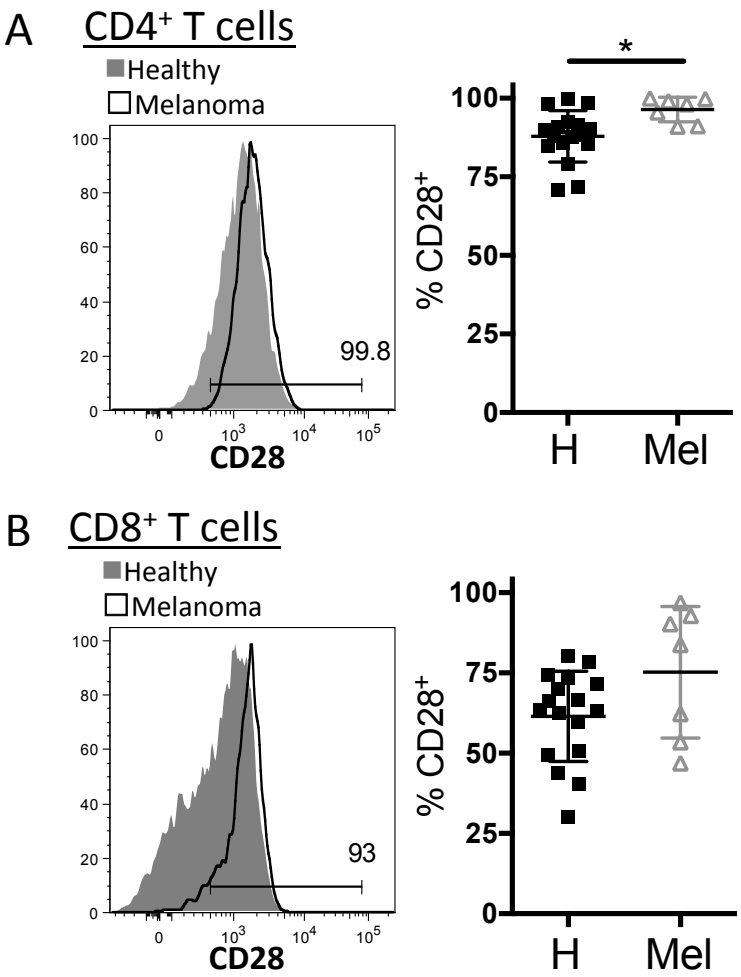


Fig. 5-7: Comparison of CD28 expression in T cells derived from the skin of melanoma patients and healthy individuals.

CD28 expression was measured by flow cytometry ex vivo in T cells derived from the skin from melanoma patients (n=7) and healthy controls (n=17) across all age groups. H= healthy skin. Mel= melanoma skin.
A: representative graph and cumulative data showing CD28 expression in $CD4^+$ T cells.
B: representative graph and cumulative data showing CD28 expression in $CD8^+$ T cells.
Mean and standard deviation are shown. Cohorts were compared using the standard T-test.
*=$p<0.05$

5.3.2.3 **Skin derived $CD8^+$ T cells of melanoma patients show an increase in CD57 and KLRG1 expression.**

As described in the previous chapters, KLRG1 and CD57 are expressed on highly differentiated cells in the blood. KLRG1 and CD57 coexpression were measured in skin derived T cells of melanoma patients and compared to healthy individuals. Although expression of both markers increases with age in blood T cells, this was not found to be the case in the skin. Participants were therefore compared across all age groups (Fig. 5-8A,B). $CD4^+$ T cells in the skin of melanoma patients expressed similar levels of CD57 and KLRG1 compared to healthy controls and were predominantly $CD57^-KLRG1^-$. Skin derived Skin $CD8^+$ T cells from melanoma patients showed a small but significant increase of $CD57^+KLRG1^+$ expressing cells, as 21±20% of $CD8^+$ T cells were positive for both markers among the patients compared to 9±12% in healthy individuals ($p=0.0169$). This was accompanied by a significant reduction in cells not expressing either marker (45±18% compared to 65±15%, $p=0.0083$). $CD57^+KLRG1^+$ $CD8^+$ T cells were further analysed for CD45RA and CD27 coexpression in order to determine which T cell subset they belonged to (Fig. 5-8C). Whilst in healthy skin these $CD57^+KLRG1^+$ $CD8^+$ T cells clustered in both, the T_{CM} and T_{EMRA} compartments. $CD57^+KLRG1^+$ $CD8^+$ T cells in melanoma patients belonged predominantly to the T_{CM} compartment. Therefore, a significant increase of KLRG1 and CD57 coexpressing cells could be found in the skin $CD8^+$ T cells of the melanoma patients. However, these cells belonged predominantly to the T_{CM} compartment and not to the T_{EM} or T_{EMRA} compartments, which are commonly associated with increased differentiation and CD57 and KLRG1 expression in the blood.

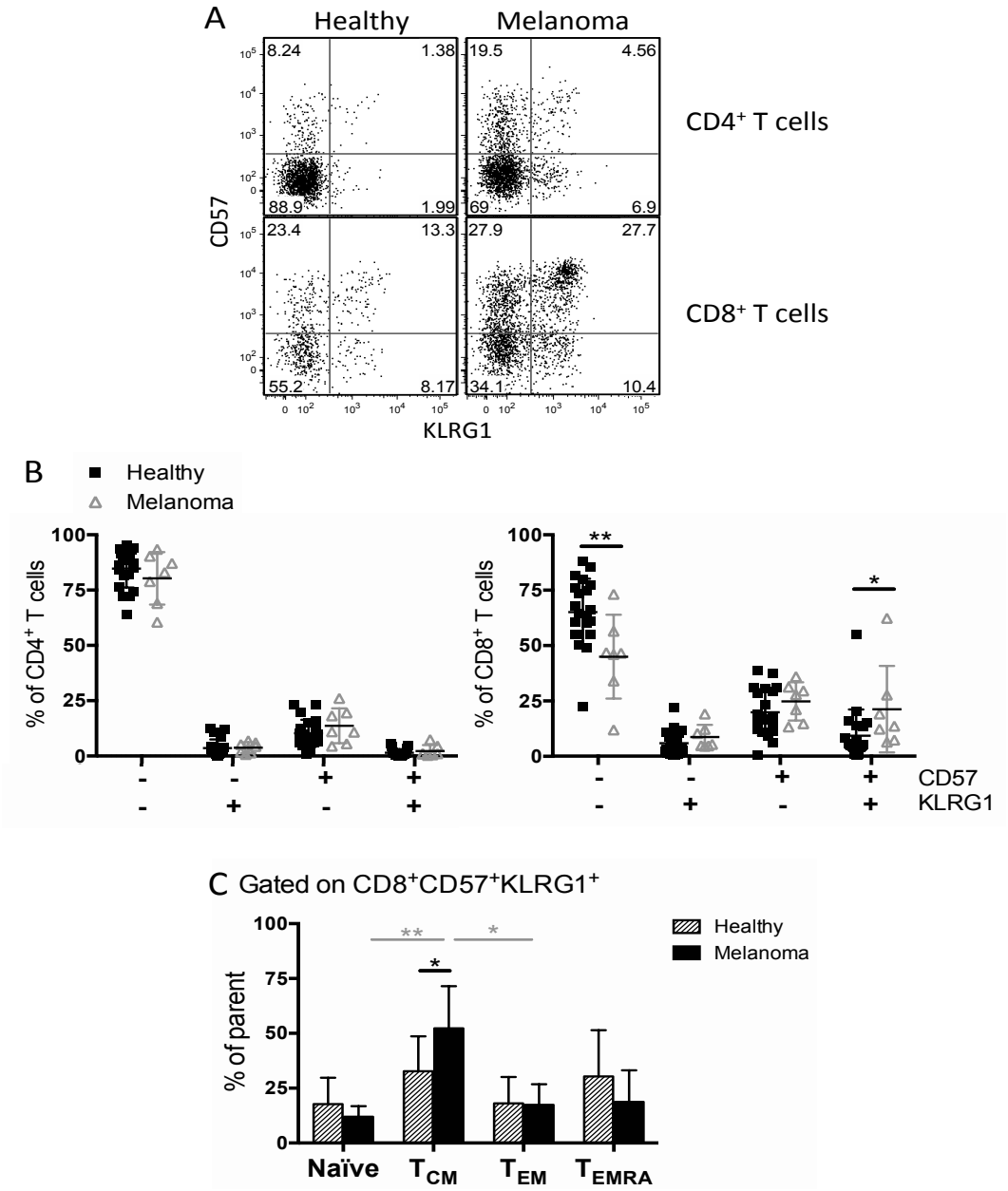


Fig. 5-8: KLRG1 and CD57 expression in CD4$^+$ and CD8$^+$ T cells derived from the skin of melanoma patients and healthy controls.

CD57 and KLRG1 and other surface markers were measured *ex vivo* by flow cytometry in cells extracted from the skin of melanoma patients (n=21) and healthy controls (n=21).
A: Representative FACS dot plot showing KLRG1 and CD57 expression in skin CD4$^+$ and CD8$^+$ T cells in a healthy individual and melanoma patient.
B: CD4$^+$ and CD8$^+$ T cells were divided into four subgroups according to KLRG1 and CD57 expression and frequencies of each subgroup were compared between patients (n=7) and healthy controls (n=21) using the Student t-test.
C: CD45RA/CD27 subset distribution of CD57$^+$KLRG1$^+$ CD8$^+$ T cells in the skin of healthy individuals and patients with melanoma. Horizontal bars in grey highlight significant differences in the melanoma patients' subset frequencies, calculated using one-way ANOVA.
Mean and standard deviation are shown. Differences between patients and healthy controls were compared using the Student T-test or the Mann-Whitney test. *=p<0.05; **=p<0.005

5.3.2.4 **PD-1 is increased in the skin of young but not old melanoma patients compared to healthy controls.**

PD-1 is an inhibitory marker often associated with immune exhaustion and with reduced T cell function in anti-tumour immune responses (Chapon et al., 2010). Surface expression of the inhibitory marker PD-1 was measured on skin derived T cells from melanoma patients and healthy controls via flow cytometry (Fig. 5-9). In healthy individuals, PD-1 increased with age on skin T cells ($p=0.0060$ for $CD4^+$ and $p=0.0017$ for $CD8^+$ T cells). Young melanoma patients were therefore found to have significantly increased PD-1 expression compared to healthy controls (31±14% versus 54±8%; $p=0.0177$ for $CD4^+$ T cells and 38±14% versus 67±8%; $p=0.0039$ in the $CD8^+$ T cells), whilst PD-1 was not increased further in old melanoma patients compared to healthy age matched controls (49±8% of $CD4^+$ and 65±18% of $CD8^+$ T cells in old healthy individuals expressed PD-1, compared to 51±20% of $CD4^+$ and 60±19% of $CD8^+$ T cells in old melanoma patients).

This meant that although PD-1 levels were high on skin derived T cells of melanoma patients across all age groups, PD-1 levels were only significantly increased compared to healthy controls in the younger age groups.

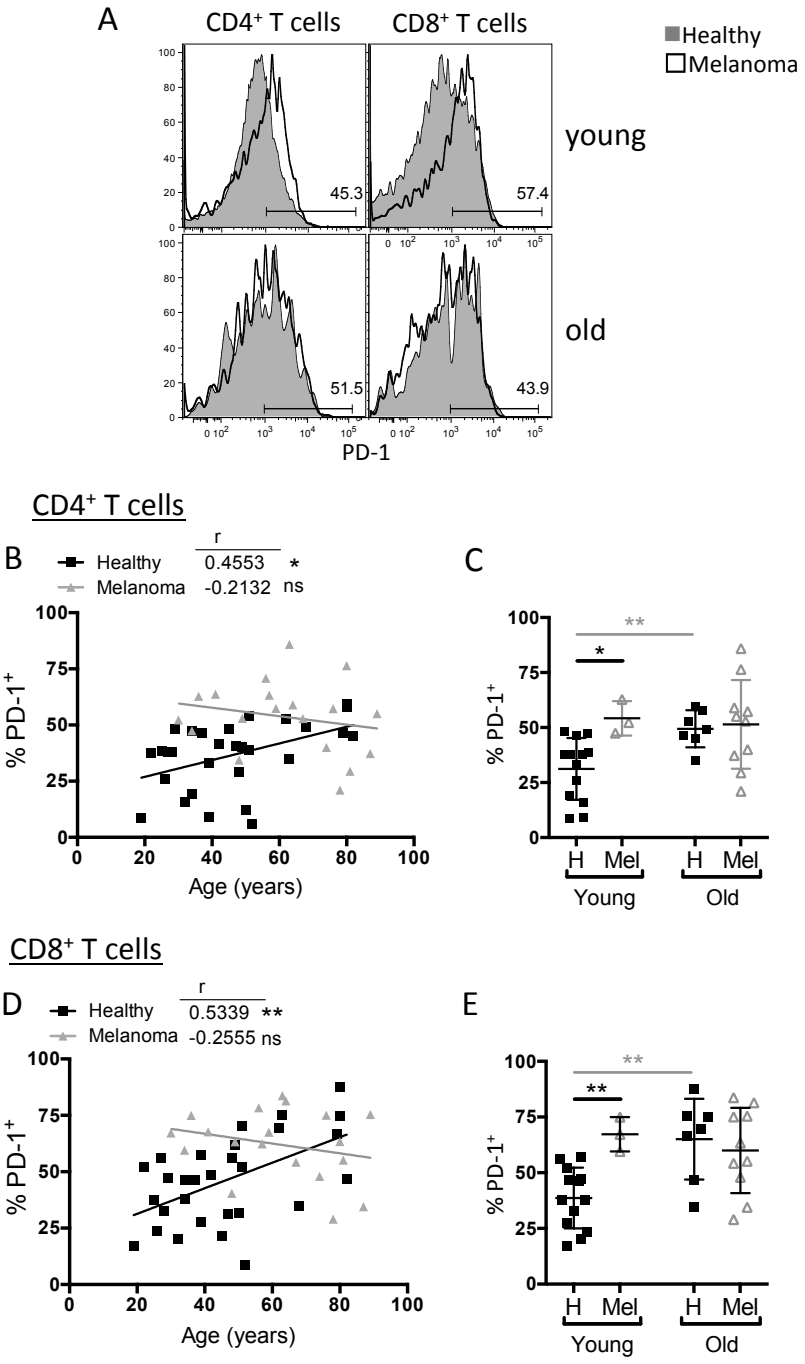


Fig. 5-9 *Ex vivo* PD-1 expression in CD4$^+$ and CD8$^+$ T cells derived from the skin of melanoma patients and healthy controls.

Surface PD-1 expression was measured by flow cytometry in T cells derived from the skin of melanoma patients and healthy controls. H= healthy skin. Mel= melanoma skin.
A: Representative histograms showing PD-1 expression in CD4+ and CD8+ T cells derived from the skin of young and old melanoma patients and healthy controls.
B, **D:** Percentage of PD-1 expressing cells by age in the CD4$^+$ and CD8$^+$ T cells derived from the skin of melanoma patients (n=19) and healthy controls. Lines of best fit were generated by linear regression and the correlation (r-value) and significance were assessed by Pearson rank.
C, **E:** Percentage of PD-1 positive cells in the CD4$^+$ and CD8$^+$ T cells derived from the skin of young (aged under 40) melanoma patients (n=3) and healthy controls (n=13) and old (aged 60 or above) melanoma patients (n=10) and healthy controls (n=7).
*=p<0.05; **=p<0.005

5.3.2.5 **CTLA-4 expression is increased in $CD4^+$ and $CD8^+$ T cells in the skin of patients with melanoma**

Similar to PD-1, CTLA-4 is a receptor associated with immune regulation and immune exhaustion and with reduced T cell function among melanoma infiltrating T cells (Curran et al. 2010). CTLA-4 was measured on stimulated $CD4^+$ and $CD8^+$ T cells extracted from the skin of melanoma patients and levels were compared to cells from healthy skin. Due to the low numbers of participants, it was not possible to assess differences of expression with age and participants were therefore compared across all age groups. We found that CTLA-4 expression was significantly increased in both $CD4^+$ and $CD8^+$ T cells of melanoma patients compared to healthy controls. 44±26% of melanoma patients' $CD4^+$ T cells expressed CTLA-4 compared to only 16±8% of $CD4^+$ T cells in healthy controls ($p=0.0012$). In healthy skin, only 5±5% of $CD8^+$ T cells express CTLA-4, which was increased to 23±26% in melanoma ($p=0.0023$). CTLA-4 expressing $CD4^+$ and $CD8^+$ T cells were further analysed for their CD45RA and CD27 expression in order to identify if they were more likely to associate with a particular differentiation subset. Indeed, CTLA-4^+ T cells were detected more frequently in the T_{CM} and T_{EM} subsets than the Naïve and T_{CM} subsets. CTLA-4 was therefore increased on the skin T cells of patients with melanoma.

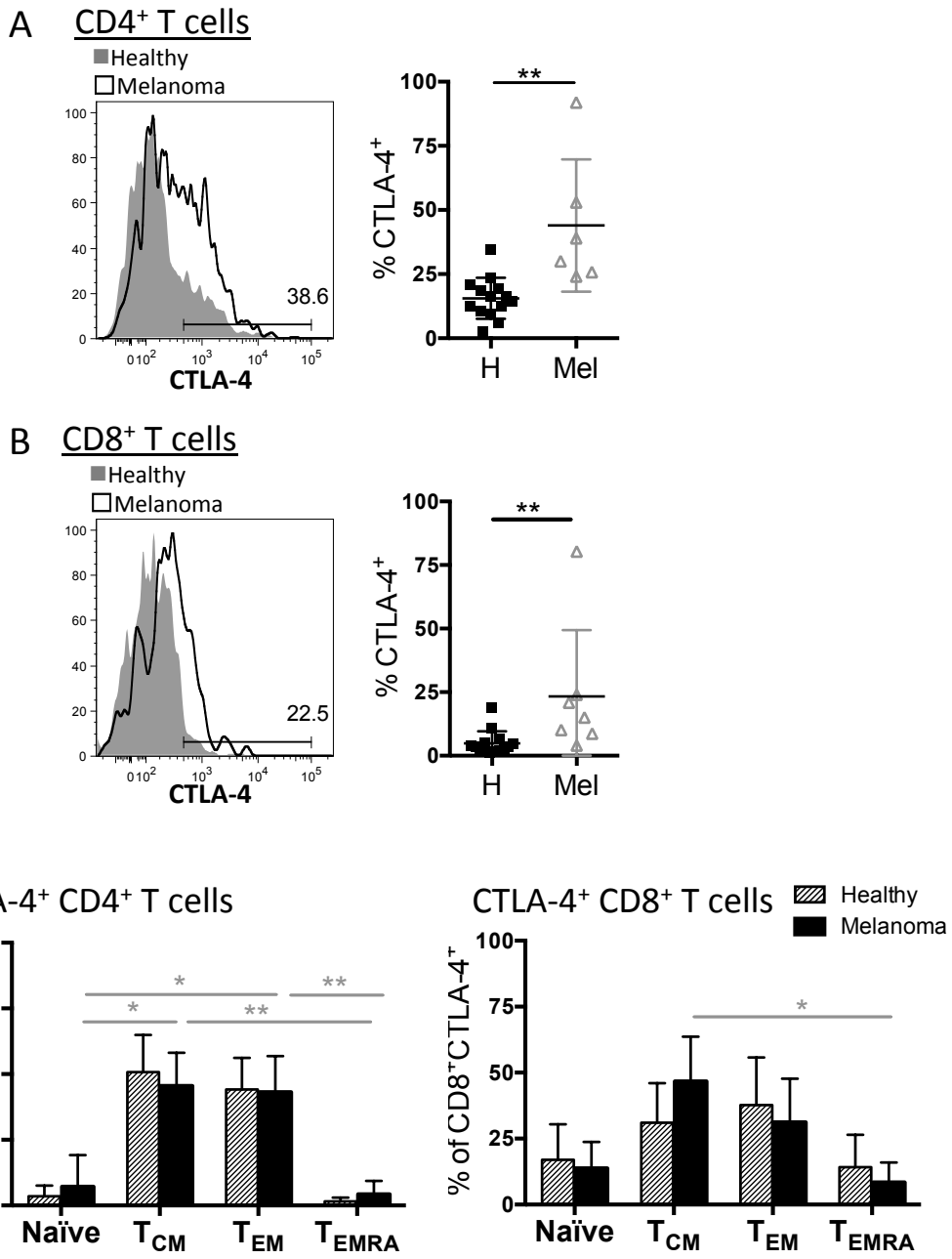


Fig. 5-10: Comparison of CTLA-4 expression in T cells derived from the skin of melanoma patients and healthy individuals.

CTLA-4 expression was measured following overnight stimulation with immobilized anti-CD3 antibody in the skin from melanoma patients (n=6) and healthy controls (n=14) across all age groups. H= healthy skin. Mel= melanoma skin.
A: representative graph and cumulative data showing CTLA-4 expression in $CD4^+$ T cells.
B: representative graph and cumulative data showing CTLA-4 expression in $CD8^+$ T cells.
C: CD45RA/CD27 subset distribution of CTLA-4 expressing $CD4^+$ and $CD8^+$ T cells in the skin of healthy individuals and patients with melanoma. Horizontal bars in grey highlight significant differences in the melanoma patients' subset frequencies, calculated using one-way ANOVA. Mean and standard deviation are shown. Melanoma and healthy cohorts were compared using the Student t-test.
**=p<0.005

5.3.2.6 **CD69 expression is decreased in the skin of patients with melanoma across all age groups**

We next examined cell surface expression of CD69, a marker for tissue retention, in the $CD4^+$ and $CD8^+$ T cells derived from the skin of melanoma patients and healthy controls using flow cytometry (Fig. 5-11). CD69 expression did not change significantly with age but was significantly decreased in melanoma patients compared to healthy controls. 58±14% of $CD4^+$ T cells of melanoma patients expressed CD69 compared to 80±8% in healthy controls ($p< 0.0001$), as did 66±19% of $CD8^+$ T cells in melanoma patients compared to 91±5% among controls ($p< 0.0001$).

As CD69 negative cells in tissues are thought to be more mobile compared to those that express the receptor (Mackay et al. 2013), we asked next whether the CD69 negative cells in melanoma patients were phenotypically distinct from the CD69 positive cells. CD27, PD-1 and KLRG1 surface expression were compared between CD69 positive and CD69 negative cells in the melanoma patients (Fig. 5-12). In the $CD4^+$ T cells of melanoma patients, CD69 negative cells expressed significantly more CD27 ($p< 0.0001$) and less PD-1 ($p< 0.0001$), whilst KLRG1 expression was similar between the two populations. Conversely, CD69 negative cells of the $CD8^+$ skin T cells of melanoma patients displayed no change in CD27 expression compared to CD69 positive cells, whilst showing a significant reduction in PD-1 ($p< 0.0001$) and increase in KLRG1 ($p=0.0026$).

Therefore T cells in the skin of melanoma patients showed an increase in cells negative for the tissue retention marker CD69 compared to healthy controls. These CD69 negative cells expressed less PD-1, and more CD27 among the $CD4^+$ and more KLRG1 among the $CD8^+$ T cells compared to CD69 negative cells.

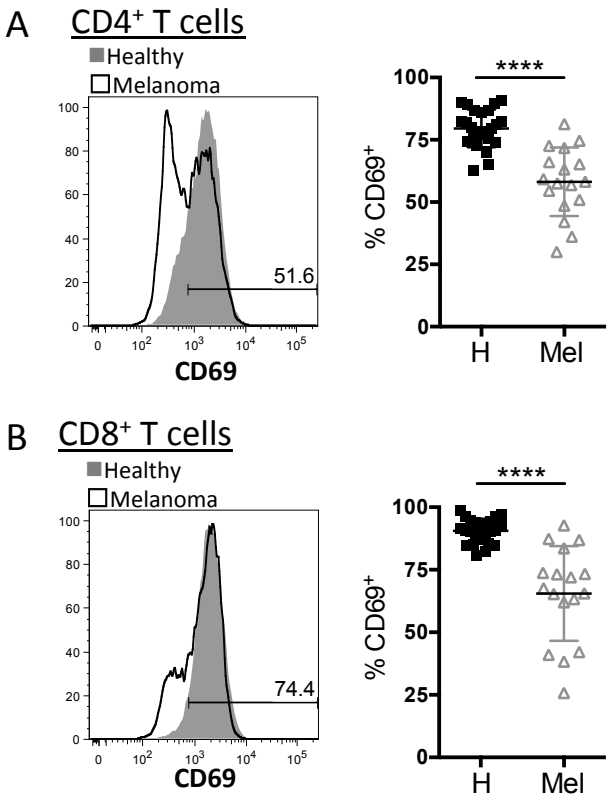


Fig. 5-11: CD69 expression on skin derived $CD4^+$ and $CD8^+$ T cells in melanoma patients compared to healthy controls

CD69 expression was measured ex vivo in T cells extracted from the skin of patients with melanoma (n=17) or healthy individuals (n=24) across all age groups. H= healthy skin. Mel= melanoma skin.
A: Representative graph and cumulative data showing CD69 expression in $CD4^+$ T cells.
B: Representative graph and cumulative data showing CD69 expression in $CD8^+$ T cells.
Mean and standard deviation are shown. Cohorts were compared using the standard T-test.
**=p<0.005

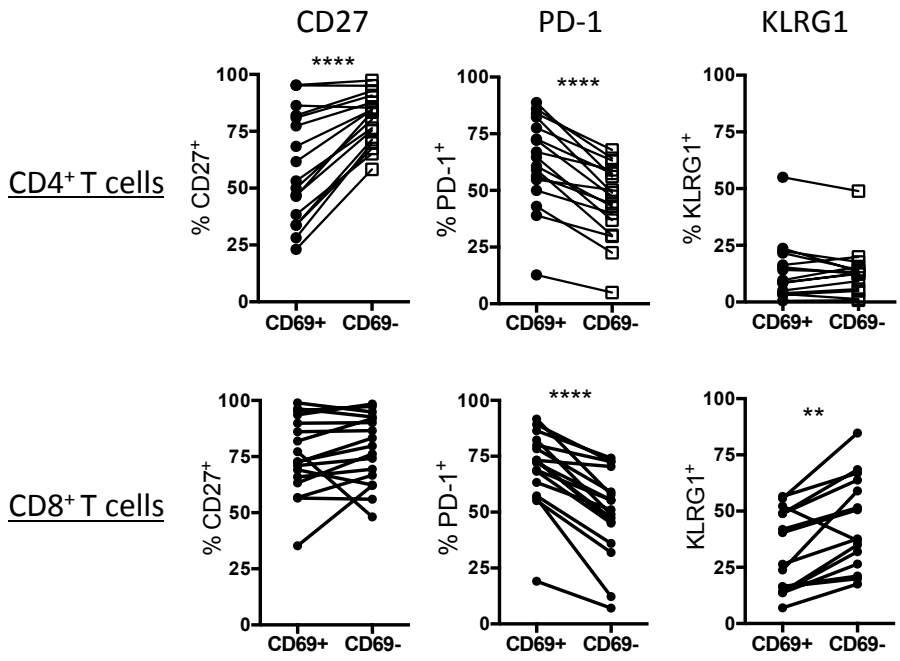


Fig. 5-12 Comparing CD27, PD-1 and KLRG1 expression between CD69 positive and CD69 negative $CD4^+$ and $CD8^+$ T cells derived from the skin of melanoma patients.

Surface receptors were measured by flow cytometry in cells extracted from the skin of melanoma patients of all ages. Statistical differences of surface marker expression between CD69 positive and CD69 negative cells were calculated using the paired T-test or Wilcoxon test. **=p<0.005; ****=p<0.0001

5.3.2.7 **Skin $CD8^+$ T cells in patients with melanoma have low cytotoxic potential**

The cytotoxic granule components perforin and granzyme B are thought to be important for tumour cell killing (Medema et al. 2001; Cullen et al. 2010). We assessed intracellular expression of these proteins in skin derived T cells of melanoma patients by flow cytometry and compared measurements to healthy controls.

Although granzyme B and perforin expressing T cells increase in frequency with age in the circulation, this was not found to be the case in the skin in both healthy and patients groups. We therefore compared expression in $CD8^+$ skin T cell of participants across all ages (Fig. 5-13). Whilst only 8±5% of skin resident $CD8^+$ T cells expressed granzyme B in healthy individuals, 23±6% $CD8^+$ T cells in melanoma patients were positive for this protease ($p< 0.0001$). Conversely, perforin expression was found to remain low in melanoma patients' skin (4±3%) and at a similar level to healthy individuals (3±3%; $p=0.1915$).

To test how these results based on wide local excision skin compared to cytotoxic potential in primary lesions, we performed histological staining for granzyme B or perforin in combination with CD8 on a primary melanoma section of a lesion. As can be seen in Fig. 5-14, the $CD8^+$ T cells infiltrating in this melanoma lesion did not express granzyme B or perforin.

We were also interested in finding out whether the few granzyme B expressing cells in wide local excision skin of melanoma patients showed increased differentiation compared to the granzyme B negative cells of the same patients. We compared CD27 and CD28 expression by flow cytometry, but did not find a significant difference in these receptors levels between granzyme B positive and negative $CD8^+$ T cells in the melanoma patients (Fig. 5-15).

$CD8^+$ T cells in the skin of melanoma patients therefore appeared to express more granzyme B but not perforin compared to healthy controls. Further, granzyme B expressing cells in the patients maintained high levels of CD27 and CD28.

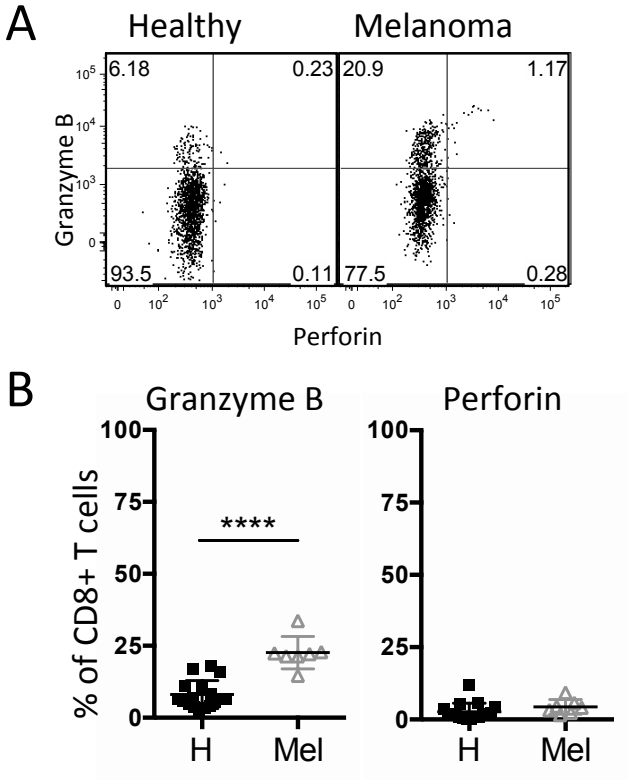


Fig. 5-13: *Ex vivo* expression of the cytotoxic granule components perforin and granzyme B in the CD8$^+$ T cells derived from the skin of melanoma patients and healthy controls.

Skin cells were extracted using collagenase digestion and analysed using flow cytometry. H= healthy skin. Mel= melanoma skin.
A: Representative flow cytometry dot plot showing granzyme B and perforin expression in the CD8$^+$ T cells extracted from the skin of a melanoma patient and a healthy individual.
B: Cumulative data showing granzyme B and perforin expression in the skin of melanoma patients (n=7) and healthy controls (n=17). Mean and standard deviation are shown. Groups were compared using the standard t-test. Participants were from all age groups.
****=p<0.0001

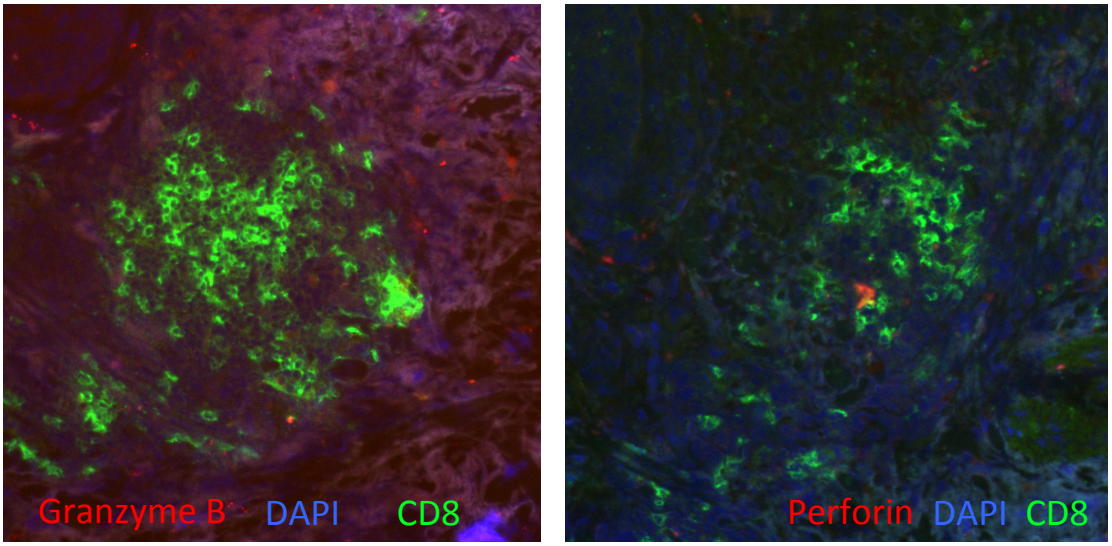


Fig. 5-14: Immunofluorescent staining for granzyme B or perforin in CD8$^+$ cells infiltrating a primary melanoma lesion.

Immunofluorescence staining was performed on frozen sections of a primary melanoma lesion using CD8 (green), DAPI (blue) and either granzyme B, shown on the left, or perforin, shown on the right (both red).

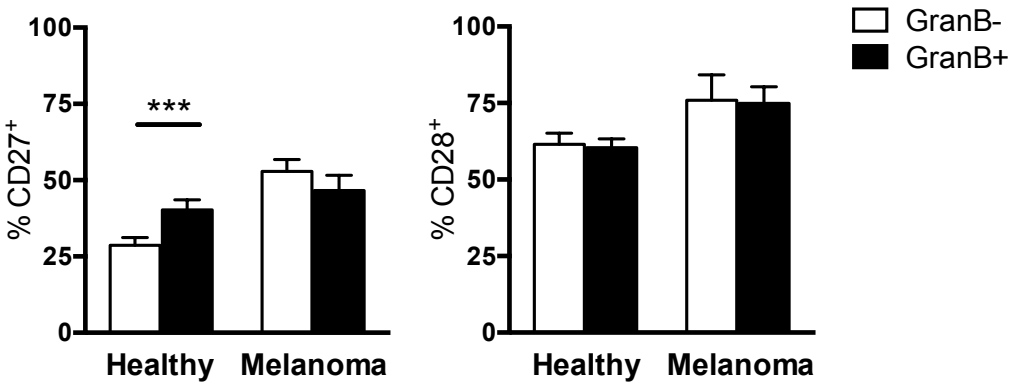


Fig. 5-15: CD27 and CD28 expression in granzyme B positive and negative CD8$^+$ T cells derived from the skin of healthy individuals and patients with melanoma.

CD27, CD28 were measured extracellularly and granzyme B intracellularly by flow cytometry in CD8$^+$ T cells derived from the skin of melanoma patients (n=7) and healthy individuals (n=17). Surface marker expression on granzyme B positive cells was compared to granzyme B negative cells using the paired T-test.
Mean and standard error are shown.
***=p<0.0005

5.4 Discussion

T cells extracted from wide local excisions of young melanoma patients displayed increased expression of the inhibitory receptors PD-1 and CTLA-4 and the costimulatory receptors CD27 and CD28, whilst showing reduced levels of the tissue retention marker CD69 compared to age matched healthy controls. Uninvolved skin in old melanoma patients showed similar changes. PD-1 levels were high in the T cells of these individuals but not significantly different from age matched healthy controls, as healthy individuals expressed increased levels of PD-1 with age. Proportions of T_{CM} were decreased in the skin of old compared to young melanoma patients. However, cells extracted from the primary melanoma of old individuals did display high levels of T_{CM}-like cells, suggesting that an increase in T_{CM} is inherent to T cells associated with primary melanoma lesions of all ages. This is further supported by published data showing high CD27 expression levels (>90%) in T cells of primary melanomas (Pepe et al. 2011).

Perforin and granzyme B are normally absent from T cells in healthy skin. However, cells expressing granzyme B and perforin have been shown to occur in T cells during vitiligo, which involves autoimmune destruction of healthy melanocytes (van den Wijngaard et al. 2000). Further, a retrospective study showed that absence of granzyme B in primary melanoma infiltrating T cells correlated with occurrence of metastases in draining lymph nodes and distant organs (van Houdt et al. 2009). These observations suggest that granzyme B and perforin are important for T cell mediated killing of healthy or malignant melanocytes in the skin. In this study, 20% of $CD8^+$ T cells in the skin of patients with melanoma expressed granzyme B but less than 5% expressed perforin. This is interesting, as perforin is absolutely essential for granzyme mediated cytoloytic activity (Browne et al. 1999). Therefore, $CD8^+$ T cells in the skin of melanoma patients seem to lack effector molecules essential to kill the local tumour cells. It is possible that skin resident T cells in the patients expressed low intracellular cytotoxic granule components due to recent degranulation events. Measurement of perforin mRNA in these cells could address this.

Acquisition of effector functions such as cytotoxic granule components involves differentiation and is associated with phenotypic changes in T cells. In the blood, this includes increased expression of KLRG1 and CD57 and a decrease in CD27 and CD28 (Speiser et al. 1999; Appay et al. 2008). Resting T cells in healthy skin expressed high levels of CD28 and literature suggests that CD28 can be downregulated in T cells during the inflammatory autoimmune condition psoriasis (De Rie et al. 1996). $CD8^+$ T cells in the skin of melanoma patients expressed high levels of CD27 and CD28, but also showed increased expression of KLRG1,CD57 and granzyme B in absence of perforin. This suggests that full maturation to an effector phenotype does not occur in skin T cells of melanoma patients. Similar findings on a lack of effective T cell differentiation into cytotoxic effectors have been published on $CD8^+$ T cells extracted from melanoma lymph node metastases, where the majority of cells had a non-differentiated $CCR7^+CD45RA^+CD27^+CD28^+$perforin- phenotype and on $CD8^+$ T cells derived from distant metastases, which were $CD27^+CD28^+CD57^+GranzymeB^+$ and expressed little or no perforin (Mortarini et al. 2003; Wu et al. 2012). The findings in this chapter therefore indicate that $CD8^+$ T cells in the skin of melanoma patients fail to mature into potent cytotoxic T cells that might mediate tumour cell killing.

The inhibitory receptors PD-1 and CTLA-4 were found to be significantly increased on skin derived $CD4^+$ and $CD8^+$ T cells of patients with melanoma compared to healthy controls. Expression of both PD-1 and CTLA-4 on the surface of $CD8^+$ T cells has been associated with reduced proliferative capacity and cytokine production upon challenge (Duraiswamy et al. 2013). This functional state, maintained by inhibitory receptor signalling, is known as T cell exhaustion and has been shown to occur in the context of chronic antigen stimulation during persistent viral diseases and in a number of tumours including melanoma (Ahmadzadeh et al. 2009; Duraiswamy et al. 2013; Baitsch et al. 2011). Upon ligation, PD-1 and CTLA-4 are thought to inhibit T cell activation through separate intracellular signalling pathways, as dual blockade of the receptors shows synergistic effects *in vitro* and in mouse models *in vivo* (Duraiswamy et al. 2013; Parry et al. 2005; Curran et al. 2010). PD-1 and CTLA-4 mediated inhibition might therefore have contributed to the lack of

cytotoxic granule components observed in the skin resident T cells in the melanoma patients.

High expression of CD27 and CTLA-4 observed on skin T cells in patients with melanoma might also be associated with an increase in Tregs. Skin resident $CD4^+$ Tregs are restricted to the CD27 expressing T_{CM} compartment in healthy individuals and express high levels of CTLA-4 but low levels of CD69 (see section 4.3.2.7). Investigating the frequency and suppressive activity of Tregs in the skin of the melanoma patients was beyond the scope of this work because of limited sample availability. However, previous reports suggest increased levels of $CD4^+$ $FoxP3^+$ Tregs in melanoma patients' primary lesions and metastases (Jacobs et al. 2012). Tregs might therefore contribute to the observed increase in T_{CM} in melanoma patients and might inhibit effector T cell maturation through CTLA-4 mediated signalling (Takahashi et al. 2000).

Changes in T cell phenotypes in melanoma patients could be explained by increased T cell infiltration and/or local modulation of cells. CD69 is a marker for tissue retention in T cells. Mouse experiments have shown that T cells which have recently entered the skin during inflammation do not initially express CD69 but will do so upon memory formation (Mackay et al. 2013). In this chapter, we observed a reduction in CD69 expression among skin derived T cells from melanoma patients, suggesting an increase in T cell infiltration. The overall increase in T_{CM}-like cells that expressed high levels of PD-1, CD27 and CD28 in the skin of melanoma patients might therefore be due to preferential recruitment from the circulation of cells with this phenotype. However, the CD69 negative cells expressed less PD-1 than CD69 positive resident cells in the same patient. PD-1 is low on cells in the circulation but increased in skin resident T cells of healthy individuals, suggesting that additionally to being recruited, cells infiltrating into the skin of the melanoma patients are undergoing local modulation, possibly as a result of the skin microenvironment created directly or indirectly by the tumour.

A number of cytokines and chemokines are differentially expressed in stressed skin or by melanoma cells and might actively inhibit T cell differentiation or promote expression of inhibitory receptors. The immunosuppressive cytokine TGFβ is often increased in the tumour microenvironment and can prevent the expression of cytotolytic genes in $CD8^+$ T cells or enhance cell death in

cyototoxic T cells (D. A. Thomas and Massagué 2005; Cumont et al. 2007). Indeed, exogenous addition of TGFβ prevented differentiation and upregulation of granzyme B and perforin in melanoma metastases derived stimulated T cells *in vitro* (Wu et al. 2012).

It is also possible that the stimulatory signals required for cytotoxic differentiation are simply not present in the skin of the melanoma patients. CD27 can be initially upregulated on T cells upon CD3 ligation, but exposure to IL-2 and CD70 ultimately lead to CD27 downregulation (Hintzen et al. 1993; Huang et al. 2006). Partial activation through the TCR but suboptimal levels of CD70 or IL-2 in the skin of the melanoma patients might therefore explain the high levels of CD27 observed on the T cells in this study. Immature dendritic cells, which occur in melanoma lesions and normal skin, may be responsible for such signals or lack thereof (Polak et al. 2012; Vermi et al. 2003). It would be interesting to include staining for IL-2 and CD70 in future histological studies of primary melanoma lesions.

Finally, it should be noted that the skin obtained in this study was generally derived from sun exposed sites (such as arms, legs, head and neck) and a mixed gender pool in the case of melanoma patient samples and from sun protected sites (breast, abdomen) from a healthy volunteer pool that was mostly female. It has been shown that sun exposure can alter composition of immune cells in the skin and might therefore play a role in the results obtained (Di Nuzzo et al., 2009). Other studies however reported no significant difference between phenotypes in T cells derived from sun exposed and sun unexposed skin (Clark et al. 2006). In order to confirm that the results presented here were not due to sun exposure on the melanoma patient skin, the panels used could be repeated on skin samples from sun exposed and sun-unexposed sites, preferably within the same individual.

A further point of consideration is the fact that the majority of data described in this chapter is based on skin derived from wide local excisions. This skin was taken from areas that were declared tumour-free, but might have been influenced by soluble factors emitted from the melanoma lesion. This means that T cells derived from the skin of wide local excisions are neither completely uninvolved, nor directly involved with the tumour. Another factor to consider is the effect of wound healing from the biopsy taken within the melanoma lesion

prior to performing the wide local excision. However, the striking increase in CD27 expressing cells observed in the wide local excision skin in many of the patients was also found to occur in T cells in primary melanoma lesions by us and others (Pepe et al. 2011), suggesting that this phenomenon is a true attribute of melanoma rather than a side effect from the wound healing.

In summary, the results presented in this chapter indicate increased T cell infiltration in the skin of patients with melanoma. These skin derived T cells expressed the hallmarks of immune exhaustion, with high levels of costimulatory and inhibitory receptors but few cytotoxic granule components. This suggests that T cells are being attracted into the skin of patients but are unable to undergo full cytotoxic maturation. The following chapter will therefore investigate what signals might directly modulate effector functions in skin-derived T cells (such as inflammatory cytokines or PD-1 signalling).

6 Modulation of T cell functionality in the blood and skin

6.1 Introduction

Standard treatments for melanoma currently rely on surgery, radio- and chemotherapy and are often insufficient to treat advance stage disease. Whilst melanoma is highly immunogenic and can lead to melanoma specific immune responses in the patients, various levels of immune dysfunction may occur, such as immune exhaustion and increased frequencies of regulatory cells (Robert et al. 2011). Recent years have therefore seen an increasing interest in immune based therapies that aim to restore anti-melanoma immune function in patients.

Although many recent clinical trials for melanoma immunotherapies have focussed on blocking inhibitory receptors associated with immune exhaustion, such as PD-1 and CTLA-4 (Ahmadzadeh et al., 2009, Fourcade et al. 2009), these markers were not found to be markedly increased on the surface of overall circulating $CD8^+$ T cells in melanoma patients (see chapter 3). Instead, we identified increased frequencies of highly differentiated T_{EMRA} cells in the blood of old patients, which expressed high levels of effector molecules but showed aberrant proliferation and IL-2 production. Although it could not be determined whether these cells were melanoma specific, melanoma patients might therefore benefit from restoring these functions in this cell subset. The highly differentiated T_{EMRA} cells express high levels of p38 and show increased proliferation upon p38 inhibition in healthy individuals (Di Mitri et al, 2011). T_{EMRA} cells in old melanoma patients might therefore similarly respond to p38 blockade. The first part of this chapter will therefore address if p38 blockade can boost proliferation and cytokine production in the $CD8^+$ T cell compartment of old melanoma patients.

The previous chapters in this thesis have also highlighted that both, melanoma and healthy skin $CD8^+$ T cells only express low levels of the cytotoxic granule components granzyme B and perforin. Because these molecules are important in T cell mediated tumour killing (Medema et al. 2001; Cullen et al. 2010), it was

of interest to identify mechanisms that can license skin resident T cells to become effective killers. The second part of this chapter therefore investigates the role of positive and negative signals that might influence effector functions in skin derived T cells.

6.2 **Aims**

The aim of this chapter was to investigate ways to manipulate blood and skin derived $CD8^+$ T cells in order to boost T cell function and therefore potentially enable anti-tumour immune responses in patients with melanoma.

6.3 Results

6.3.1 Restoring T cell function in blood derived CD8$^+$ T cells of old melanoma patients

As described in chapter 3, old melanoma patients showed increased markers of highly differentiated T_{EMRA} cells in the circulation compared to healthy controls. These cells display high levels of p38 phosphorylation (Fig. 6-1), whose signalling is thought to be actively involved in the maintenance of their senescent state (Henson et al. 2014). This is supported by the fact that the small molecule inhibitor for p38, BIRB 796, which inhibits all four isoforms of p38, was previously shown to partially restore proliferation and telomerase activity in CD4$^+$ and CD8$^+$ T_{EMRA} cells (Di Mitri et al. 2011; Henson et al. 2014). This section therefore explores if p38 blockade can restore T_{EMRA} CD8$^+$ T cell function of old melanoma patients.

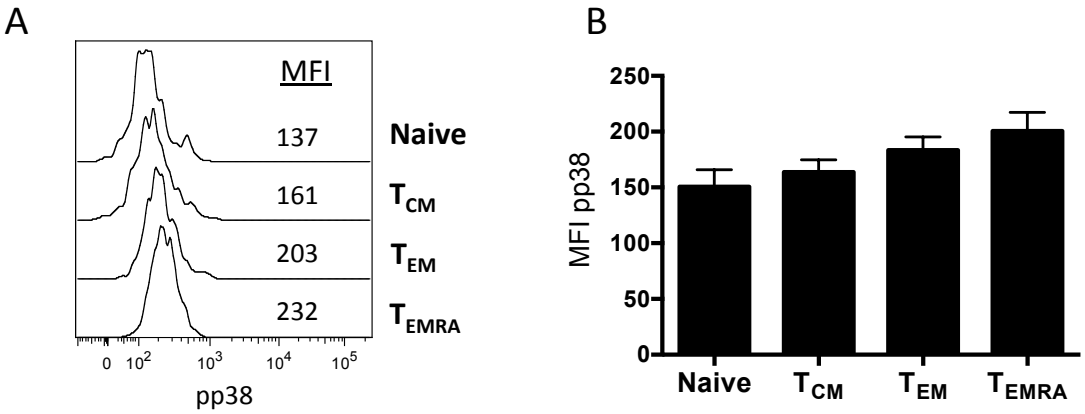


Fig. 6-1: P38 phosphorylation levels in CD8$^+$ T cell subsets in the blood of melanoma patients

Levels of phosphorylated p38 (pp38) were measured via phosphoflow as described in the material and methods section in the CD8$^+$ T cell subsets of patients with melanoma (n=3). The antibody used recognizes phosphorylation of the conserved T180/Y182 in all four p38 isoforms: α, β, γ, and δ.
A: Representative flow cytometry histograms showing expression and median fluorescence intensity (MFI) for intracellular pp38 levels in CD8$^+$ T cell subsets of a melanoma patient.
B: Cumulative data showing average and standard error for pp38 MFI, measured in 3 melanoma patients.

6.3.1.1 **Effect of p38 signalling blockade on T cell proliferation**

As p38 blockade has previously been shown to reinstate proliferation in highly differentiated T cells, it was investigated whether this would also be true for the highly differentiated T cells in old melanoma patients (Di Mitri et al. 2011; Henson et al. 2014). Proliferation was measured via ki67 expression in total PBMCs or sorted $CD8^+$ T cell subsets stimulated overnight with anti-CD3 antibody in the presence or absence of the p38 inhibitor BIRB796 (Fig. 6-2). Cell sorting before stimulation was essential to analyse proliferation in the $CD8^+$ T cell subsets, as the expression of the markers used to identify these cells (CD45RA and CD27) can be altered after 3 days in culture. A significant increase in ki67 was measured in total $CD8^+$ T cells incubated with the p38 inhibitor ($p=0.0055$). However, changes in Ki67 expression following p38 inhibitor treatment in sorted $CD8^+$ T cell subsets varied among patients, with some showing an increase and others a decrease in proliferation in T_{EMRA} cells following treatment. P38 blockade was therefore shown to boost proliferation of total $CD8^+$ T cells in melanoma patients, but did not significantly increase proliferation in the T_{EMRA} compartment.

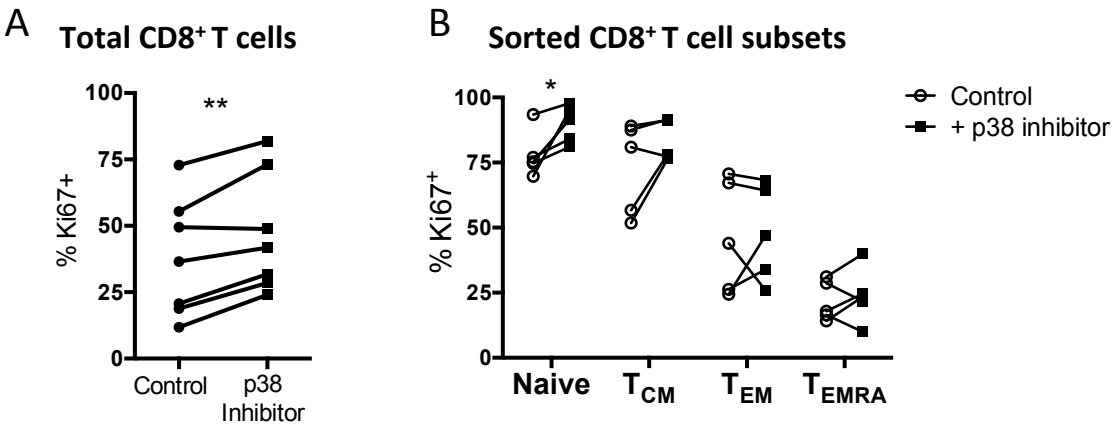


Fig. 6-2: Changes in proliferation in response to p38 blockade in blood-derived CD8$^+$ T cells derived from old melanoma patients.

Ki67 expression was measured by intracellular flow cytometry as marker of proliferation in whole PBMCs or sorted CD8$^+$ T cell subsets stimulated for 3 days with 0.5µg/ml immobilized anti-CD3 in the presence of 500nM of the p38 inhibitor BIRB796 or 0.1% DMSO as control. Sorted T cells further required the presence of autologous irradiated antigen presenting cells as source of co-stimulation. All participants were old melanoma patients.
A: Percentage of ki67 expressing cells in total CD8$^+$ T cells. Whole PBMCs were stimulated in this assay (n=7).
B: Percentage cells expressing ki67 in CD8$^+$ T cells subsets. CD8$^+$ CD45RA/CD27 T cell subsets of old melanoma patients were FACS sorted and incubated for 3 days with anti-CD3 and irradiated autologous antigen presenting cells (n=5).
P38 treated samples were compared to control samples using the paired T-test.
*=p<0.05; **=p>0.01

6.3.1.2 p38 signalling blockade enhances IL-2 and IFNγ cytokine production in T_{EMRA}

As described in the introduction and chapter 3, highly differentiated T_{EMRA} cells in melanoma patients and healthy individuals express the lowest levels of IL-2 out of all the differentiation subsets, whilst maintaining production of effector cytokines such as IFNγ and TNFα. We therefore investigated whether p38 blockade might also affect cytokine production in these cells.

PBMCs from old melanoma patients were stimulated overnight with immobilized anti-CD3 in the presence or absence of the p38 blocking agent and cytokine production was measured the following day via intracellular flow cytometry (Fig. 6-3). Whilst TNFα levels were not significantly altered in any $CD8^+$ T cell subsets following p38 blockade, we observed that both IL-2 and IFNγ production were significantly enhanced in the T_{EM} and T_{EMRA} subsets following treatment ($p=0.0151$ for T_{EM} and $p=0.0122$ for T_{EMRA} IL-2 production and $p=0.0024$ for T_{EM} and $p=0.0171$ for T_{EMRA} IFNγ production).

Therefore, IL-2 and IFNγ but not TNFα production could be enhanced in differentiated $CD8^+$ T cells of old melanoma patients by p38 blockade during stimulation.

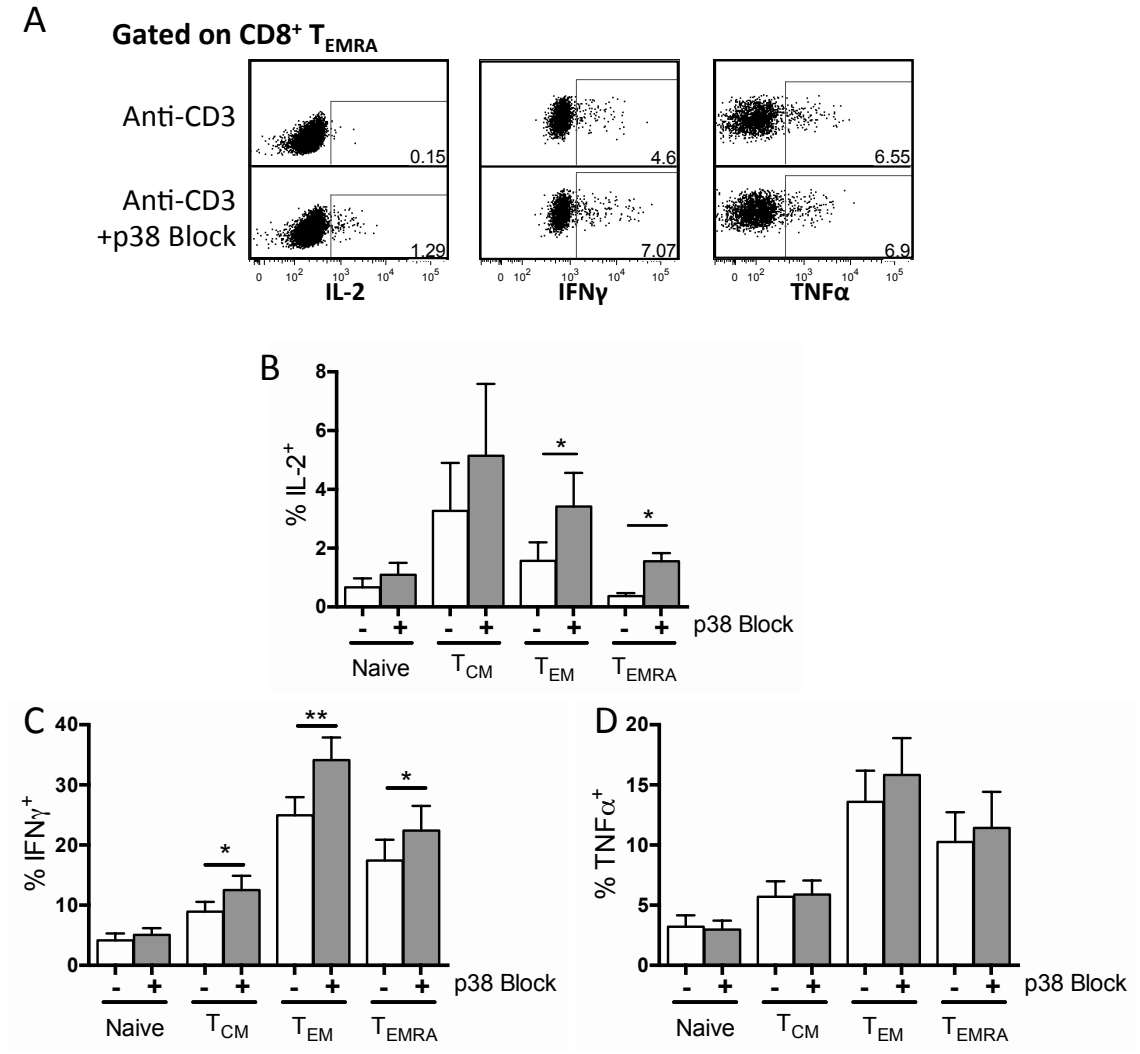


Fig. 6-3: Changes in cytokine production in stimulated, blood-derived CD8$^+$ T cell subsets of old melanoma patients following p38 blockade

PBMCs of old melanoma patients were incubated overnight with immobilized 0.5µg/ml anti-CD3 and the presence of 500nM of p38 inhibitor BIRB796 or 0.1% DMSO as control. Intracellular IL-2, IFNγ and TNFα levels were measured via flow cytometry in the CD8$^+$ T cell subsets.
A: Representative FACS dot plots showing changes in IL-2, IFNγ and TNFα expression in CD8$^+$ T_{EMRA} after treatment with the p38 inhibitor compared to the control.
B,C,D: Cumulative data showing IL-2 (n=7), IFNγ (n=13) or TNFα (n=13) production in CD8$^+$ T cell subsets in untreated or p38 inhibitor treated cells. Populations were compared statistically using the paired t-test or Wilcoxon test. Mean and standard error are shown.
*=p<0.05; **p<0.01

6.3.1.3 **p38 signalling blockade enhances degranulation during anti-CD3 stimulation**

In the previous chapter, T_{EMRA} cells were also shown to have the highest levels of cytotoxic granule components out of all the $CD8^+$ T cell subsets but failed to show equally high levels of degranulation as measured by CD107a staining following overnight stimulation with anti-CD3. The potential of p38 blockade to enhance degranulation in highly differentiated T cells of old melanoma patients was therefore investigated. PBMCs from patients were stimulated with anti-CD3 overnight in the presence of CD107a antibody and in the presence or absence of the p38 inhibitor BIRB796. CD107a and granzyme B levels were measured by flow cytometry. P38 inhibition during *in vitro* stimulation was found to cause a small but significant decrease in granzyme B ($p=0.0391$ for T_{EM} and $p= 0.0416$ for T_{EMRA}) and a marked increase in CD107a ($p=0.0004$ for T_{EM} and $p=0.0002$ in T_{EMRA}) in $CD8^+$ T_{EM} and T_{EMRA} of old melanoma patients.

Therefore, inhibition of p38 signalling during *in vitro* stimulation resulted in an increase in degranulation and might therefore be beneficial in enhancing cytotoxic function in highly differentiated T_{EMRA} cells in patients with melanoma.

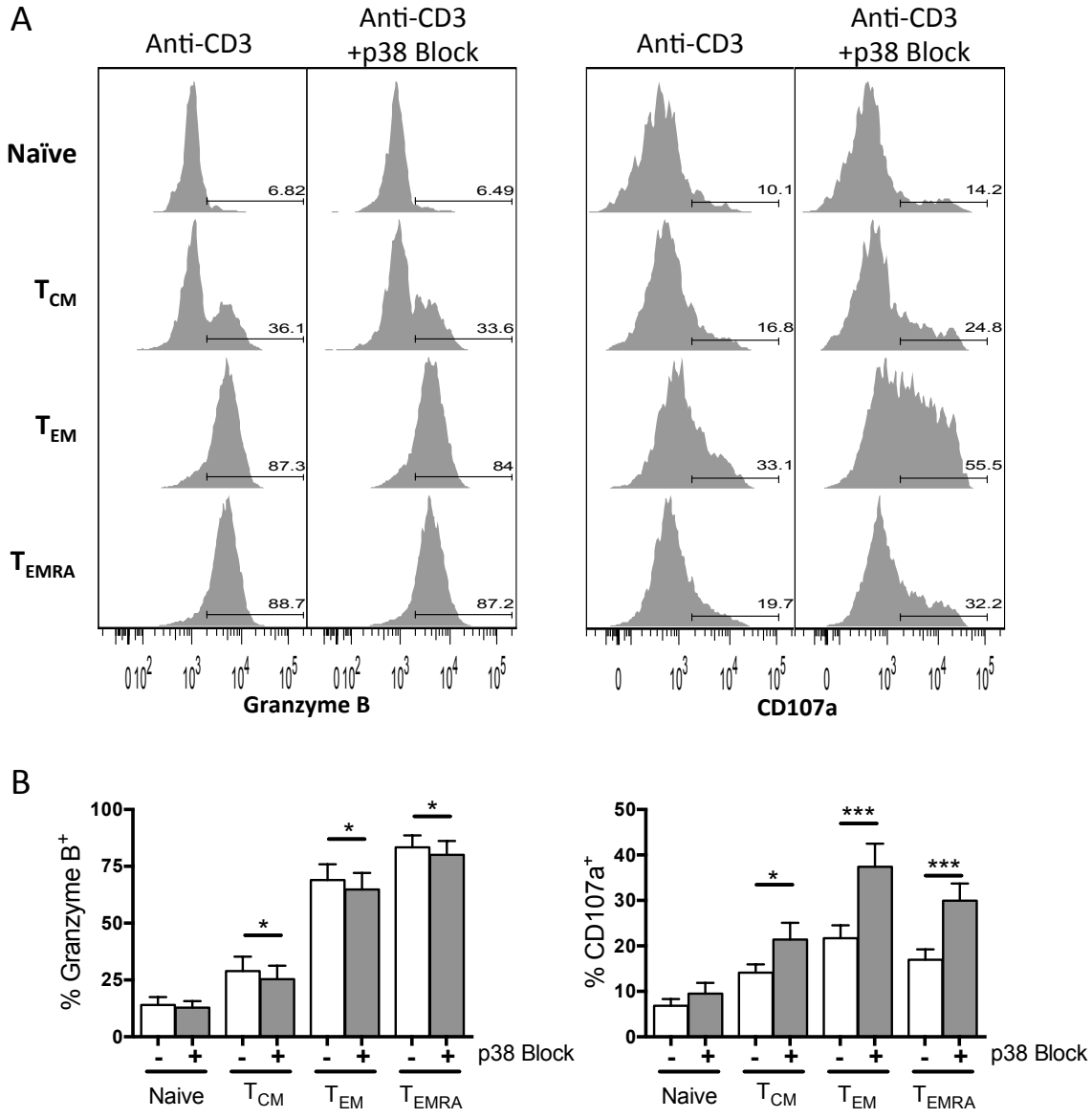


Fig. 6-4 Changes in expression of cytotoxicity markers CD107a and granzyme B in stimulated, blood-derived CD8$^+$ T cell subsets from old melanoma patients induced by p38 signalling blockade.

PBMCs from old melanoma patients (n=8) were stimulated overnight with plate-bound 0.5µg/ml anti-CD3 in the presence of 500nM of the p38 inhibitor BIRB796 or 0.1% DMSO as control. CD107a antibody was added to the cells prior to stimulation. Samples were harvested the following day and stained extra- and intracellularly. Percentage cells expressing CD107a and granzyme B in CD8$^+$ T cells subsets were measured via flow cytometry.
A: Representative flow cytometry histograms showing granzyme B and CD107a staining in stimulated T cell subsets treated with or without p38 inhibitor.
B: Cumulative data showing changes in granzyme B and CD107a expression in stimulated p38 treated CD8$^+$ T cell subsets compared to untreated samples. Treatment groups were compared using the paired T-test or Wilcoxon test. Mean and standard error are shown.
*=p<0.05; ***=p<0.001

6.3.2 **Increasing cytotoxic potential in skin derived T cells**

Results shown in previous chapters indicate that although old melanoma patients present with increased T cell differentiation in the blood, T cells in their skin fail to differentiate into cytotoxic effectors. Cytotoxic granule components are important for T cell mediated tumour cell killing (Browne et al. 1999), but T cells in healthy skin do not normally express perforin or granzyme B. We therefore explored what signals might influence cytotoxic maturation of T cells in the skin in order to identify mechanisms that might boost T cell function in the skin of patients with melanoma. First, we investigated the role of inflammatory cytokines and then the role of PD-1 signalling in the cytotoxic maturation of skin resident T cells. Because skin from patients with melanoma was not available in sufficient amounts to perform the assays required, skin from healthy individuals was used for this purpose.

6.3.2.1 **IL-2, IL-15 and CD3/CD28 induce granzyme B and perforin expression in skin T cells *in vitro***

Although cytotoxic effector-like T cells are not present in healthy skin, previous studies have shown that T cells bearing cytotoxic granule components are present in this organ under certain inflammatory conditions (van den Wijngaard et al. 2000; Yawalkar et al. 2001). We therefore hypothesised that the inflammatory cytokines IL-2 and IL-15 as well as strong signalling through the CD3 and CD28 receptors may induce cytotoxic maturation in skin resident T cells, especially as these signals have previously been shown to induce cytotoxic T cell functions in blood-derived cells (Salcedo et al. 1993; Alves et al. 2003; Geginat et al., 2003).

In order to test whether these signals would also induce granzyme B and perforin expression in T cells derived from the skin, PBMCs or skin derived cells were incubated with recombinant TNFα, IL-2 or IL-15 or CD3/CD28 beads. After 4 days in culture, intracellular perforin and granzyme B were analysed by flow cytometry (Fig. 6-5). 21±15% of untreated blood-derived $CD8^+$ T cells were on average granzyme B positive and remained unchanged after treatment with TNFα, but increased slightly to 26±16% in cells treated with IL-2 ($p=0.0017$) and to 34±16% in cells treated with IL-15 ($p=0.0001$), as well as showing a very high increase to 68±30% after treatment with anti-CD3/CD28 ($p=0.0001$). Granzyme B expression in skin derived T cells averaged 10±7% and was also unaffected by TNFα but increased to 19±11% after treatment with IL-2 ($p=0.0042$), to 37±20% with IL-15 ($p=0.0006$) and to 60±20% in response to anti-CD3/CD28 stimulation ($p<0.0001$).

Perforin expression followed similar patterns but remained overall lower: Untreated blood derived $CD8^+$ T cells expressed on average 4±4% perforin and this increased to 8±11% after IL-2 ($p=0.0371$), to 12±15% after IL-15 ($p=0.0059$) and to 12±12% after anti-CD3/CD28 treatment ($p=0.0039$). Similarly, only 2±2% of untreated skin $CD8^+$ T cells expressed perforin, but this increased slightly to 3±4% after IL-2 ($p=0.0078$) and to 4±4% after IL-15 ($p=0.0039$) treatment and increased greatly to 19±17% after anti-CD3/CD28 treatment ($p=0.0078$).

Although not expressing perforin and granzyme B under steady state, skin derived T cells were therefore shown to upregulate these cytotoxic granule components given the appropriate signals *in vitro*.

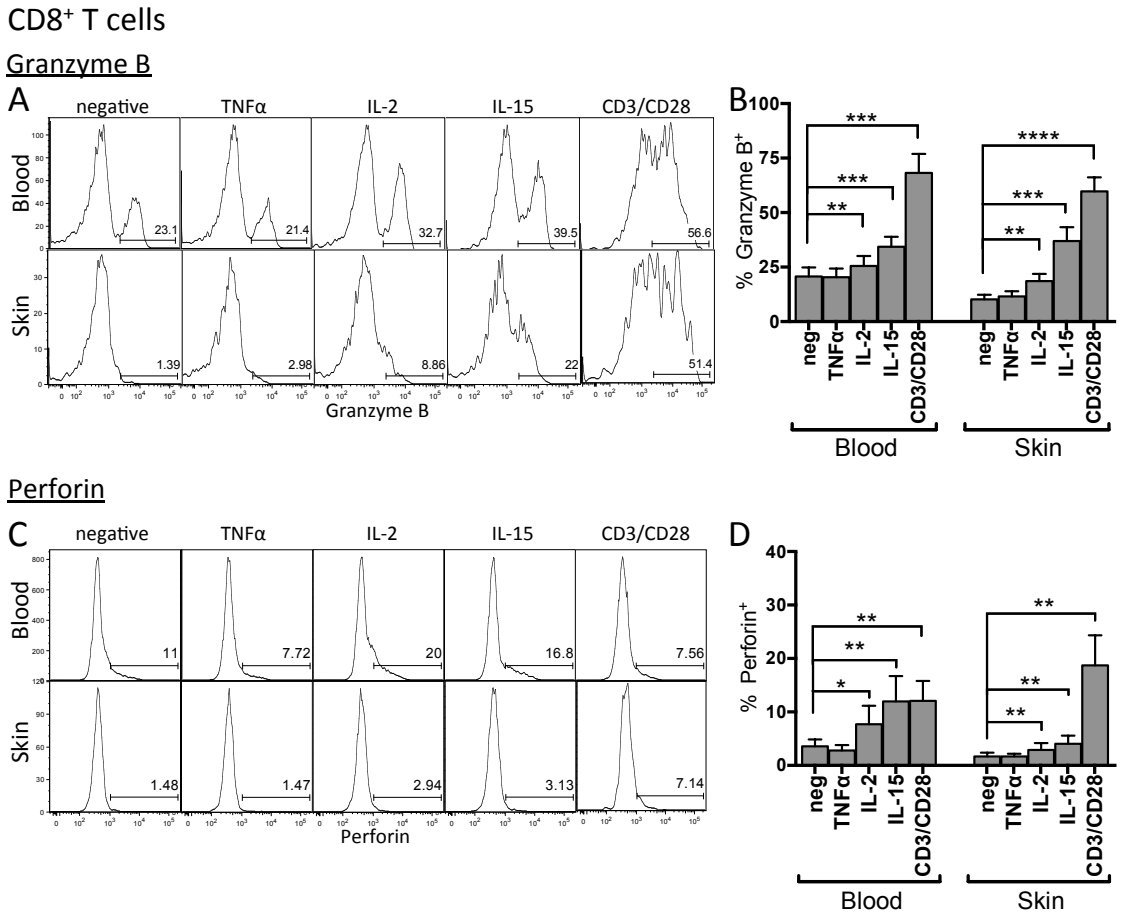


Fig. 6-5: Changes in Granzyme B and Perforin expression in blood and skin-derived CD8$^+$ T cells of healthy individuals in response to cytokines or anti-CD3/CD28 stimulation.

PBMCs or collagenase digested skin samples from healthy individuals were incubated for 4 days in the presence or absence of 5ng/ml IL-2, 10ng/ml IL-15, 10ng/ml TNFα or anti-CD3/CD28 microbeads, before being analyzsed by flow cytometry.
A, C: Representative histograms for granzyme B and perforin expression in blood and skin derived CD8$^+$ T cells following incubation with the various stimuli.
B: Cumulative data showing average granzyme B expression following the various treatments in the CD8$^+$ T cells derived from the blood (n=12) and skin (n=10) of healthy donors
D: Cumulative data showing average perforin expression following the various treatments in the CD8$^+$ T cells derived from the blood (n=10) and skin (n=9) of healthy donors.
Data was analysed by one-way ANOVA.
*=p<0.05; **p>0.01, ***p>0.001, ****p>0.0001

6.3.2.2 **Skin derived T cells acquire granzyme B in the absence of proliferation**

Increased frequencies of granzyme B expressing cells can be caused by proliferation of cells that already express the marker or alternatively by an increase of granzyme B expression in cells that previously did not. In order to test which of these cases was true in skin derived T cells, cells were labelled with CFSE before stimulation with IL-2, IL-15 or CD3/CD28 for 4 days (Fig. 6-6A).

The percentage of proliferating cells among the granzyme B expressing skin $CD8^+$ T cells was compared to the relative increase (compared to the untreated control) in granzyme B expressing cells for each treatment (Fig. 6-6B). Proliferation among the granzyme B expressing cells remained low, with only 6±4% of IL-2, 5±4% and 22±3% of granzyme B treated cells showing reduced CFSE in response to IL-2, IL-15 and anti-CD3/CD28 bead treatment respectively. These proliferation rates were lower than the relative gain in granzyme B expressing cells, which were 16±5% for IL-2, 30±5% for IL-15 and 35±15%. The increase in granzyme B expression among skin T cells was therefore not due to increased proliferation of granzyme B expressing cells. Instead, it seems that IL-2, IL-15 and anti-CD3/CD28 stimulation induced granzyme B expression in the majority of skin cells *de novo*.

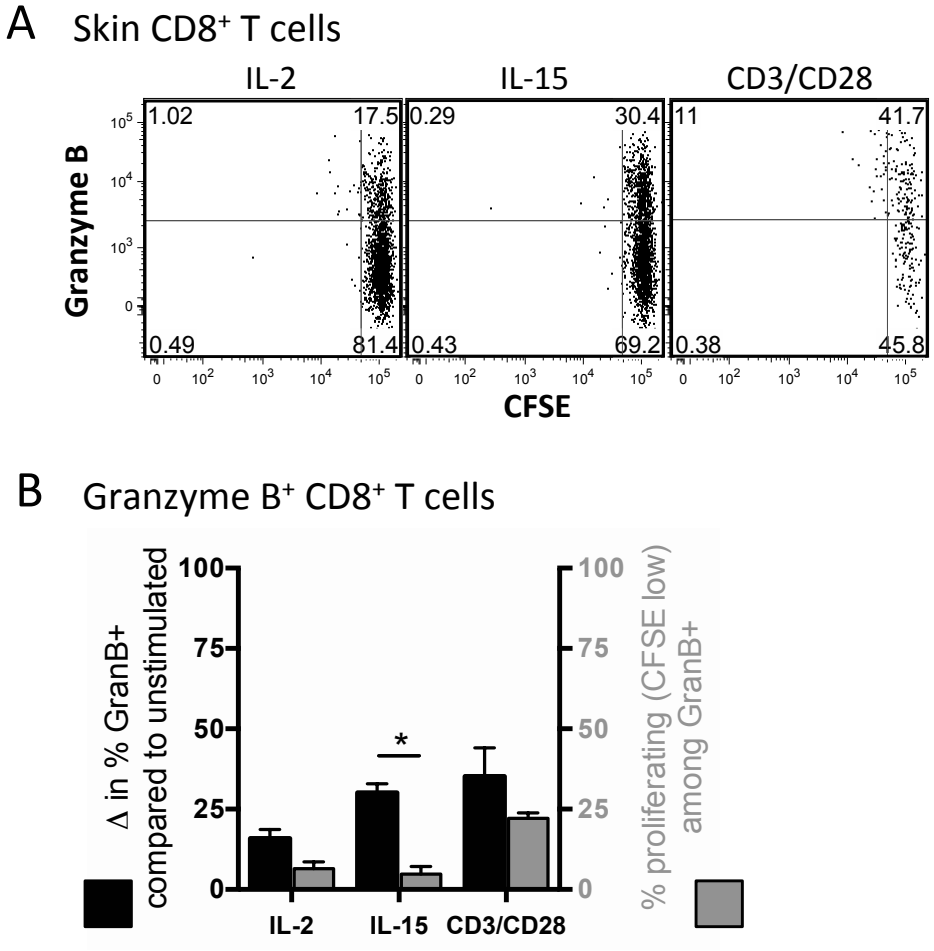


Fig. 6-6: Proliferation among granzyme B expressing cells derived from the skin of healthy individuals, after stimulation with IL-2, IL-15 or anti-CD3/CD28

CFSE labelled skin derived cells were cultured for 4 days in the presence of 5ng/ml IL-2, 10ng/ml IL-15 or anti-CD3/CD28 beads. Loss of CFSE staining indicates cell proliferation.
A: Representative flow cytometry scatter dot plots showing CFSE and Granzyme B staining in skin derived $CD8^+$ T cells following treatment.
B: Cumulative data showing the increase of granzyme B expressing cells per treatment relative to the unstimulated control (in black) compared to the percentage of proliferating cells amongst total Granzyme B expressing $CD8^+$ T cells (in grey) in samples derived from the skin (n=3). Populations were compared using the paired T-test. Mean and standard error are shown.

6.3.3 **The role of PD-1 signalling in T cell function in the skin**

Whilst skin resident $CD8^+$ T cells of melanoma patients and healthy controls expressed low levels of cytotoxic granule components, levels of the inhibitory receptor PD-1 were high among these cells. We therefore hypothesised that PD-1 signalling might play a role in blocking acquisition of effector functions in skin resident T cells. In order to explore the role of PD-1 signalling in skin T cell function, skin derived T cells were treated with anti-PDL-1 and PDL-2 blocking antibodies during anti-CD3 stimulation *in vitro*. A suboptimal dose of 0.05µg/ml of plate coated anti-CD3 antibody was chosen for this purpose as previous studies have shown that saturating conditions of TCR activation may overcome the effects of PDL-1 blockade (Freeman et al. 2000).

The production of granzyme B and perforin as well as proliferation were analysed in blood and skin derived $CD8^+$ T cells in response to PD-1 ligand blockade. Again, these assays used cells derived from healthy skin due to a lack of sufficient amounts of skin of melanoma patients. Additionally, skin blister cells were used and skin samples obtained by collagenase digestion needed to undergo FACS sorting due to the technical implications discussed below.

6.3.3.1 **Skin debris interferes with functional assays**

Collagenase digestion allows the generation of single cell suspensions of immune cells that can be manipulated *in vitro*. Whilst cells extracted using this method responded to soluble or microbead-based stimuli, these cells did not respond to stimuli that relied on cell-to-cell contact or contact with plate-bound antibody. The reason for this was found to be the high amount of debris present in the cell preparations (Fig. 6-7A).

Further filtration, magnetic-bead based sorting and density gradient centrifugation techniques (using Ficoll or Percoll) were tested, but did not yield the desired debris-free single cell suspensions required (data not shown). Finally, FACS-based sorting of live $CD3^+$ and antigen presenting cells was found to successfully isolate viable cells that could be used for PD-1 ligand blocking assays (Fig. 6-7B). Simultaneously to using FACS-sorted skin cells, we tested PD-1 ligand blockade on skin suction blister derived cells, as this method also allowed effective isolation of viable, debris-free immune cells from human skin without the need for cell sorting.

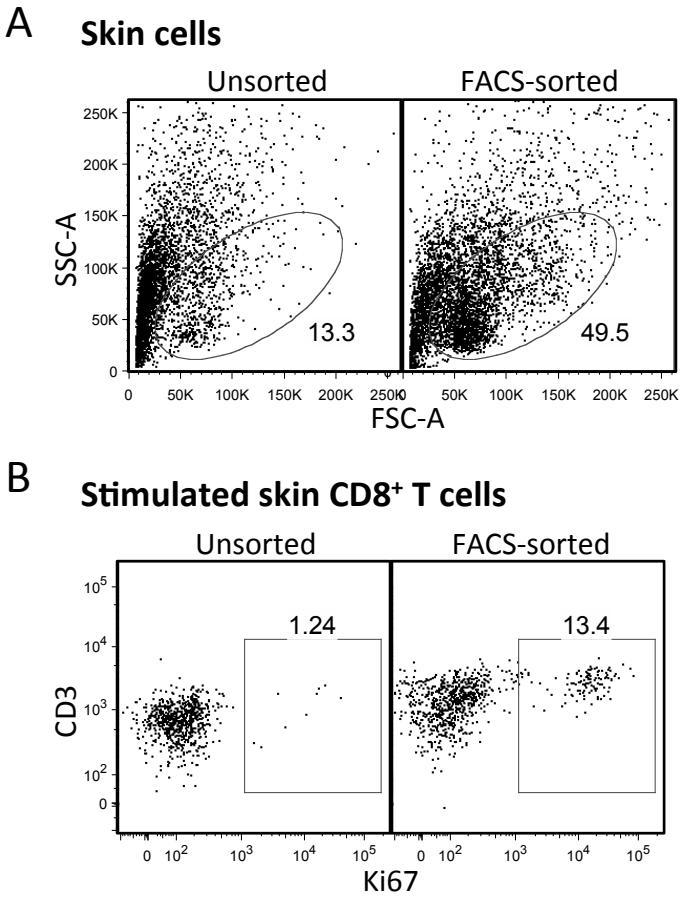


Fig. 6-7: Effect of skin debris on functional assays.

Cell suspensions extracted via collagenase digestion from healthy skin underwent FACS-based cell sorting in order to remove skin debris. Sorting involved selection of viable cells suitable for functional assays via staining using a live/dead dye, anti-CD3 antibodies and a cocktail of antibodies selecting for antigen presenting cells in the skin as described in detail in the material and methods section. Skin samples were obtained from healthy donors.
A: Comparison of forward scatter (FSC-A)– side scatter (SSC-A) profiles of skin cell preparations before and after cell sorting.
B: Whole digested skin or FACS sorted digested skin cells from a healthy individual were incubated for 3 days in the presence of 0.05µg/ml plate-bound anti-CD3 antibody. Intracellular Ki67 staining was performed as measure of proliferation.

6.3.3.2 **Granzyme B expression is increased in stimulated skin CD8$^+$ T cells after PDL-blockade.**

After optimizing conditions to successfully stimulate skin-derived T cells *in vitro* in a contact dependent manner, we tested whether blockade of the PD-1 ligands PDL-1 and PDL-2 during T cell stimulation might influence cytotoxic granule component production in skin derived T cells. For this purpose, we stimulated PBMCs, blister-fluid derived cells and sorted skin cells from healthy individuals with anti-CD3 antibody in the presence of PDL-1 and PDL-2 blocking antibodies or the appropriate isotype control. After 3 days of stimulation, intracellular levels of granzyme B and perforin were measured using flow cytometry in the CD8$^+$ T cells and treatment groups were compared statistically (Fig. 6-8).

Granzyme B was significantly increased after PD-1 ligand blockade compared to the isotype control in CD8$^+$ T cells derived from the blood ($p= 0.0198$), blister ($p=0.0316$) and skin ($p= 0.0170$). Conversely, perforin was only significantly increased after PD-1 ligand blockade in blood derived CD8$^+$ T cells ($p= 0.0234$), but not in blister or skin derived cells.

Therefore, PD-1 signalling seems to negatively regulate granzyme B expression in stimulated skin derived CD8$^+$ T cells.

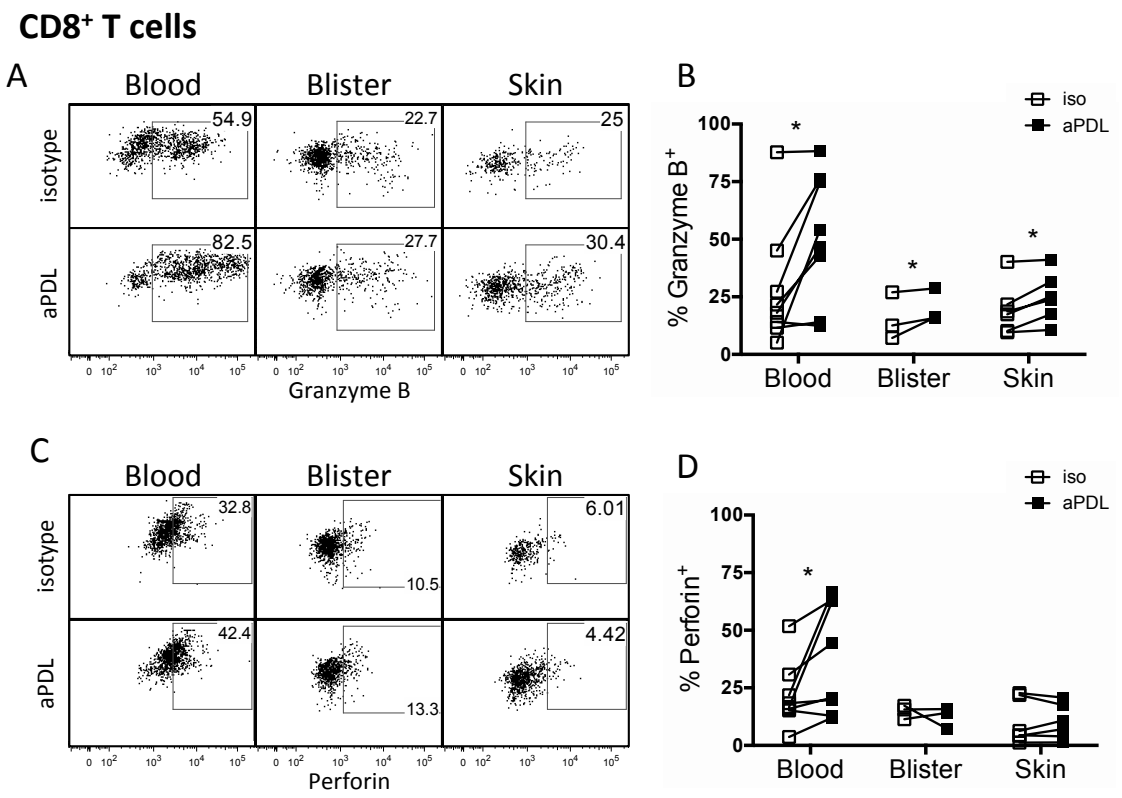


Fig. 6-8: Granzyme B and perforin expression after PD-1 ligand blockade in CD8$^+$ T cells derived from blood, blister fluid or skin of healthy individuals.

Whole PBMCs, whole blister cells and sorted skin cells (sorted for CD3 positive and antigen presenting cells; described in detail in the material and methods section) from healthy donors were stimulated with 0.05µg/ml plate coated anti-CD3 antibody in the presence of 10µg/ml of anti-PDL-1 and anti-PDL2 antibodies or isotype control antibody. Cells were harvested after 3 days of stimulation and stained for flow cytometric analysis. Perforin and granzyme B were detected using intracellular staining.

A: Representative flow cytometry plot showing Granzyme B expression in anti-PDL or isotype treated, stimulated CD8$^+$ T cells derived from blood, blister or skin of healthy donors.

B: Cumulative data showing changes in Granzyme B expression in anti-PDL treated CD8$^+$ T cells compared to isotype controls derived from the blood (n=8), blister (n=3) and skin (n=6).

C: Representative flow cytometry plot showing Perforin expression in anti-PDL or isotype treated, stimulated CD8$^+$ T cells derived from blood, blister or skin of healthy donors.

D: Cumulative data showing changes in Perforin expression in anti-PDL treated CD8$^+$ T cells compared to isotype controls derived from the blood (n=8), blister (n=3) and skin (n=6).

Treatment and control groups were compared using the paired T-test or Wilcoxon test.

*p<0.05

6.3.3.3 **Effects of PD-1 blockade on T cell proliferation**

PD-1 is an inhibitory receptor that downmodulates contact-dependent signalling during T cell activation (Freeman et al. 2000). In order to find out if PD-1 regulates functions other than cytotoxic granule formation in skin derived T cells, $CD8^+$ T cell proliferation was also examined in the context of PD-1 ligand blockade. Proliferation was therefore measured indirectly via flow cytometric detection of intracellular Ki67 levels in stimulated blood, blister and skin derived T cells (Fig. 6-9).

Ki67 levels were compared between anti-PDL-1/anti-PDL-2 or isotype treated cells in the $CD8^+$ T cell compartment in various tissues. Whilst blood derived $CD8^+$ T cells of half of the donors responded to PD-1 ligand blockade by showing increased proliferation, no overall significant difference was detected in the proliferation in blood $CD8^+$ T cells after incubation with PDL-1 and PDL-2 blocking antibodies. Further, PD-1 ligand blockade did not affect ki67 levels significantly in the $CD8^+$ T cells derived from blisters or skin. Unlike granzyme B expression, proliferation was therefore not affected by PD-1 signalling in skin derived $CD8^+$ T cells.

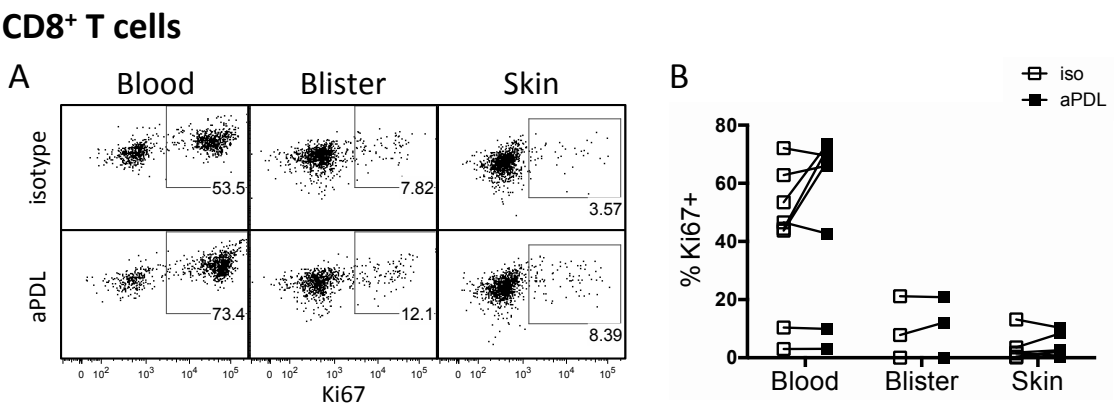


Fig. 6-9: Proliferation in response to PD-1 ligand blockade in stimulated CD8$^+$ T cells derived from blood, blister fluid or skin of healthy individuals.

Whole PBMCs, whole blister cells and sorted skin cells (sorted for CD3 positive and antigen presenting cells; described in detail in the material and methods section) from healthy donors were stimulated with 0.05µg/ml plate coated anti-CD3 antibody in the presence of 10µg/ml of anti-PDL-1 and anti-PDL-2 antibodies or isotype control. Cells were harvested after 3 days of stimulation and stained for flow cytometric analysis. High intranucellular levels of ki67 were used as markers for proliferation.
A: Representative flow cytomtry plot showing Ki67 expression in anti-PDL or isotype treated, stimulated CD8$^+$ T cells derived from blood, blister or skin of healthy donors.
B: Cumulative data showing changes in Ki67 expression in anti-PDL treated CD8$^+$ T cells compared to isotype controls derived from the blood (n=8), blister (n=3) and skin (n=6).

6.3.3.4 **PDL-1 and PDL-2 expression in the skin**

After having shown that PD-1 expression is high on skin derived $CD8^+$ T cells in healthy individuals and melanoma patients, and that PD-1 signalling might regulate their function, we investigated PD-1 ligand expression in the skin. For this, frozen sections of skin biopsies from healthy individuals and primary melanoma lesions were stained separately by immunohistochemistry using antibodies against the PD-1 ligands PDL-1 and PDL-2.

Expression of PDL-1 and PDL-2 was considered in young and old healthy individuals separately, as PD-1 expression on healthy skin resident T cells was previously found to be age-dependent (see section 5.3.2.4). PDL-1 and PDL-2 expressing cells were successfully detected in the skin of some but not all healthy individuals, and was restricted to individual cells in the dermis (Fig. 6-10). Three out of five young and three out of eight old healthy individuals presented with PDL-1 positive cells in their skin. The average frequency of PDL-1 expressing cells in the skin of healthy individuals was 2±2 per mm^2 in both age groups. PDL-2 expressing cells could be detected in all young skin sections and in four out of five old individuals. The average frequency of $PDL\text{-}2^+$ cells also remained similar between both age groups, with 5±3 $PDL\text{-}2^+$ cells per mm^2 in young and 6±5 in old donors. Overall, $PDL\text{-}2^+$ cells were found more frequently than $PD\text{-}1^+$ cells in healthy skin and this difference was significant if cell frequencies were compared irrespective of donor age ($p=0.0125$)

PDL-1 and PDL-2 expression was also investigated in frozen skin sections of primary melanoma lesions of old patients (Fig. 6-11). Primary melanoma presented with 27±15 $PDL\text{-}1^+$ cells per mm^2 compared to 2±2 cells in the skin of old healthy individuals ($p=0.0108$). PDL-2 expression was similarly increased in the melanoma patients, who displayed 42±27 $PDL\text{-}2^+$ cells per mm^2 compared to 7±5 in the healthy controls ($p=0.0146$). Within the primary melanoma lesions, $PDL\text{-}2^+$ cells were found more frequently than $PDL\text{-}1^+$ cells ($p=0.0374$).

In summary, PD-1 ligand expressing cells were more abundant in primary melanoma lesions than in healthy skin. Further, PDL-2 was more abundantly expressed than PDL-1 in both, healthy skin and primary melanoma.

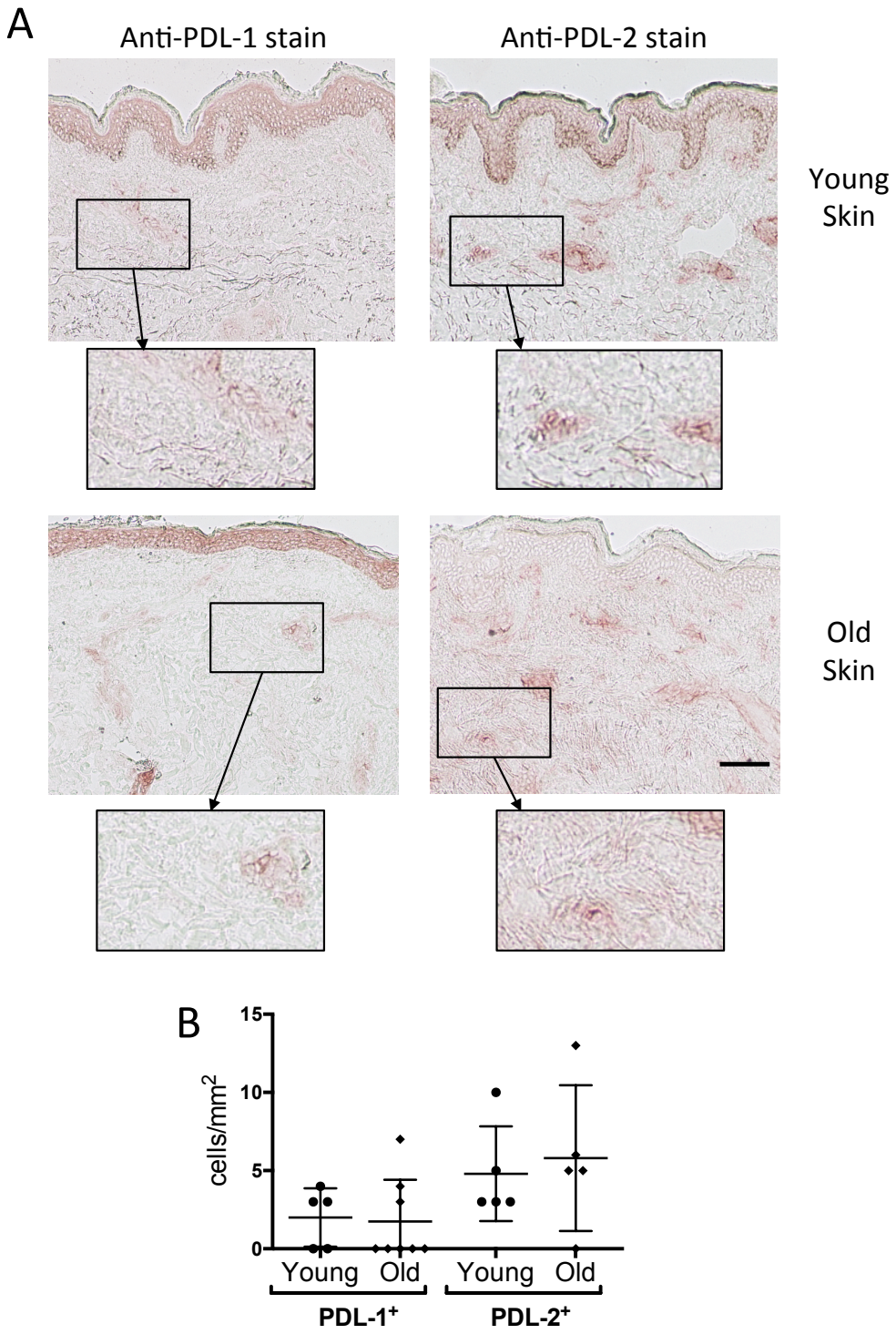


Fig. 6-10: PDL-1 and PDL-2 expression in skin sections of young and old healthy donors

Punch biopsies were taken from the skin of young and old healthy donors and indirect immunoperooxidase staining was performed on frozen healthy skin sections for PDL-1 and PDL-2 separately. PDL-1 and PDL-2 expressing cells were identified via marked red-brown circular staining.

A: Representative stains for PDL-1 and PDL-2 in skin sections from young and old healthy donors. Boxed areas are shown in two-fold magnification in order to highlight individual positive staining cells. Only the representative skin section shown for anti-PDL-1 staining in a young donor highlights an area of intense staining that was not counted as staining was nut circular.

B: Cumulative data showing frequency of cells expressing PDL-1 and PDL-2 in the skin of young (n=5 for both) and old donors (n=8 for PDL-1 and n=5 for PDL-2). Scale Bar =100µm

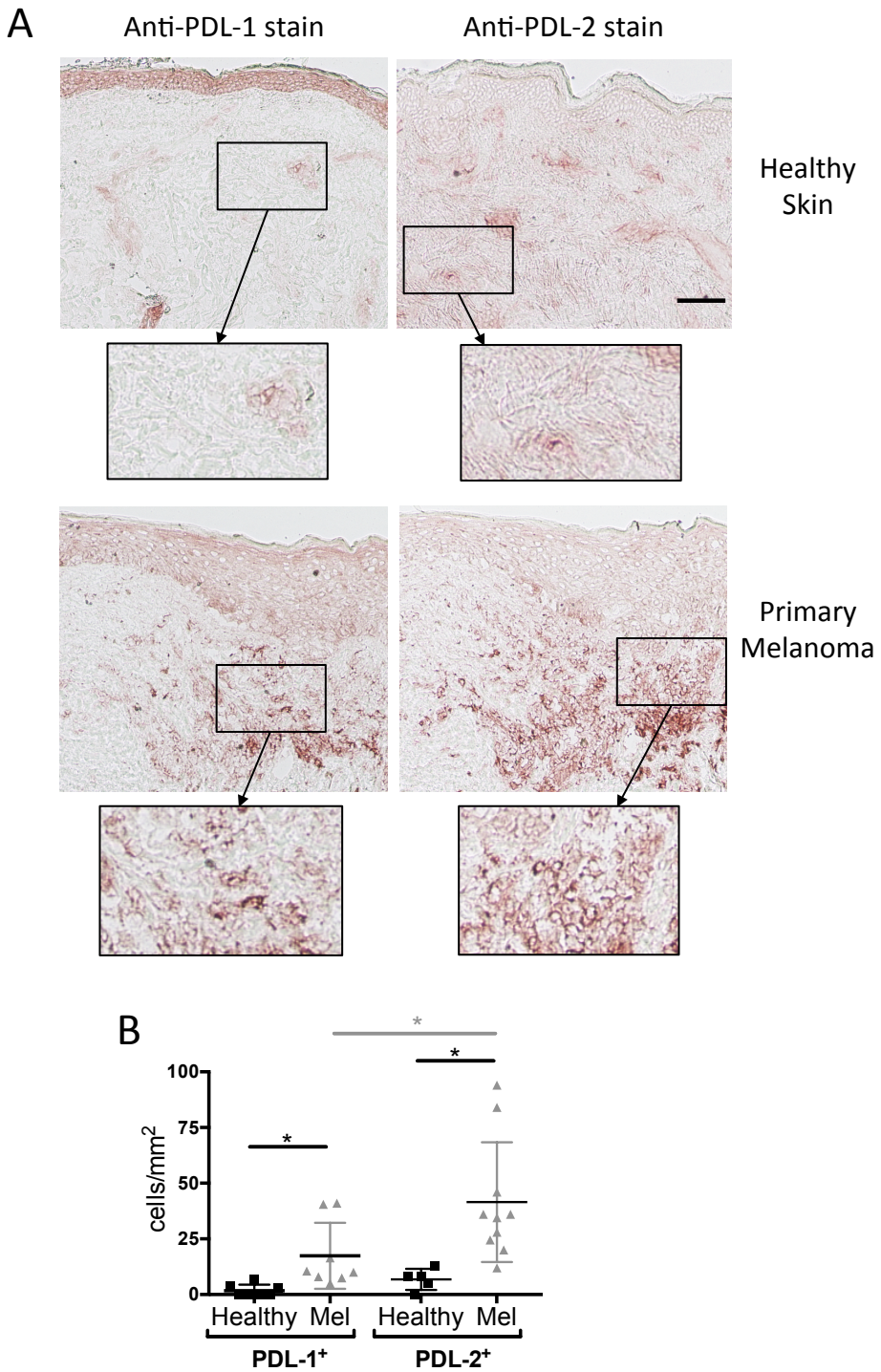


Fig. 6-11: PDL-1 and PDL-2 expression in the primary melanoma lesions of old melanoma patients compared to age matched healthy controls.

Indirect immunoperooxidase staining was performed for PDL-1 and PDL-2 on frozen skin sections from old healthy individuals and primary melanoma lesions from old patients. PDL-1 and PDL-2 expressing cells were identified via marked red-brown circular staining.
A: Representative stainings for PDL-1 and PDL-2 in the skin of healthy donors and lesions of melanoma patients. Boxed areas are shown in two-fold magnification in order to highlight individual positive staining cells.
B: Cumulative data showing frequency of cells expressing PDL-1 and PDL-2 in the skin of old healthy donors (n=8 and n=5, respectively) and primary melanoma lesions of old patients (n=8 for PDL-1 and n=10 for PDL-2).
Patients and controls were compared using the Student t-test, PDL-1 and PDL-2 levels within participant groups were compared using the paired t-test. *p<0.05; Scale Bar =100µm

6.4 Discussion

6.4.1 Restoring circulating T_{EMRA} $CD8^+$ T cell function in melanoma patients

P38 signalling is actively involved in maintaining a state of replicative senescence in highly differentiated T cells, as previous reports have shown an increase in T cell proliferation in $CD4^+$ and $CD8^+$ T_{EMRA} in healthy individuals after p38 inhibition (Di Mitri et al. 2011; S. M. Henson et al. 2014). Whilst proliferation was boosted after p38 blockade in melanoma patients' total $CD8^+$ T cells when they were stimulated with anti-CD3 in PBMC preparations, this effect was not observed in FACS sorted $CD8^+$ T_{EMRA} of all donors. Indeed, some donors displayed a decrease in proliferation after p38 blockade. This discrepancy in results might be due to the fact that p38 does not control T cell proliferation in some patients as it does in healthy controls. Alternatively, reduced proliferation in the T_{EMRA} cells of melanoma patients after p38 blockade might have been caused by technical constraints of the assay as excessive stimulation can cause a decrease in cell viability *in vitro* (Shi et al. 2013).

Previous reports mention that blockade of P38 signalling is associated with a decrease in TNFα production in T cells of healthy individuals (S. M. Henson et al. 2014). Here, TNFα production was not significantly affected by the blockade in $CD8^+$ T cells of patients with melanoma. However, melanoma patients' stimulated T_{EMRA} cells displayed increased IL-2 and IFNγ production as well as increased degranulation (as measured by CD107a surface detection) upon p38 blockade. Targeting the p38 pathway might therefore improve functionality in the highly differentiated cells in old melanoma patients and might therefore be beneficial for improving anti-tumour immune responses.

It should be noted that growth arrest in senescence is thought to be a physiological mechanism to prevent malignancy and blockade of p38 might therefore promote tumour growth. Interestingly, a number of melanoma cell lines display aberrant intracellular signalling pathways and use p38 signalling to promote growth and migration (Estrada, Dong, and Ossowski 2009). P38 inhibition in melanoma patients might therefore affect the tumour directly as well as T cell functions.

6.4.2 **Signals inducing a cytotoxic phenotype in skin cells**

Skin resident T cells in healthy individuals were shown in previous chapters to express no perforin and only low levels of granzyme B. In the skin T cells of patients with melanoma an increase in granzyme B but not in perforin was found, suggesting insufficient cytotoxic maturation. This chapter therefore explored what signals might contribute to cytotoxic maturation of T cells in the skin and confirmed that IL-2, IL-15 and CD3/CD28 stimulation induced granzyme B and perforin expression in skin resident T cells *in vitro*. This is consistent with previous findings in the blood that showed acquisition of granzyme B and perforin and increased cytotoxic function in T cells cultured with IL-2, IL-15 and strong signalling through the TCR/CD3 complex and CD28 receptor (Janas et al. 2005; Alves et al. 2003; Grossman et al. 2004; Azuma et al. 1992)

The fact that perforin and granzyme B levels were low under steady state conditions in healthy skin suggest that these cells are not exposed to sufficient amounts of IL-2 or IL-15 to induce cytotoxic granule formation. Indeed, IL-15 and IL-2 are not normally expressed in healthy skin and the majority of skin derived cells only upregulate these cytokines during inflammatory conditions, as has been found to be the case for IL-15 in keratinocytes and dermal fibroblasts (Han et al. 1999; Rappl et al. 2001; Bouchaud et al. 2013). Similarly, only mature but not immature dendritic cells in the skin, such as Langerhans cells, will express the CD28 ligands CD80 and CD86 and therefore activate effector T cells in a contact dependent manner (Peña-Cruz et al. 2010).

High expression of IL-15 and IL-2 and presence of activated dendritic cells might therefore explain the occurrence of perforin and granzyme B in psoriasis lesions (Uyemura et al. 1993; Nestle et al. 1994; Yawalkar et al. 2001; Bouchaud et al. 2013). Conversely, T cells detected in melanoma patients might only present low levels of cytotoxic granule components due to a lack of these cytokines and dendritic cell maturation in the areas within and surrounding the tumour (El Marsafy et al. 2009).

6.4.3 **The role of PD-1 in controlling skin cells**

Results presented in previous chapters showed that although PD-1 expression is low in circulating T cells, it is constitutively expressed on a large proportion of T cells the skin in healthy individuals as well as patients with melanoma. We therefore hypothesized that signalling of this inhibitory receptor might inhibit cytotoxic marker expression. In this chapter the inhibitory role of PD-1 in skin T cells was confirmed by showing increased granzyme B expression following PD-1 ligand blockade during stimulation. However perforin expression and proliferation (as measured by ki67 expression) were not affected by the treatment.

This work shows for the first time that PD-1 signalling can control granzyme B expression upon activation in human skin cells and is consistent with previous findings showing that PD-1 signalling reduces expression of transcription factors involved in effector functions (such as Tbet and Eomes) and cytotoxicity in circulating $CD8^+$ T cells (Nurieva et al. 2006). Although blockade of the PD-1 signalling pathway is also often associated with increased proliferation (Freeman et al. 2000; Carter et al. 2002), other studies have shown changes in T cell function (such as cytokine production) in the absence of proliferation in certain animal models or at higher antigen doses (Kuipers et al. 2006; Latchman et al. 2001). PD-1 might therefore still regulate proliferation and perforin production in skin resident T cells under physiological conditions or under experimental conditions other than the one used during this work.

PD-1 signalling is only effective during stimulation and ligation of PD-1 in absence of TCR signalling has no significant effect on T cell functions (Freeman et al. 2000). Therefore, PD-1 binding to its ligands is physiologically only relevant in the context of concomitant T cell activation. This is consistent with our finding that the PD-1 ligands PDL-1 and PDL-2 were only expressed at low levels in healthy skin (where T cell activation does presumably not occur), whilst being upregulated in melanoma lesions. This work did not discern whether PDL-1 and PDL-2 were expressed by healthy or malignant cells. Previous studies have shown that melanoma cells can express PDL-1 to evade immune responses and that inflammatory conditions promote PDL-1 and/or PDL-2 in skin derived Langerhans cells and keratinocytes (Peña-Cruz et al. 2010; Freeman et al. 2000; Gadiot et al. 2011). Increased PD-1 ligand expression in

melanoma lesions may therefore indicate the presence and activation of immune cells or tumour immune evasion.

It should be noted that although the increase in granzyme B expression in skin resident T cells after PD-1 ligand blockade was significant, it was not as pronounced as in the blood, suggesting that additional mechanisms prevent cytotoxic T cell maturation in these tissue resident cells.

6.4.4 **Points of consideration**

One point of consideration is that increased granzyme B and perforin expression *in vitro* do not necessarily translate into increased killing capacity, as even cells that do express high levels of cytotoxic granule components might show impaired cytolytic activity (Lee et al. 1999). However, identifying mechanisms that allow skin resident T cells to acquire cytotoxic granule components is a first step in understanding how skin resident T cells in melanoma patients can be induced to become effective at killing melanoma cells *in vivo*.

Whilst cells in healthy skin express only low levels of cytotoxic granule components, we identified in this chapter positive and negative signals that might control their cytotoxic maturation. The low expression of cytotoxic granule components in skin resident T cells of melanoma patients might therefore be explained by an imbalance of these signals.

7 Overall Discussion

The results presented in this thesis do not only contribute to our understanding of T cell differentiation in the blood and skin of patients with melanoma, but also to our appreciation of the phenotypic properties of T cells residing in healthy skin.

7.1 T cells in healthy skin

7.1.1 An imbalance between skin and blood T cells

Recent studies have shown that skin resident T cells are phenotypically and transcriptionally distinct from cells in the blood and recognize different targets (Clark, Chong, Mirchandani, Brinster, et al. 2006; L. K. Mackay et al. 2013). However, human skin resident T cells remain poorly characterized to this day. This work confirmed that T cells in the skin are at an imbalance with T cells in the blood in humans, as both $CD4^+$ and $CD8^+$ T cells are phenotypically distinct in both organs. Further, surface markers that define T cell differentiation in the blood were not co-expressed in the same manner in skin resident T cells and could therefore not be used in the same manner to define skin resident T cell differentiation stages. This also suggests that some of these surface receptors may have different physiological roles and may be differentially regulated in the skin compared to the blood. The role of markers for differentiation, such as CD45RA, CD27, CD28, CD57 and KLRG1, should therefore be considered with care in the context of tissue resident T cells.

It is often stated that tissue resident T cells are T_{EM}-like because they lack lymph node homing receptors and are at the site of potential pathogen encounter, making them important immune sentinels (Jiang et al. 2012; Ariotti et al. 2014). However, the data presented in this thesis suggests that skin resident T cells are in fact profoundly distinct from circulating T_{EM}. For example, circulating T_{EM} readily displayed effector functions such as pre-formed cytotoxic granule components, whilst the majority of T cells derived from healthy skin did not, although they could be induced to do so *in vitro*. This, together with the observation that skin resident T cells express high levels of the costimulatory

receptor CD28 and the inhibitory receptor PD-1, suggests that T cell functions in the skin are heavily regulated.

7.1.2 **The skin as a tolerogenic environment**

The concept that the skin is an environment that favours immune tolerance over activation during steady state conditions is supported by the detection of the inhibitory receptor PD-1 on resident T cells as well as the presence of inhibitory cell types in the skin.

The role of PD-1 in peripheral tolerance has been highlighted in PD-1 knock out mice that developed spontaneous systemic autoimmunity (Nishimura et al. 1999). T cells obtained from healthy skin expressed high levels of PD-1, suggesting that PD-1 may also be an important factor in skin tolerance. Indeed, we observed that PD-1 signalling during CD3 activation decreased acquisition of granzyme B in skin derived T cells *in vitro*. The role of PD-1 in controlling T cell functions in the skin has been further exemplified in a mouse model, where OVA-expressing keratinocytes upregulated PDL-1 during inflammation and were more likely to be killed and contribute to immunopathology when exposed to PD-1 deficient compared to wild-type OVA-specific T cells (Okiyama and Katz 2014).

Inhibitory $CD4^+$ FoxP3 Treg cells are also important in immune tolerance in the skin and other organs(Dudda et al. 2008) and FoxP3 Treg frequency was found to be increased in the skin compared to the blood in this and previous studies (Booth et al. 2010; Vukmanovic-Stejic et al. 2013; Sanchez Rodriguez et al. 2014). Other immunomodulatory cell types in the skin may include immature dendritic cells which patrol healthy skin and have been shown to readily promote *in vitro* Treg formation and proliferation rather than effector T cell differentiation (Seneschal et al. 2012; Chu et al. 2012).

The skin is an organ that is constantly exposed to environmental insults, most of which might be innocuous or rapidly contained by innate immune mechanisms. In order to maintain tissue integrity, it is therefore important to limit immune mediated tissue damage through the mechanisms listed above, including PD-1 signalling. However, during infection or malignancy, mechanisms must exist to promote inflammation and induction of T cell effector functions.

7.1.3 **Induction of effector T cell responses in the skin**

Whilst not expressing perforin and granzyme under steady state conditions, skin derived T cells were shown to acquire perforin and granzyme B when exposed to IL-2, IL-15 or CD3/CD28 stimulation *in vitro*. This suggests that skin resident T cells can acquire effector functions given the appropriate signals and is supported by published observations that confirm granzyme B and perforin expression in the skin during autoimmune conditions such as psoriasis and vitiligo (van den Wijngaard et al. 2000; Yawalkar et al. 2001).

The cue for the conversion of resting skin resident T cells to potent effector cells might simply be TCR-ligation through antigen recognition on a target cell, but is likely to further require costimulatory signals in form of receptors or cytokines provided by either target or bystander cells. The importance of cell help for effector T cell licensing in the skin has been demonstrated in a recent murine graft-versus-host model, where T cell infiltration into the skin graft was not sufficient for rejection but required local Langerhans-mediated help for T cell mediated killing of the mismatched graft (Bennett et al. 2011). Other cell types might also be involved in promoting effector T cell functions in the skin, such as activated dermal dendritic cells or keratinocytes, when exposed to danger signals (El Marsafy et al. 2009; Igyarta et al. 2011; Kupper 1990). In absence of receptor-mediated stimulation, increased local levels of inflammatory cytokines might be sufficient to induce effector functions in resident T cells and therefore contribute to a tissue-wide state of “readiness” when skin integrity is breached (Ariotti et al. 2014).

A balance of pro- and anti-inflammatory signals therefore seems to determine effector function acquisition of T cells in the skin (as visualised in Fig. 7-1). In healthy skin, this balance is tilted in favour of the negative signals, possibly by virtue of missing inflammatory signals under steady state conditions and high prevalence of suppressive Tregs and PD-1. This balance must however be shifted in favour of T cell effector maturation in order for $CD8^+$ T cell mediated immune responses to occur during infection and malignancy.

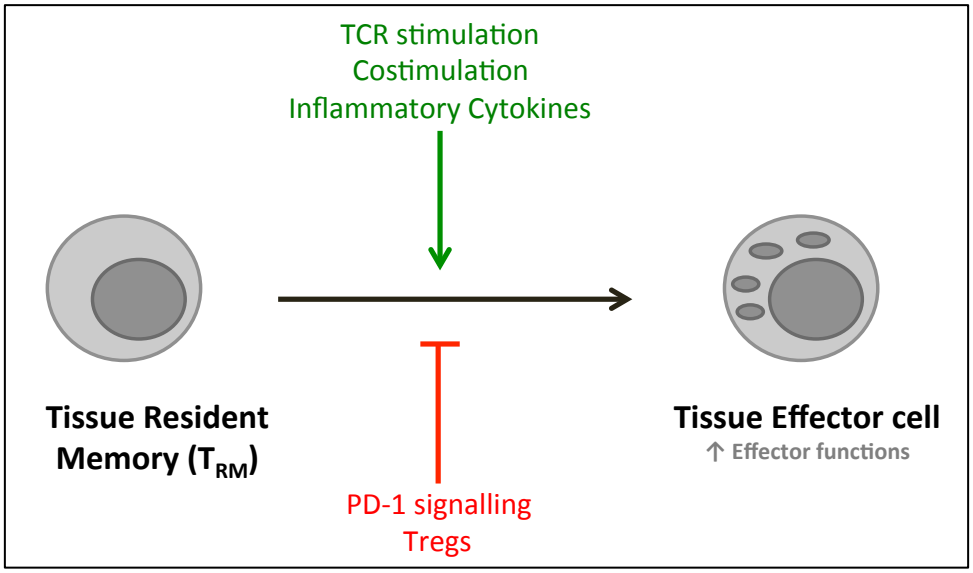


Fig. 7-1: Schematic diagram visualising the proposed model for cytotoxic T cell differentiation in the skin.

In healthy skin, T cells do not express cytotoxic granule components. Positive signals, such as TCR stimulation, costimulation with CD28 and inflammatory cytokines such as IL-2 and IL-15 can induce differentiation into a cytotoxic phenotype. Negative signals such as the inhibitory receptor PD-1 and Treg cells may inhibit acquisition of effector functions.

7.2 T cells in patients with melanoma

7.2.1.1 Lack of effector T cells in the skin of patients with melanoma

T cells in the skin of patients with melanoma showed phenotypic differences in the skin compared to healthy controls, which might have been caused by increased infiltration and/or local modulation of the cells. Interestingly, no increase in cytotoxic granule components was observed in the tissue $CD8^+$ T cells of the patients. This suggests that a lack of inflammatory signals or prevalence of inhibitory signals prevent T cells in the patients from acquiring potent effector functions in order to mediate tumour rejection. High expression of PDL-1 and PDL-2 on melanoma or melanoma infiltrating cells in the primary lesion, as well as high levels of PD-1 expression on melanoma skin T cells, suggest that the tumour has hijacked the tolerogenic properties of the skin in order to avoid effective T cell activation and killing. Additional inhibitory mechanisms might prevent the development of effector functions in the melanoma microenvironment: This is supported by reports of increased levels of local Tregs, of inhibitory cytokines such as TGFβ and IL-10 and absence or lack of maturation of antigen presenting cells (Stene et al. 1988; Toriyama et al. 1993; Vermi et al. 2003; Jacobs et al. 2012; Díaz-Valdés et al. 2011). In

summary, the balance of signals in the skin of patients with melanoma seems to be in tilted favour of immune tolerance and therefore prevent acquisition of effector molecules in local T cells.

7.2.1.2 T cell differentiation in blood and skin of melanoma patients

End stage differentiated and senescent $CD8^+$ T_{EMRA} cells were increased in old patients with melanoma and certain functions in these cells could be boosted through blockade of p38 signalling during activation. However, the origin and role of these cells in melanoma could not be established. Instead, melanoma-specific T cells recognizing Melan-A and NY-ESO-1 derived peptides had a T_{CM}-like phenotype. Similarly, the majority of T cells accumulating in the skin of the patients expressed surface receptors consistent with a T_{CM} phenotype. A number of these cells also expressed high levels of the inhibitory receptors PD-1 and CTLA-4, suggesting that these cells were exhausted.

End stage differentiation, even during senescence, is usually associated with high effector functions at the expense of proliferative capacity and generally occurs in cells after repeated intermittent stimulation, particularly through strong TCR signalling or can be induced by bystander exposure to high levels of IL-15 (Griffiths et al. 2013; van Baarle et al. 2005; Arne N. Akbar and Henson 2011). Exhaustion on the other hand is associated with immune dysfunction, both at the level of proliferation and effector functions and is caused by persistent stimulation and maintained by inhibitory receptor signalling (Wherry et al. 2007; Nakamoto et al. 2009). As the phenotype of skin resident T cells in melanoma patients suggests that they belong to the latter, it makes sense to design melanoma treatments that diminish inhibitory signalling and simultaneously boost acquisition of effector functions.

7.3 Clinical relevance of this work

The data presented in this thesis suggests that T cells in the skin of melanoma patients fail to differentiate into potent effector cells, therefore potentially impairing melanoma cell killing. IL-2, IL-15 or CD3/CD28 ligation were shown to induce expression of cytotoxic granule components, whilst PD-1 signalling inhibited it, suggesting targets that could be exploited to improve immune function in the patients. Indeed, various studies have already examined the

efficacy of boosting anti-melanoma immune function through administration of IL-2 and IL-15 or blocking antibodies that target inhibitory receptors such as PD-1:

Based on promising animal models (Rosenberg et al. 1985), administration of IL-2 was one of the first immunotherapies tested as treatment for melanoma. In 270 metastatic melanoma patients treated with high dose recombinant IL-2, 16% were partial or complete responders of which 12% remained disease free during the follow up period. However, 6 of the patients treated died of treatment toxicity (Atkins et al. 1999). Interestingly, frequency of perforin and granzyme B expressing cells increased upon treatment with IL-2 in the circulation of patients (Leger-Ravet et al. 1994), but IL-2 treatment also induced Treg expansion, with patients that showed particular high Treg expansions having worse clinical outcome than the other participants (Sim et al. 2014).

IL-15 has been shown to improve disease outcome in a melanoma mouse model where adoptively transferred transgenic melanoma specific $CD8^+$ T cells were pre-treated with the cytokine (Klebanoff et al. 2004). IL-15 was also efficacious in boosting T cell function in a number of non-melanoma mouse and *in vitro* models (Pagliari et al. 2013). A recent trial in metastatic melanoma patients and renal cell carcinoma patients showed that administration of recombinant IL-15 was safe and induced lymphocyte redistribution and expansion in patients. However, no objective clinical responses were recorded after treatment, despite a decrease in tumour metastases sizes in some patients (Conlon et al. 2014). It should also be noted however that IL-15 may directly act as a growth factor to the malignant cells (He et al. 2004).

Inhibition of PD-1 signalling in order to boost immune function in melanoma patients is the focus of numerous emerging clinical trials that use monoclonal blocking antibodies against PD-1 or its ligands. A recent trial showed an encouraging 28% complete or partial response rate for advance stage melanoma patients after treatment with anti-PD1 and the response lasted for over a year in more than half of the responders that were followed up (Topalian et al. 2012). In a different study, a 17% response rate was found in melanoma patients treated with anti-PDL-1 (Brahmer et al. 2012).

Combination therapy targeting PD-1 together with other inhibitory receptors associated with immune exhaustion such as CLTA-4 and TIM-3 have been

reported to rescue anti-tumour T cell responses in mouse models and *in vitro* and might therefore become the next generation of therapeutic targets in humans (Curran et al., 2010, Fourcade et al., 2010).

Anti-melanoma T cell responses are a multistep process that requires activation in the lymph node, effective migration into the tumour itself, overcoming inhibitory signals within the tumour microenvironment and finally effective killing. In this thesis we have shown that T cells in melanoma patients can be impaired through different mechanisms in the skin compared to the blood. All these factors should therefore be considered carefully when manipulating T cell functions in order to treat patients with melanoma effectively.

7.4 Technical aspects of using flow cytometry

Because the data presented in this thesis were acquired over a period of several years, great care was applied in order to ensure consistency and reliability of results obtained. The same series of experiments (such as the screening for T cell memory subset frequency in melanoma patient and healthy controls) used continuously the same protocol with antibodies matched for fluorochrome, clone and manufacturer. Further, the same flow cytometer was used for acquisition of similar experiments and all flow cytometry files were re-analysed prior of the writing of this thesis to assure a consistent gating strategy. Pure water was run between FACS tubes during acquisition to avoid spill over between samples and dead cells and duplets were excluded during analysis to avoid unspecific staining. Where possible, at least 20 000 $CD8^+$ or $CD4^+$ T cells were acquired for phenotyping purposes and at least 100 0000 $CD8^+$ T cells for enumeration of rare events such as melanoma specific T cells using MHC-Dextramers, as recommended by Britten and colleagues (Britten et al. 2009). However, a certain degree of variation between experiments could not be prevented due to unavoidable factors such as the occasional re-calibration of the flow cytometer or potential batch variation in the antibodies used. Different experiments may have involved the use of different clones for certain markers of interest (e.g. CD27 and CD45RA), further accounting for potential variation in results obtained in experiments using different antibody panels.

7.5 Future work

7.5.1 Identifying the source and role of increased T_{EMRA} in old melanoma patients

Obtaining clinical follow up data for the patients that participated in this work might reveal changes in disease outcome in relation to CMV status and T cell differentiation levels and might hint at the role of T_{EMRA} expansions in the melanoma setting. Testing for T cells recognizing alternative melanoma associated antigens to the ones used previously (using tetramer or dextramer technology) might elucidate the origin of T_{EMRA}. Tyrosinase or gp100 specific T cells for example have not been investigated in this work. An alternative method for identifying the antigen specificity of the T_{EMRA} could be to stimulate these cells with melanoma antigen peptide libraries, and measure potential cytokine responses. However, the limitation of this technique is that it relies on the T_{EMRA} cells being functional in this assay.

7.5.2 Understanding T cell differentiation in healthy skin

This work showed that surface markers commonly used in the blood to measure T cell differentiation might not be applicable in the skin. The extent of differentiation of skin resident T cells therefore remains to be elucidated. One key experiment that will help to address this question will be to measure the telomere length of the skin resident T cells and compare levels to blood derived T cells. Preliminary experiments have shown that debris from the collagenase-digested skin interfered with telomere probe binding in Flow-FISH experiments. Future experiments will therefore require FACS sorting of the skin cell preparations or could use cells from suction blisters.

7.5.3 FasL expression on skin T cells

This work showed that T cells in the skin of healthy individuals and patients with melanoma express only low levels of cytotoxic granule components, suggesting that skin resident T cells are not readily cytotoxic. However, T cell mediated killing can also be mediated through the receptor FasL (CD95L) and therefore be cytotoxic granule independent (Kägi et al. 1994). Future experiments addressing T cell cytotoxic function in the skin should therefore measure FasL expression of skin resident T cells.

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