Defining the lymphoid stress surveillance response in human skin

Woolf, Richard

Awarding institution:
King's College London

Defining the lymphoid stress surveillance response in human skin

Richard Thomas Woolf

Thesis submitted to the University of London in fulfilment of the requirements for the degree of Doctor of Philosophy

September 2015

Peter Gorer Department of Immunobiology

King's College London

2nd Floor Borough Wing

Guy's Campus

London SE1 9RT

Abstract

The skin contains many resident immune cells that play key roles in tissue homeostasis. In mouse skin, $V\gamma5V\delta1^+$ T cells have the innate-like capacity to respond rapidly and in large numbers to markers of tissue stress, rather than requiring clonal, antigen-specific activation. This lymphoid stress surveillance response (LSSR) is key to host immunity, contributing to barrier integrity and cutaneous atopic responses. In this thesis, we have sought to identify if the potential for LSSR exists in human skin. Our key and novel findings are a revised assessment of the lymphocyte content of human skin, with the discovery that the predominant γδ T cell population shows innate-like responsiveness analogous to that in the murine epidermis.

Through the adaptation of a novel skin explant protocol, we have established that the skin contains a distinct and reproducible population of γδ T cells, which predominantly express the Vδ1 and Vδ3 TCR chains. These cells displayed features of tissue-resident "memory" $TCR\alpha\beta^+$ [T_{RM}] cells, but strikingly had the capacity to become activated by ligands for the NKG2D receptor, seemingly independent of TCR antigen receptor signalling. Such responsiveness was not seen in other $NKG2D^+$ T cell subsets. Upon such NKG2D-mediated activation skin-resident γδ T cells showed robust effector responses, producing TNFα, IFNg, cytolytic mediators and additional growth factors/chemokines. Such innate-like responsiveness was dependent on both PI3K and calcineurin activity, but independent of Lck, characterising this novel mode of innate-like T cell activation. Skin-derived γδ T cells also showed profound cytolytic activity against transformed epithelial cells *in vitro*, which was in part dependent on NKG2D, demonstrating the functional potential of these cells in tissue stress-surveillance.

These data provide the first clear identification of a human innate-like T cell subset, locating them in the tissue immune compartment. This strongly argues for the existence of LSSR in human tissues and places skin-resident γδ T cells in the early afferent phase of the immune response. This offers a new perspective on tissue immune surveillance and has implications for future studies in human skin immunobiology.

Acknowledgements

I would like to acknowledge; Professor Adrian Hayday for his guidance, supervision, support, stimulation and encouragement throughout the course of this thesis – his scientific thought is truly original, incisive and inspiring – without which this work and my own development as a clinician scientist would not have been possible; Professor Jonathan Barker for his mentorship and support prior to and throughout his co-supervision of this thesis; past and present members of the Hayday laboratory at both King's College London and the Francis Crick Institute for both their collaboration, and practical and intellectual input – especially Dr Oliver Nussbaumer, who has become a close colleague and friend, and Dr Fernanda Kyle, Dr Maria-Luisa Iannitto and Dr Robin Dart; Professor Frank Nestle, Ms Isabella Tosi and the team of research nurses at the St John's Institute of Dermatology who provided access to skin material; the Division of Immunology, Infection and Inflammatory Disease, King's College London, and the NIHR Biomedical Research Centre, Guys' and St Thomas' NHS Foundation Trust, for their provision of core research facilities; and funding bodies for their financial support, notably the Medical Research Council, who sponsored my Clinical Research Training Fellowship, and the Wellcome Trust and Cancer Research UK, who provide programme funding for the laboratory. Lastly, I would like to acknowledge the love and support provided from my family and friends throughout the course of this thesis, particularly that of my wife Katie, who is pregnant with our first child, and whom I would like to dedicate this work to.

Table of Contents

List of Figures

Chapter One: Introduction

Chapter Two: Characterisation of the skin-resident lymphocyte compartment in healthy human skin

List of Tables

List of Abbreviations

α-GalCer	α-Galactosylceramide
AHR	Arylhydrocarbon receptor
APC	Antigen presenting cell
BFA	Brefeldin A
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte antigen
CMV	Cytomegalovirus
DC	Dendritic cell
DETC	Dendritic epidermal T cell
DMSO	Dimethyl sulfoxide
DN	Double negative
DP	Double positive
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
FCS	Foetal calf serum
FOXP3	Forkhead box P3
GM-CSF	Granulocyte macrophage colony stimulating factor
GWAS	Genome-wide association study
GROα	Growth related oncogene α
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMB-PP	(E)-4- hydroxy-3-methyl-but-2-enyl pyrophosphate
HHV8	Human herpes virus 8
HSCT	haematopoietic stem cell transplantation
ICAM-1	Intercellular adhesion molecule-1

IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgE	Immunoglobulin E
IGF	Insulin-like growth factor
IL	Interleukin
IL-1RA	IL-1 receptor antagonist
ILL	Innate-like lymphocyte
IMDM	Iscoves Modified Dulbecco's Medium
iNKT	Invariant natural killer T cell
IP-10	IFNγ-inducible protein 10
IPP	Isopentenyl pyrophosphate
JAK	Janus kinase
KGF	Keratinocyte growth factor
LC	Langerhans cell
LSSR	Lymphoid stress surveillance response
MAIT	Mucosal-associate invariant T cell
MCP-1	Monocyte chemoattractant protein 1
MEP	2-C-methyl-D-erythritol 4-phosphate
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex class I
MICA/B	MHC-like class 1 related protein A or B
MIP-1α/β	Macrophage inflammatory protein 1α/β
NCR	Natural cytotoxicity receptor
NK	Natural killer
MULT-1	Murine UL16-binding protein-like transcript 1
NFAT	Nuclear factor of activated T cells
NKT	Natural killer T cell
NKG2D	Natural killer group 2D
OVA	Ovalbumin
PALP	Phosphorylated tyrosine–rich aggregates located on projections
PAMP	Pathogen-associated molecular pattern

PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PRR	Pattern recognition receptor
PMA	Phorbol 12-myristate 13-acetate
RAE-1	Retinoic acid early inducible-1
RANTES	Regulated on activation, normal T cell expressed and secreted
RORC	RAR-related orphan receptor C
RPMI	Roswell Park Memorial Institute
S1P	Sphingosine-1-phosphate
SALT	Skin associated lymphoid tissue
SD	Standard deviation
SDF-1α	Stromal cell-derived factor 1α
SEM	Standard error of the mean
SIS	Skin immune system
TBX21	T-box 21
T_C	$CD8^+$ cytotoxic T cell
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EFF}	Effector T cell
T_{EM}	Effector memory T cell
T_{EMRA}	Terminally differentiated effector memory T cell
TEWL	Trans-epidermal water loss
T_H	$CD4^+$ T helper cell
T_M	Memory T cell
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
T_{reg}	Regulatory T cell
T_{RM}	Resident memory T cell
ULBP	UL16-binding protein
VACV	Vaccinia virus
ZAP70	Zeta-chain-associated protein kinase 70

Chapter One: Introduction

The skin provides a physical barrier to the environment and is exposed to constant challenges. It has key roles in maintaining body homeostasis such as temperature regulation, in maintaining hydration, as a sensory organ, vitamin D synthesis and in certain endocrine functions. The skin also forms a protective barrier to pathogens and environmental challenges. The immune system has adapted to this such that the skin is populated with specialised immune cells that are key to excluding infection and maintaining tissue integrity. Loss of this protective function, as seen in chronic immunosuppression, is associated with an increase in cutaneous infection and malignancy (Hogewoning et al., 2001; O'Reilly Zwald and Brown, 2011; Ramsay et al., 2002). However, maintaining tissue homeostasis is a fine line between health and disease, with inappropriate activation of the skin immune system associated with disease processes, such as allergy, psoriasis and atopic dermatitis (Nestle et al., 2009a).

The vertebrate immune system has evolved to sense microbial challenges that may develop into pathological infections. Specialized myeloid cells, such as dendritic (DCs) cells, detect microbial products through innate pattern recognition receptors (PRRs) (Janeway, 1989; Medzhitov and Janeway, 2000). This results in their rapid local activation, the recruitment other immune cells, and the subsequent migration of DCs to local lymphoid tissues (Banchereau and Steinman, 1998). In lymphoid tissues they present processed microbial peptides, as antigens, to T (thymus-derived) lymphocytes that bear a highly diversified pool T cell receptors (TCR), generated by productive somatic rearrangement of TCR genes during T cell development. T cells recognize a wide range of potential antigens, and upon recognition of their cognate antigen become activated – leading to their clonal expansion and differentiation. Intracellular antigens are mostly bound to major histocompatibility (MHC) class I (MHC-I) molecules and activate $CD8^+$ cytotoxic T cells (CTLs), whereas extracellular antigens presented bound to MHC class II activate $CD4^+$ T-helper cells. Upon activation-dependent differentiation, T cells acquire distinct effector functions and undergo migrational imprinting, which permits their homing to the peripheral site of infection, leading to an antigen-specific "adaptive" immune response and eradication of the pathogen. On resolution of the infection, some lymphocytes

persist as memory cells, which enables a rapid and effective immune response on re-exposure to the same pathogen.

Much of the work to describe the adaptive immune system has been undertaken in lymphoid tissues, which has shaped our understanding of tissue immunobiology. However, by studying tissue-resident lymphocytes, such as in the skin, it is now appreciated that following microbial challenges a distinct population of tissue-resident memory T (T_{RM}) cells are generated. These cells have the capacity to elicit effective adaptive immune responses on re-exposure to tissue pathogens within the periphery, independent of the circulating memory T cell compartment.

In addition, the view that tissue-resident T cells are only involved in the effector arm of the adaptive immune response has been challenged by the concept of the lymphoid stress surveillance (Hayday, 2009). The lymphoid stress surveillance response (LSSR) considers that certain lymphocytes have the capacity to directly interact with epithelial or stromal cells to recognise tissue dysregulation ("stress"). This leads to the rapid and synchronous activation of these "unconventional" lymphocytes, independent of MHC-restricted activation, resulting in direct tissue repair and cross talk with components of the adaptive immune response (Hayday, 2009; Strid et al., 2011). Although this mode of immune activation has been described in murine epithelial tissues, such as the epidermis, there may be a tissue-resident T cell compartment in the human skin that has the capacity to directly sense danger "signals" in dysregulated tissues, and to participate in the early immune responses compatible with LSSR. Addressing this uncharacterised aspect of human skin immunobiology will be the focus of this thesis.

In considering such innate-like T cell biology, I shall first consider the established paradigm of adaptive immunity in the skin, with the establishment of tissue-resident immunological memory. I will then address alternate modes of immune activation in the tissues, largely focusing on prototypic innate-like lymphocytes, and then consider key cells that may mediate such responses in human skin.

1.1. The skin immune system

The skin is a large and complex organ – for an average adult human it has an area of approximately 1.8 m^2. The skin is composed of various different cellular components that meet the diverse functions demanded of it. This includes many different cells that have specialised immune functions. It has been claimed that in healthy human skin there are approximately one million lymphocytes per square centimetre, which equates to around 20 billion lymphocytes in the entire skin of an adult (Clark et al., 2006a). This is twice the number of lymphocytes found circulating in the blood, highlighting that skin-resident immune cells form a major part of the human immune system.

The outermost layer of the skin is the epidermis, which is a stratified squamous epithelium composed mainly of keratinocytes. Human keratinocytes differentiate from epidermal stem cells that are in the stratum basale to form the outermost stratum corneum, which is composed of flattened dead keratinocytes known as corneocytes (Figure 1.1). Keratinocytes are firmly attached to each other through tight junctions and desmosomes. As they differentiate through the layers of the epidermis their physical and biochemical properties change, such that they form the primary hydrophobic barrier to the external environment (Madison, 2003).

Additional specialised cell types are found in the basal layers of the epidermis that include; melanocytes, which produce melanin skin pigments; neuroendocrine Merkel cells; and immune cells, which include Langerhans cells (LCs) and T cells (Bos et al., 1990; 1987; Foster et al., 1990). LCs form a highly dendritic network throughout the epidermis, with approximately 45 thousand cells per square centimetre (Berman et al., 1983; Girolomoni et al., 2002; Steinman et al., 2003). Conversely, T cells are rare in the human epidermis and account for just 2 % of all skin-resident T cells in healthy tissue (Bos et al., 1987). Human epidermal T cells predominantly express an αβ TCR and are $CD8^+$ ($CD8^+$ αβ T cells) (Bos et al., 1987; Foster et al., 1990; Spetz et al., 1996). γδ TCR-bearing cells (γδ T cells) are also found in the epidermis, and may make up approximately 25 % of the human epidermal T cell compartment (Bos et al., 1990; Foster et al., 1990; Groh et al., 1989).

The human dermis is much thicker than the overlying epidermis but is more sparsely populated with cells. Despite this, the dermis contains a wide range of cell types and tissues. It is a structurally important, as the dermis is largely composed of complex proteins such as collagen and elastin that form bundles embedded in the dermal extracellular matrix. These components are produced and maintained by dermal fibroblasts. The dermis also contains an extensive network of cutaneous nerves and appendageal structures, such as hair follicles and sweat glands. It is also supplied with an extensive network of blood and lymphatic vessels, which maintain the oxygenation and nutrition of the skin and allow the migration of immune cells to and from the tissue.

The human dermis contains the vast majority of skin-resident immune cells (Figure 1.1). These include T lymphocytes, natural killer (NK) lymphocytes, innate lymphoid cells and B lymphocytes; and myeloid DCs, macrophages and mast cells (Nestle et al., 2009a). Dermal immune cells have an anisotropic distribution – in the superficial dermis (<60 μm from the dermal-epidermal junction) lymphocytes, DCs and macrophages form an extensive interstitial immune network, whereas in the deeper dermis they localise around appendageal and vascular structures (Bos et al., 1987; Wang et al., 2014). In the healthy dermis the majority of T cells are αβ T cells, with an equal representation of $CD4^+$ T-helper (T_H) and $CD8^+$ T cells (T_C) of multiple subtypes, including $FOXP3^+$ regulatory T cells (Bos et al., 1987; Clark and Kupper, 2007; Clark et al., 2006a; Hijnen et al., 2012). Additional human resident dermal lymphocytes include γδ T cells, NK cells, and other innate lymphoid cells, which are less common than αβ T cells and contribute approximately 5 %, 10 % and <5 % of the dermal lymphocyte compartment, respectively (Bos et al., 1990; Clark et al., 2006a; Ebert et al., 2006; Kim et al., 2013).

Many functional studies of the skin immune system have been undertaken using mouse models. Although many aspects of mouse and human skin anatomy are comparable, there are key differences (Gudjonsson et al., 2007). The murine epidermis and dermis are much thinner than in human skin, and mice have a much greater hair follicle density. In murine skin there is also an additional layer of subcutaneous muscle called the panniculus carnosus. These physical differences

may, respectively, lead to higher cell turnover, protection from environmental insults and wound contraction after injury – factors that may influence the response of murine skin to cutaneous challenges (Di Meglio et al., 2011). In addition, there is a striking difference in murine skin-resident T cells. Murine epidermis contains a prominent population of T cells that are highly dendritic in morphology and form close contacts with LCs and keratinocytes. These cells are called dendritic epidermal T cells (DETC) and express a γδ TCR (Koning et al., 1987; Kuziel et al., 1987; Stingl et al., 1987a; 1987b).

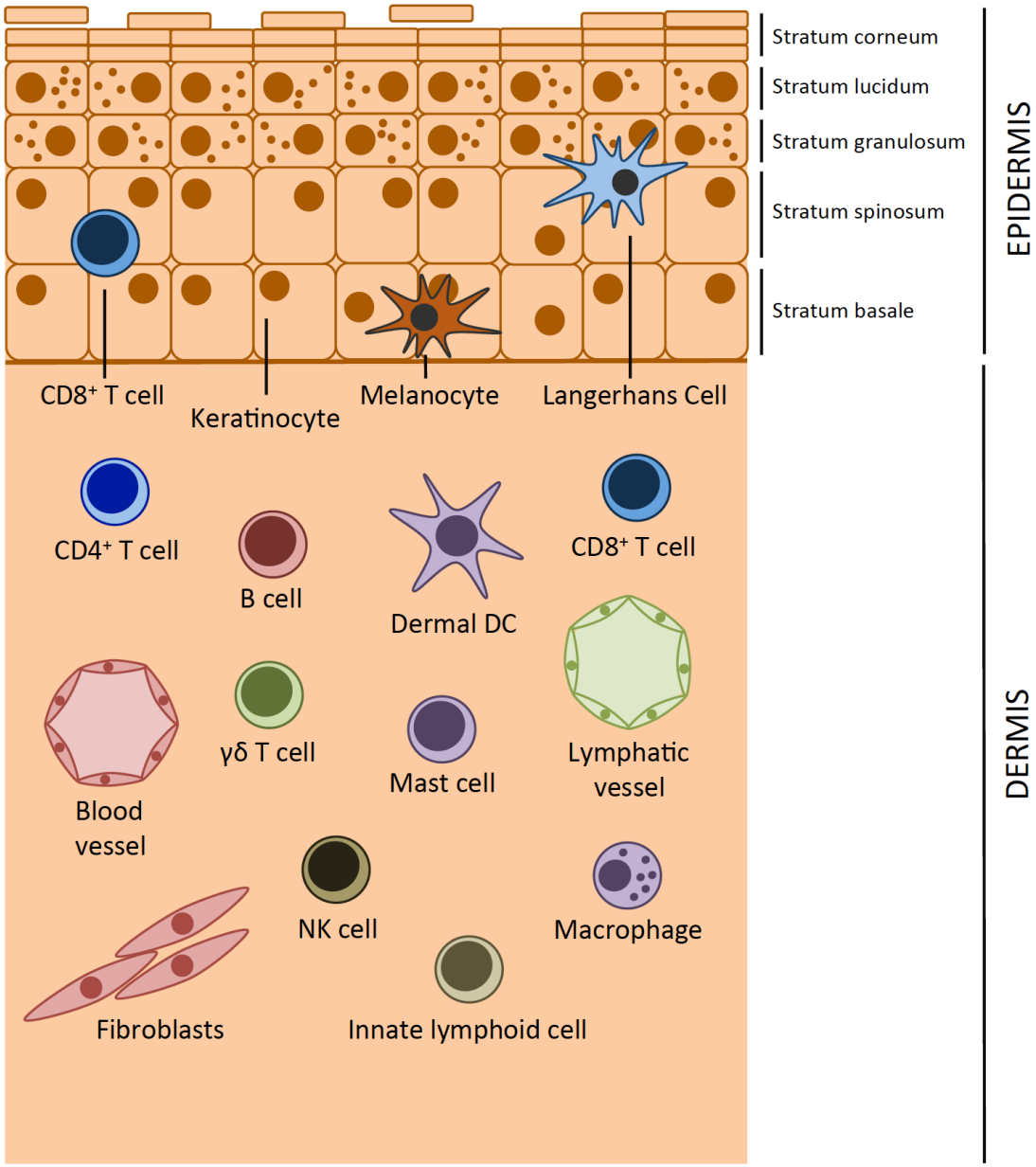

Figure 1.1. Human skin contains many immune cells that have key roles in tissue homeostasis

The skin contains many cells with specialised immune functions that include, αβ T cells, γδ T lymphocytes, NK lymphocytes, innate lymphoid cells and B lymphocytes; and LCs, dermal DCs, macrophages and mast cells (adapted from Nestle *et al.* (2009a)).

1.2. The skin as a model of adaptive immunity

It has been appreciated for many years that immune cells present in the skin play key roles in the surveillance of infections and cancer. The concepts of "skin associated lymphoid tissue" (SALT) and the "skin immune system" (SIS) were proposed approximately 30 years ago (Bos and Kapsenberg, 1986; Streilein, 1983). Although these concepts have continued to evolve, they have already established the paradigm of cutaneous adaptive immunity. Through studies in mice and humans, often using models of infection or cutaneous allergy (contact hypersensitivity), it has been demonstrated that the skin contains specialised cells, such as DCs, that can innately sense danger signals in the skin that may indicate challenges such as infection (Nestle et al., 2009a). Recruited αβ T cells subsequently mediate effector responses, with some lymphocytes persisting within the skin on resolution of the challenge. These cells can mount effective immune responses to future challenges, but have also been implicated in the pathology of certain inflammatory skin conditions.

1.2.1. Skin-resident DCs initiate adaptive immune responses

Skin-resident DCs act as "professional APCs" and can be subdivided by their anatomical location within the skin – LCs are found within the epidermis and dermal DCs in the dermis. Skin-resident DCs are conventionally considered immunogenic, with an important role in initiating adaptive immune responses following cutaneous challenges (Girolomoni et al., 2002).

LCs form an extensive network in the suprabasal layers of the epidermis of both mouse and human skin, and it was following the demonstration that they expressed MHC Class II, Fc and C3 receptors that their potential role as professional APCs was appreciated (Klareskog et al., 1977; Rowden et al., 1977; Stingl et al., 1977). LCs have a highly dendritic morphology, extending their dendrites apically between keratinocytes, which enables them to sample the external environment and take up antigens (Kissenpfennig et al., 2005; Kubo et al., 2009; Ouchi et al., 2011). LCs also express a range of PRRs that innately recognise conserved pathogen-associated molecular patterns (PAMPs). These PRRs include toll-like receptors (TLRs) that bind microbial lipids, lipoproteins, proteins and nucleic acids, leading to a diverse

range of pro-inflammatory effects (Medzhitov and Janeway, 2000). LCs are thus rapidly activated by innate stimuli, such as microbial products or the cytokines interleukin (IL) 1β, IL18 and tumour necrosis factor (TNF) α, which results in their migration to skin-draining lymph nodes (Cumberbatch et al., 1997; 2001; Flacher et al., 2006; Peiser et al., 2008; Stoitzner et al., 1999). Upon activation, LCs also down-regulate the expression of E-cadherin, which facilitates their migration away from the epidermis, and they upregulate the secondary lymphoid-homing marker CCR7 (Jakob and Udey, 1998; Saeki et al., 1999). Activated LCs also have increased expression of MHC class II proteins and co-stimulatory molecules that are required for T cell priming (Jakob and Udey, 1998; Kissenpfennig et al., 2005; Stoitzner et al., 2003; Witmer-Pack et al., 1987). *In vitro* LCs have the capacity to activate naïve T cells in a MHC-dependent manner, both activating $CD4^+$ T cells and cross-presenting antigen to $CD8^+$ CTLs (Klechevsky et al., 2008; Romani et al., 1989; Schuler and Steinman, 1985; Stingl et al., 1978; Stoitzner et al., 2006).

These data helped established the "LC paradigm", whereby LCs act as prototypic professional APCs that could sample and process antigen following infection in the peripheral tissues and migrate to draining lymph nodes to prime naïve T cells (Girolomoni et al., 2002). However, this has been challenged by studies *in vivo* that indicate LCs may also have an immune-regulatory role. For example, in certain murine models of contact hypersensitivity, the loss of LCs either had no effect on allergic responses or amplified certain immune responses (Kaplan et al., 2005; Kissenpfennig et al., 2005). In addition, in certain models of skin infection (both viral and protozoan), LCs have a less prominent role than other skin-resident DC subsets in eliciting T cell responses (Allan et al., 2003; Bedoui et al., 2009; Brewig et al., 2009).

The dermis contains a heterogeneous group of DCs that are highly mobile and constantly circulating through the tissue (Ng et al., 2008). In mice, the main dermal DC population expresses CD11b but is CD103-negative ($CD11b^{hi}CD103^-$), but there are also $CD11b^{lo}CD103^+Langerin^+$ and $CD11b^-CD103^-$ subsets (Bursch et al., 2007; Ginhoux et al., 2007; Malissen et al., 2014; Poulin et al., 2007; Tay et al., 2013). Human dermal DCs are broadly classified into 3 subsets based on their expression of CD1c, CD14 and $CD141^{hi}$ (Haniffa et al., 2015). In response to microbial stimuli,

dermal DCs are rapidly activated to undergo terminal differentiation; upregulate their expression of MHC and co-stimulatory molecules; and are mobilised from the skin, arriving at skin-draining lymph nodes within 48 hours (Kissenpfennig et al., 2005; Malissen et al., 2014; Shklovskaya et al., 2008). Here, dermal DCs promote the clonal expansion of naïve antigen-specific T cells leading to their acquisition of effector T cell functions (Kissenpfennig et al., 2005; Malissen et al., 2014; Shklovskaya et al., 2008).

Although the functional specialisation of dermal DC subsets is largely unknown, it may be that certain subsets are activated under different conditions to drive different effector T cell responses. In murine experiments *in vivo*, using both infective and allergic challenges, it has been established that $CD103^+$ DCs have a key role in the induction of both $CD4^+$ T_H1-type responses, and antigen cross-presentation to elicit a $CD8^+$ CTL response (Brewig et al., 2009; Henri et al., 2010; Igyártó et al., 2011). Human $CD141^{hi}$ dermal DCs are also highly efficient at cross-presenting antigen to $CD8^+$ T cells, and they are considered functionally equivalent to murine $CD103^+$ DCs (Haniffa et al., 2012). Conversely, certain murine $CD103^-$ dermal DCs can efficiently present antigen to $CD4^+$ T cells to induce T_H2 responses (Kumamoto et al., 2013; Murakami et al., 2013).

1.2.3. Skin-resident αβ T cells are effectors of the adaptive immune response

αβ T cells are considered effector cells of the adaptive immune response and show highly specialised functions, with multiple different subtypes described, characterised by their distinct effector (cytokine) responses (Iwasaki and Medzhitov, 2015). Certain T cell responses are associated with different types of microbial challenge, such as $CD8^+$ CTL and $CD4^+$ T_H1 responses to intracellular bacteria, protozoa and viral infection characterised by cytotoxic degranulation and interferon (IFN) γ production; T_H2 responses to helminth infections characterised by IL-4 and IL-13 production; and T_H17 responses to extra-cellular bacteria and fungal pathogens characterised by IL-17 production (Iwasaki and Medzhitov, 2015).

During activation-dependent differentiation in the lymph node, T cells alter their expression of tissue-homing receptors. In mice, E-selectin ligands (ESL) and P-selectin ligands (PSL) facilitate the homing of activated lymphocytes to the skin, which bind key selectin adhesion molecules expressed on the endothelial surface of inflamed dermal blood vessels (Campbell and Butcher, 2002; Tietz et al., 1998). In humans the counter-part skin-homing receptors include cutaneous leukocyte antigen (CLA), which binds E-selectin, and chemokine receptor (CCR)4, which binds both chemokine ligand (CCL)17 and CCL22 (Berg et al., 1991; Campbell et al., 1999; Fuhlbrigge et al., 1997; Picker et al., 1990).

On clearance of the acute challenge most effector T (T_{EFF}) cells will apoptose with a subsequent contraction of the lymphocyte population; however, a heterogeneous subset of "memory" T cells (T_M) will persist. T_M cells studied in the blood and lymphoid tissues have been divided into "central memory" (T_{CM}) and "effector memory" (T_{EM}) subsets based on their expression of secondary lymphoid homing markers (Sallusto et al., 1999). T_{CM} cells express both CCR7 and CD62L (L-selectin), which bind molecules expressed on the vascular endothelium of lymph nodes and permits T_{CM} to traffic between secondary lymphoid organs and the circulation (Sallusto et al., 1999). T_{EM} lose CCR7 and CD62L expression and thus show limited trafficking between the circulation and the periphery. In keeping with their distinct anatomical localisation and migratory capacity, T_{CM} and T_{EM} differ in their functional response upon re-activation; T_{CM} actively proliferate and produce the T cell growth factor IL-2, whereas T_{EM} show limited proliferative capacity but produce multiple effector cytokines (Sallusto et al., 1999).

Studies of tissues have identified a third population T_M that persist in the tissue after resolution of the primary challenge. Tissue-resident memory (T_{RM}) lymphocytes show limited capacity to migrate out of the tissue, and confer effective immunological memory upon re-exposure to microbial challenges (Figure 1.2) (Gebhardt et al., 2012; Jiang et al., 2012; Masopust, 2001; Schenkel et al., 2013). Studies to characterise the function of T_{RM} cells have been performed primarily in mice, with most focusing on $CD8^+$ αβ T cells. T_{RM} populations have been identified in multiple non-lymphoid tissues that include the skin, gut, lung, brain and reproductive tract (Jiang et al., 2012; Masopust, 2001; Masopust et al., 2010;

Schenkel et al., 2013; Wakim et al., 2010). There is compelling evidence that equivalent T_{RM} populations exist in human tissues, including the skin.

1.2.4. T cell memory within the skin

In murine models of cutaneous infection with vaccinia virus (VACV), $CD8^+$ T_{RM} cells were shown to be rapidly recruited to the skin, dependent on the skin-homing markers PSL and ESL, and persisted for at least 6 months following resolution (Jiang et al., 2012). On re-infection of the skin with VACV, following treatment with a sphingosine-1-phosphate inhibitor that prevents T_{CM} cell egress from lymph nodes, skin-resident $CD8^+$ T_{RM} cells alone could rapidly and effectively clear the virus. Conversely, using a parabiotic mouse model, the conjoined infection-naïve mouse that gained only a T_{CM} compartment showed a delayed response to cutaneous VACV infection with impaired viral clearance. Comparable T_{RM} responses have also been shown following cutaneous immunisation with protein antigen, chemical haptens and other viral pathogens, such as the poxvirus modified Vaccinia Ankara (Gaide et al., 2015). In these studies, using high-throughput sequencing of the gene encoding the TCR-beta (*TRB*) chain complementary determining region (CDR)3, the authors were able to demonstrate identical TCR clones in both the skin-resident T_{RM} and lymph node-derived T_{CM} populations, indicating that these cells shared the same naïve T cell precursor (Gaide et al., 2015).

T_{RM} are also characterised by their expression of additional distinct cell surface markers that are thought to be associated with their function. Expression of the C-type lectin CD69 is conventionally considered a marker of early lymphocyte activation; however, expression persists and may play a role in lymphocyte egress from secondary lymphoid organs and T_{RM} retention in the skin by antagonising the function of sphingosine-1-phosphate (S1P) receptor $S1P_1$ (Mackay et al., 2015; Schenkel and Masopust, 2014; Shiow et al., 2006). Expression of the integrin CD103 ($\alpha e\beta 7$) has also been associated with the migration of murine T_{RM} into epithelial tissues, including the epidermis (Jiang et al., 2012; Masopust et al., 2006; Schenkel and Masopust, 2014).

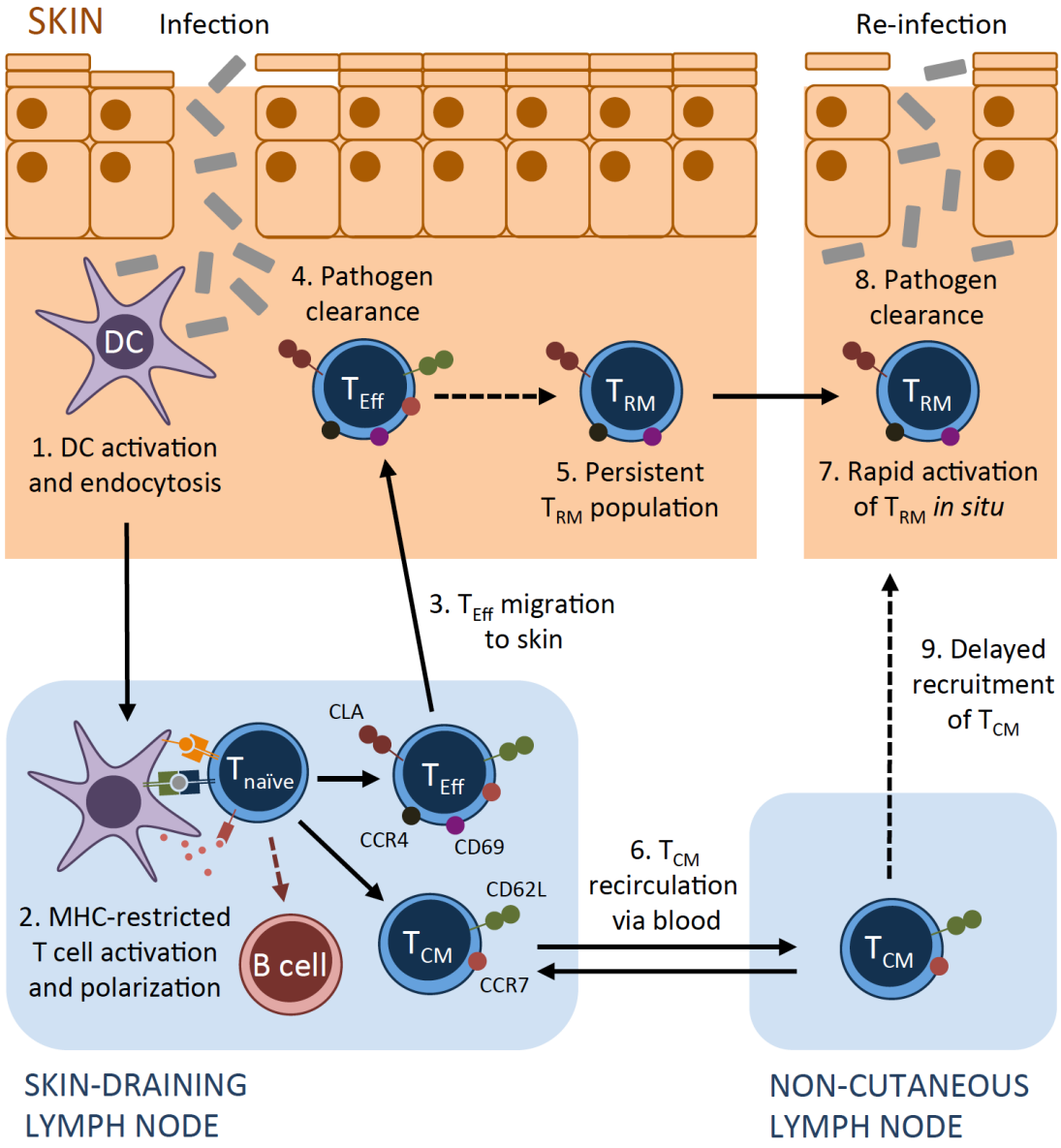

Figure 1.2. Skin as a model of adaptive immunity

(1) Following skin infection activated skin-resident DCs migrate to regional lymph nodes, where they present cognate antigens, in association with MHC, to naïve T cells (2). This leads to the activation and polarisation of T_{EFF} cells that upregulate activation and skin-homing markers. (3) T_{EFF} cells migrate to the skin where they orchestrate a targeted adaptive immune response against the pathogen (4). (5) On resolution of the infection, some T cells lose secondary lymphoid-homing markers and persist in the skin as T_{RM}. (6) T_{CM} cells circulate between lymphoid and peripheral tissues. (7) On re-challenge by the same pathogen, T_{RM} cells are rapidly activated locally to mount an effective immune response (8). (9) T_{CM} cell activation occurs but recruitment to the tissue is slower.

Although T cells will localise primarily to the peripheral site of infection, T_{RM} also populate distant sites throughout the skin – albeit less effectively than at the site of primary challenge (Jiang et al., 2012; Mackay et al., 2012). However, repeated exposure to the primary infection increases the number of T_{RM} found at distant sites, where they can confer some memory upon re-infection (Jiang et al., 2012). In addition to eliciting an antigen-specific response, $CD8^+$ T_{RM} re-activation following infection also leads to the induction of broadly active antiviral and antimicrobial genes, initiating a general anti-microbial response within the skin (Ariotti et al., 2014). T_{RM} activation in other tissues is associated with the rapid recruitment of non-specific memory $CD8^+$ T cells from the blood and initiation of other innate immune responses (Schenkel et al., 2013; 2014). In a model of cutaneous herpes simplex virus (HSV) infection, it has been shown that $CD4^+$ T_{RM} can also be found in the skin (Gebhardt et al., 2012). Interestingly, in this model, $CD8^+$ and $CD4^+$ T_{RM} cells showed distinct migration patterns; $CD8^+$ T_{RM} cells localised to the epidermis and were sessile, whereas $CD4^+$ T_{RM} cells localised to the dermis but were more dynamic, trafficking through the tissue and showed some recirculation *via* the blood (Gebhardt et al., 2012).

Thus, mouse models have demonstrated that the accumulation of antigen-experienced T_{RM} cells in the skin clearly forms an important immune compartment for future challenges to the tissue. Along with the discovery that healthy human non-lymphoid tissues contain large T cell populations, this has revised our view of T cell responses in human skin.

1.2.5. T_{RM} in human skin health and disease

In normal healthy skin there is a large number of resident lymphocytes, many of which have features of T_{RM} cells. αβ T cells isolated directly from normal human skin highly express the skin-homing markers CLA and CCR4 (Clark et al., 2006b). Although some CLA^+ lymphocytes are found in the blood, it has been estimated that 98 % of CLA^+ cells are located in the skin (Clark et al., 2006b). Most skin-resident αβ T cells express the activation marker CD45RO, and no longer co-express CCR7 and CD62L (Clark et al., 2006b; Spetz et al., 1996). This indicates that the skin

contains a large population of skin-homing memory αβ T cells that have a limited capacity to re-circulate, especially *via* secondary lymphoid organs. Human skin-resident T cells also express additional markers associated with T_{RM}, such as CD69 and CD103 (Clark et al., 2006a; Watanabe et al., 2015). Comparable populations of tissue-tropic αβ T cells expressing these T_{RM} markers have also been isolated from other human tissues, including the lung and gastrointestinal tract (Purwar et al., 2011; Sathaliyawala et al., 2013).

The differential migratory properties of human skin-homing αβ T cells have been demonstrated in studies of cutaneous T cell lymphoma (CTCL), which are a group of non-hodgkin lymphomas. Mycosis fungoides (MF) is the most common CTCL and presents with discrete inflammatory lesions in the skin. Sézary syndrome (SS) is a leukemic variant of CTCL, which results in ill-defined erythematous patches of skin, with malignant T cells found in the blood and lymphoid tissues. In a study of low-dose systemic alemutuzumab (an anti-CD52 monoclonal antibody) in CTCL, good responses were seen in patients with SS but it was ineffective in MF (Clark et al., 2012). Alemtuzumab depleted $CD52^+$ leukocytes by antibody–dependent cellular cytotoxicy (ADCC) within the circulation. Sézary cells expressed both skin-homing adressins (CLA and CCR4) and secondary lymphoid-homing markers (CCR7 and CD62L), thus representing malignant clones of skin-homing T_{CM} cells. In these patients all circulating T_{CM} cells, including Sézary cells, were depleted by alemtuzumab when recirculating through the blood (Clark et al., 2012). Conversely, malignant cells in MF cells were CLA^+ but $CCR7^-CD62L^-$. The lack of response to alemtuzumab in MF indicated that these cells failed to recirculate through the blood, consistent with malignant cells of T_{RM} origin (Clark et al., 2012). In keeping with this, MF is most effectively managed with skin-targeted therapies, such as potent topical steroids or low-dose skin-targeted irradiation (Trautinger et al., 2006).

Demonstrating the contribution of human skin-resident T_{RM} cells towards protective immunological memory has been experimentally more difficult. In a study of human HSV infection, a subset of specialised $CD8^+$ T cells, which express the CD8αα homodimer, were found to persist at the dermal-epidermal junction at sites of previous HSV-2 infection (Zhu et al., 2007; 2013). These cells, isolated by laser capture microdissection and analysed by transcriptomic profiling, were shown to

lack chemokine receptors required for secondary lymphoid homing and to have an effector phenotype, with increased expression of IFNγ, TNFα and granzyme genes (Zhu et al., 2013). In addition, in biopsies where there was evidence of subclinical HSV reactivation, cytotoxic $CD8^+$ T cells were clustered around infected keratinocytes with significantly lower levels of HSV DNA detected – implying early immune containment of the infection. In patients where sequential skin biopsies were taken, using high-throughput sequencing of *TRB* gene CDR3 regions, there was evidence that overlapping dominant clonotypes persisted at times of infection and resolution, indicating the existence of long-lived T_{RM} cells within the skin (Zhu et al., 2013).

In addition to the potential role of human skin-resident T_{RM} in memory responses to cutaneous infection, there is now evidence that dysregulated T_{RM} may have a role in many common auto-inflammatory skin diseases. In many ways this has been broadly appreciated for years, as a wide range of commonly used topical and systemic therapies target lymphocytes in the skin. However, the specific role of T_{RM} in inflammatory skin diseases, such as allergic contact dermatitis, fixed drug eruptions and psoriasis, is only just emerging.

Exposure to certain contact allergens leads to sensitisation with the subsequent development of an allergic contact dermatitis (ACD) on re-exposure to the allergen. As with murine contact hypersensitivity, ACD was thought to be elicited by the recruitment of antigen-specific T_{CM} cells to the site of cutaneous allergen exposure (Vocanson et al., 2009). However, cutaneous sensitisation leads to the development of T_{RM} cells within human skin. Using similar *TRB* gene CDR3 sequencing methods as used by Zhu *et al*. (2013) in their study of cutaneous HSV infection, following exposure to the contact allergen diphenylcyclopropenone (DPCP), shared TCR clones were found in both DPCP-challenged and placebo-challenged skin, implying the development of T_{RM} at both local and distant site to the primary sensitisation (Gaide et al., 2015). These clones increased over time and persisted for up to 4 months after the initial challenge, mirroring the kinetics of antigen-specific T_{RM} seen in a mouse model of allergic contact dermatitis used in the same study (Gaide et al., 2015) In fixed drug eruptions, oral exposure to a chemical allergen leads to an antigen-specific T cell response in the skin that manifests as discrete inflamed lesion.

Upon resolution a persistent population of $CD69^+$ $CD103^+$ $CD8^+$ T cells can be found limited to the previous site of inflammation (Mizukawa et al., 2002). Re-exposure to the oral allergen leads to rapid activation of epidermal $CD8^+$ T_{RM} cells, with rapid IFNγ induction within 2 – 3 hours of the challenge (Mizukawa et al., 2002).

Although the exact nature of adaptive immune responses, notably key antigens, in many chronic inflammatory skin conditions remains unresolved, there is increasing evidence that much of the pathology is due to the dysregulated activation of T_{RM} within the skin.

Chronic plaque psoriasis is a common chronic auto-inflammatory skin condition that is characterized by the development of well-demarcated, erythematous thickened plaques with scale, which histologically show evidence of T cell infiltration in the dermis and epidermis (Nestle et al., 2009b). The role of T cells as drivers of psoriasis was initially established in therapeutic studies of drugs that specifically targeted T cells; such as a IL-2-diphtheria toxin fusion protein that selectively depleted activated $CD25^+$ T cells (Gottlieb et al., 1995). These treatments lead to a striking improvement in clinical disease but also a significant reduction in T cells seen at the site of psoriatic plaques (Gottlieb et al., 1995). In contrast there was a surprising lack of response to an anti-E selectin monoclonal antibody that blocked T cell homing to the skin (Bhushan et al., 2002). This indicated that in psoriasis blocking T cell migrating to the skin was insufficient to prevent disease, and that targeting T cells within the tissue was paramount.

A further indication that T_{RM} cells could initiate psoriasis was made following the observation that transplantation of uninvolved skin from patients with psoriasis to immune-deficient mice lead to the development of spontaneous psoriatic lesions in the transplanted tissue (Boyman, 2004). Thus, transplanted T_{RM} cells alone could establish disease. In the same model, blocking T cell effector function with anti-TNFα treatment was sufficient to prevented the formation of psoriatic lesions (Boyman, 2004). One striking feature of psoriasis is that plaques often preferentially recur at sites of previous inflammation, with the same distinct well-demarcated morphology. This may in part be supported by the concept of pathological skin-

resident T_{RM}. At sites of resolved plaques (following treatment with etanercept, a TNFα receptor fusion protein) disease-related genes, including those for T cell proteins, cytokines and chemokines, can remain upregulated for up to 3 months (Suárez-Fariñas et al., 2010). In addition, increased disease-associated IFNγ- and IL17-producing T cells have been found in the uninvolved skin of patients with psoriasis, which may indicate the widespread dissemination of pathogenic T_{RM} (Hijnen et al., 2012).

Many other inflammatory dermatoses, such as chronic atopic dermatitis, share clinical features with psoriasis, and it has been speculated that this may indicate a that skin-resident T_{RM} also contribute to these processes (Clark, 2015). Such features include the recurrence of inflammatory pathology at the same anatomical sites, discrete and well-demarcated lesions, rapid onset of inflammation and increasing severity of the rash with increasing exposure (Clark, 2015).

Thus, there is compelling evidence that the skin, following repeated microbial and environmental challenges, becomes progressively populated with T_{RM} cells, which act as key effectors of adaptive immune responses. Human skin contains key components of the adaptive immune system, and harbours a major αβ T cell population that has features of T_{RM} cells. However, additional immune cells are present in the skin, which include certain "unconventional" lymphocytes such as γδ T cells, and their contribution to human skin health and disease is by contrast very poorly understood.

1.3. Innate-like lymphocytes in the skin

A model of skin immunobiology has been formed whereby myeloid cells innately sense challenges to the skin, such a microbial infection, to initiate αβ T cell responses. However, such a model largely overlooks the direct role of tissue cells in the immune response – especially as these cells form the bulk of barrier tissues such as the epidermis, and are often the primary site of infection or transformation. In addition, barrier tissues often contain conspicuous intra-epithelial lymphocytes (IEL) that include populations of "unconventional" or "innate-like" lymphocytes (ILL).

ILL have certain features that make them suited to a role in primary immune-surveillance; they are enriched within barrier tissues and show "unconventional" modes of MHC-unrestricted activation – which enable them to directly sense tissue dysregulation and to initiate rapid effector responses (Hayday, 2009). Although ILL are T cells and express a TCR, they do not show the same diversity as "conventional" naive αβ T cells, expressing a limited repertoire of TCRs (Hayday, 2009). Much ILL biology has been informed by studies on murine γδ TCR^+ IEL in the skin and gut. The human epidermis lacks such a conspicuous IEL compartment, with most T cells located in the dermis; however, the description of murine skin-resident ILL raises the possibility that functionally equivalent lymphocytes exist within human skin.

In addition to γδ T cells, other "unconventional" lymphocyte subsets exist, which are also found in the blood and tissues of mice and humans. These include natural killer T (NKT) cells and mucosal-associated invariant T (MAIT) cells, which are characterized by their expression of a limited repertoire/invariant αβ TCRs that can be engaged in a MHC-unrestricted manner, leading to lymphocyte activation (Gober et al., 2007; Mattarollo et al., 2010; Teunissen et al., 2014b; Van Kaer et al., 2015; Willberg, 2014). Innate lymphoid cells, which include NK cells, also show unconventional modes of activation, by either natural cytotoxicity receptors (NCRs) or cytokine stimulation. As these cells do no express classical antigen receptors, such as the TCR, they do not have the capacity to be regulated by "checkpoints" that are central to T cell development. Therefore, their activation is considered truly innate (Artis and Sonnenberg, 2015; Kim, 2014; Lanier, 2005; Spits et al., 2013).

1.3.1. Murine epidermal γδ T cells are prototypic ILLs

γδ T cells are distinct from αβ T cells as they express a TCR that is a heterodimer of the alternative γ and δ TCR chains, which defines them as a second type of T cell that has been conserved during evolution (Hayday et al., 2001). Mouse skin contains several distinct populations of γδ T cells that localise to different anatomical compartments within the skin. Epidermal γδ T cells are considered prototypic ILL, with dermal γδ T cells showing certain innate-like features (Hayday, 2009).

During early embryogenesis, following γδ T cell development in the thymus, rather than migrating to lymphoid tissues like naïve αβ T cells, most γδ T cells home directly to the periphery where they associate with barrier tissues (Carding and Egan, 2002). This is true of murine epidermal γδ T cells, which are the first γδ TCR^+ population to appear in the embryonic thymus at days 14 – 15 of gestational life before migrating to the epidermis by days 17 – 19 of gestation (Havran and Allison, 1988). In the epidermis they form a major immune cell network, in close contact with LCs and keratinocytes, and because of their highly dendritic morphology they are termed dendritic epidermal T cells (DETC) (Koning et al., 1987; Kuziel et al., 1987; Stingl et al., 1987a; 1987b). Despite having the potential to form extensive TCR diversity, murine γδ T cells express a limited repertoire of TCRs encoded by specific Vγ and Vδ segments, and the vast majority of DETC express the monoclonal $V\gamma5V\delta1^+$ TCR (Asarnow et al., 1988; Carding and Egan, 2002) (using Heilig and Tonegawa nomenclature (Heilig and Tonegawa, 1986)). Similarly, γδ T cells in the dermis express clonal or oligoclonal γδ TCR repertoires, with additional clonal/oligoclonal γδ T cell populations found in the intestine, lung and reproductive tract (Itohara et al., 1990).

The development of distinct clonal/oligoclonal γδ T cells within specific tissues is not compatible with the concept of adaptive immunity, where TCR diveristy enables an immunogenic response to a range of antigens through the selection of rare T cell clones following MHC-peptide interactions. This indicates an alternative selection pressure whereby γδ T cells locate to specific sites with a pre-programmed capacity to recognize either conserved pathogen-encoded antigens or self-encoded molecules that may indicate tissue dysregulation (Hayday, 2009).

1.3.1.1. DETC development is TCR-dependent and enables their skin-localization

αβ T cells undergo a complex period of MHC-restricted selection in the thymus during a "double-positive (DP) stage" when they co-express CD4 and CD8. However, γδ T cells do not pass through the DP stage, which indicates that they undergo an alternative selection process in the double-negative (DN) stage.

Vγ5Vδ1$^+$ T cell thymic selection is dependent on a tissue-specific epithelial determinant, which supports the concept that an alternative selective pressure drives the localization of DETC to the epidermis. It was noted in a specific FVB mouse strain ("Tac mouse") that there is a severe and specific loss of the Vγ5Vδ1$^+$ DETC compartment, which was replaced with γδ T cells expressing atypical γδ TCR heterodimers (Lewis et al., 2006). In the "Tac mouse" Vγ5Vδ1$^+$ thymocytes failed to mature properly, which was dependent on interactions with thymic epithelial cells and restored with TCR-mediated activation (Lewis et al., 2006). Genetic analysis of the "Tac mouse" identified a premature stop mutation in the protein Skint-1, which is a member of the butyrophilin superfamily of immunoglobulin (Ig)-like molecules (Abeler-Dorner et al., 2012; Barbee et al., 2011; Boyden et al., 2008). Skint-1 is strikingly tissue-specific, being expressed only in the thymus and epidermis (Barbee et al., 2011; Boyden et al., 2008; Lewis et al., 2006). When Skint-1 was reintroduced in "Tac mice" it restored Vγ5Vδ1$^+$ thymocyte development with normal migration of DETC to the epidermis (Barbee et al., 2011).

Additional factors are also important for the development and peripheral function of DETC. During thymic development, DETC also undergo migrational imprinting that facilitates their localization to the epidermis, dependent on E/P-selectin and CCR4 (Jiang et al., 2010). Once established in the epidermis, DETC form a sessile long-lived population capable of self-renewal, and they are not replenished from the bone marrow in adult mice (Honjo et al., 1990). DETC are also dependent on both IL-7 and IL-15, with loss of DETC seen in respective knock-out mice (De Creus et al., 2002; Edelbaum et al., 1995; Moore et al., 1996). However, the self-ligand Skint-1 defines a novel direct axis of communication between Vγ5Vδ1$^+$ T cells and epithelial cells. Although there is currently no experimental evidence demonstrating

that Skint-1 directly engages the γδ TCR, the end results of its activity are analogous to the TCR-specific selection of αβ T cells by MHC-peptide complexes. Thus Skint-1 represents a novel epithelial determinant of DETC development and peripheral function.

1.3.1.2. DETC maintain a "pre-activated" state within the skin that may facilitate unconventional modes of activation

It has been shown that γδ T cells from other tissues, such as the gut epithelium, exist constitutively in an "pre-activated" state, which may be acquired during thymic development (Jensen et al., 2008; Pennington et al., 2003; Shires et al., 2001; Silva-Santos et al., 2005). DETC have a highly dendritic morphology and at steady state constitutively interact with the tissue stroma. Vγ5Vδ1$^+$ TCRs are clustered at the tip of these dendrites and are functionally activated, forming immunological synapses that polarize and anchor DETC dendrites to keratinocyte tight junctions (Chodaczek et al., 2012). Within the TCR clusters there are phosphorylated tyrosine residues (termed "phosphorylated tyrosine–rich aggregates located on projections" or PALPs) and PALP formation is dependent on the kinase Lck – which is indicative of constitutive TCR signalling. The epidermis of TCRδ$^{-/-}$ mice is populated with dendritic αβ T cells, that are much less efficient at forming and/or stabilising PALP-like structures (Chodaczek et al., 2012). Despite the indication that the DETC Vγ5Vδ1$^+$ TCR is constitutive active, this fails to elicit full cellular activation or cytokine production (Chodaczek et al., 2012). In fact DETC freshly isolated from the epidermis show highly atypical responses when activated with TCR agonists, such as attenuated intra-cellular calcium flux and IL-2 production (Wencker et al., 2013). DETC may acquire this TCR hypo-responsiveness during thymic selection, as it is not seen in Vγ5Vδ1$^+$ thymocytes prior to maturation (Wencker et al., 2013). The ligand that tonically engages the Vγ5Vδ1$^+$ TCR in the skin has not been determined; however, given the data regarding the role of Skint-1 in thymic selection of DETC, and its limited expression in the epidermis, it can be hypothesized that it is in part responsible.

In response to tissue stress, DETC morphology radically changes with the cells rapidly losing their dendritic form and "rounding up" (Strid et al., 2008). This does not lead to an increase in signals associated with tonic TCR-mediated activation (Chodaczek et al., 2012). Thus the presence of T cells in the epidermis, with clonal TCRs that show constitutive tonic reactivity towards self-ligands, may enable these cells to respond rapidly and directly to states of tissue dysregulation in an "unconventional" manner (Hayday, 2009).

1.3.2. The lymphoid stress surveillance response

The selective focusing of "activated-yet-resting" γδ T cells to the skin places these cells in a primed state to innately respond to states of tissue stress. In response to non-microbial damage, or certain infections, epithelial cells upregulate the expression of "stress ligands". DETC, but not local myeloid cells, express activatory receptors that can bind these "stress ligands", which can result in their rapid and synchronous activation. This innate-like activation of tissue-resident T cells has been termed the lymphoid stress surveillance response (LSSR) (Hayday, 2009), and is important in maintaining tissue integrity but can also influence adaptive immune responses (Girardi et al., 2001; Strid et al., 2011; 2008). This offers a complementary mechanism whereby tissue-resident γδ T cells can innately recognise tissue dysregulation, which may not be sensed by conventional myeloid innate cells, thereby contributing to early afferent immune responses.

1.3.2.1. The NKG2D axis as a model of LSSR

An example of LSSR is the response of lymphocytes to ligands for the activatory natural killer group 2 D (NKG2D) receptor. Murine NKG2D is expressed on NK cells, certain γδ T cells and activated or memory $CD8^+$ αβ T cells; human NKG2D is expressed on NK cells, γδ T cells and $CD8^+$ αβ T cells.

Ligands for the NKG2D receptor are a group of self-encoded MHC-I-like molecules. In mice these proteins include retinoic acid early inducible-1 (RAE-1) proteins, H60 proteins and murine UL16-binding protein-like transcript 1 (MULT1) protein

(Raulet et al., 2013). In humans, the NKG2D ligands include MHC class I chain–related proteins A and B (MICA and MICB) and multiple UL16-binding proteins (ULBP1–6) (Raulet et al., 2013). Murine *RAE1* genes are orthologues of the human *ULBP/RAET1* genes; however, there are no clear murine orthologues of the human *MICA* or *MICB* genes. NKG2D ligands are considered "MHC-I-like" or "unconventional" MHC-Ib molecules, as they are structurally homologous to the "classical" MHC-Ia molecule; however, they have a distorted peptide-binding groove such that their function is not dependent on their association with peptide fragments (antigens) or any other identified cargo (Figure 1.3) (Raulet et al., 2013; Wang et al., 2012). In addition, many of the genes that encode NKG2D ligands are located within or near the MHC complex on chromosome 6.

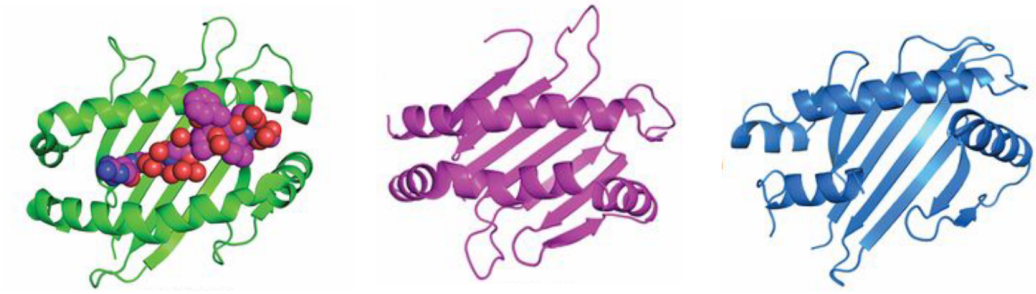

Figure 1.3. NKG2D ligands are MHC class I-like molecules

NKG2D ligands in mice and humans are structurally similar to MHC-I molecules; however, they have a distorted peptide-binding groove such that their expression is not in association with bound peptide. Murine MHC-I molecule H-2D^d shown in complex with peptide P18-I10 derived from human immunodeficiency virus (HIV) envelope glycoprotein 120. (Figure adapted from Wang *et al*. (2012)).

NKG2D ("stress") ligands act as early markers of cellular dysregulation and can be a major source of immunogenicity towards damaged cells. Many different environmental challenges lead to the increased cell surface expression of NKG2D

ligands, including physico-chemical stresses such as physical trauma, heat shock, oxidative stress, osmotic stress or ultraviolet irradiation (Borchers, 2006; Groh et al., 1996; Nice et al., 2009; Strid et al., 2011; Vantourout et al., 2014); certain infections, especially viruses (Draghi et al., 2007); and malignant transformation (Gasser et al., 2005; Girardi et al., 2001; Groh et al., 1999; Strid et al., 2008).

In mice, cytotoxic agents activate DNA damage response pathways, initiated *via* the activation of the ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3 related (ATR) kinases, which can lead to up-regulated NKG2D ligand expression (Gasser et al., 2005). However, NKG2D ligand expression is also associated with conditions in which DNA damage is not a major feature, which indicates that multiple mechanisms regulate the expression of these immunogenic self-peptides. It has been shown in human cells that increased MICA expression, following a range of challenges, was largely regulated through the activation of components of the epidermal growth factor receptor (EGFR) pathway, and was not due to DNA damage (Vantourout et al., 2014). This lead to reduced MICA mRNA degradation and therefore increased MICA cell surface expression, regulated at a post-transcriptional level. Other mechanisms implicated in the regulation of NKG2D ligands include the control of murine Rae1 transcription by the E2F transcription factor (Jung et al., 2012), and the post-transcriptional binding of micro-RNAs to MICA mRNA under certain conditions (Stern-Ginossar et al., 2008).

As previously discussed, the NKG2D receptor is predominantly expressed by NK cells, γδ T cells and $CD8^+$ αβ T cells, and stress ligands for this receptor can be a major source of immunogenicity towards damaged cells. The NKG2D receptor lacks an intracellular activation domain, but associates with the transmembrane signalling adaptor molecules DAP10 or DAP12. In mice there are two isoforms of NKG2D that differ in the length of their intra-cytoplasmic tail. The shorter isoform associates with DAP12, which upon NKG2D engagement signals *via* an immunoreceptor tyrosine-based activation motif (ITAM) to recruit Syk or Zeta-chain-associated protein kinase 70 (ZAP-70); whereas the longer NKG2D isoform can only associate with DAP10, which bears an alternative YxxM motif that activates *via* the phosphatidylinositol 3-kinase (PI3K) and Grb2-Vav1 pathways (Diefenbach et al., 2002; Gilfillan et al., 2002; Ibusuki et al., 2013; Upshaw et al.,

2006; Wu et al., 2000). Humans express only the long isoform of NKG2D, which therefore signals *via* DAP10 (Bauer et al., 1999; Upshaw et al., 2006).

Although there can be a degree of synergy between activatory natural cytotoxicity receptors, NKG2D engagement alone is sufficient to trigger NK cell cytotoxicity (Bauer et al., 1999; Bryceson et al., 2006; Cosman et al., 2001; Groh et al., 1998); whereas, NKG2D primarily acts as a co-stimulatory receptor for $CD8^+$ αβ T cells that require TCR-mediated signalling for full activation (Groh et al., 2001). There is controversy over whether certain T cell subsets seem to have the innate-like capacity to directly respond to NKG2D ligands, in the absence of other stimuli, which will be addressed later in this text. However, the function of NKG2D in certain lymphocyte subsets seems to be dependent on additional factors, such as the activation status of the T cell or the cellular environment, such as the presence of certain cytokines (Bauer et al., 1999; Groh et al., 1998; Meresse et al., 2004; Shafi et al., 2011; Verneris et al., 2004).

DETC are the major $NKG2D^+$ lymphocyte subset found in the murine epidermis. Through the study of their functional responses to innate stimuli under conditions of tissue stress, it has been identified that they act as key players in the immune response to tissue dysregulation, and serve as prototypic effector cells in LSSR.

1.3.2.2. The innate-like response of DETC to tissue stress

Although cytotoxic stressors, such as UV irradiation and chemical carcinogens, are known to up-regulate NKG2D ligand expression, they have many other pleiotropic effects on the tissue cells. In order to study the isolated response of DETC to the altered expression of NKG2D ligands a reductionist system was needed.

Using a mouse in which Rae-1 expression was specifically induced on keratinocytes, under the control of a tetracycline–dependent bitransgenic (BiTg) molecular switch, acute upregulation of epidermal Rae-I lead to the rapid (within 72 hours) activation and "rounding up" of both DETC and LCs in the epidermis (Strid et al., 2008). Tetracycline had no effect on the single transgenic (SiTg) control mouse. Only DETC express the NKG2D receptor and therefore had the capacity to directly

respond to increased Rae-1 expression. This therefore lead to the subsequent indirect activation of LCs, indicating innate-adaptive cross talk. After this acute response, there was further massive re-organisation of epidermal immune compartment, with replacement of DETC and LCs with infiltrating αβ T cells. This was in the absence of non-self antigen administration, and interestingly infiltrating αβ T cells were enriched with unconventional NKT cells (Strid et al., 2008). Such acute responses to "sterile" stress were rapidly reversible with restoration of the normal DETC and LC epidermal immune compartment within 72 hours of tetracycline withdrawal (Strid et al., 2011).

1.3.2.3. The protective role of DETC

For effective LSSR a diverse range of pre-programmed functional responses must be rapidly elicited. This is reflected in the functional pleiotropy seen across γδ T cell populations, and even within anatomical compartments. DETC have the capacity to secrete multiple growth factors, cytokines, chemokines, and cytotoxic granules when activated (Boismenu et al., 1996a; 1996b; Ibusuki et al., 2013; Strid et al., 2011); and their innate-like capacity to sense tissue stress *in vivo* determines a key role in maintaining tissue integrity.

Skin carcinogenesis can be studied using a two-step model by applying the topical carcinogen 7,12-Dimethylbenz(a)anthracene (DMBA) and tumour promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA). This system leads to the formation of epidermal papillomas that can transform to squamous cell carcinomas, with evidence of an immune cell infiltrate (Girardi et al., 2001). Chemical carcinogenesis induces the rapid upregulation of Rae-1 expression by keratinocytes in the basal and follicular epidermis, where associated $V\gamma5^{+}$ T cells appear activated with a "rounded up" morphology (Girardi et al., 2001; Strid et al., 2008). Loss of either the systemic γδ T cell compartment ($TCR\delta^{-/-}$), or typical DETC ($V\gamma5^{-/-}V\delta1^{-/-}$), increased rates of chemical-induced papilloma formation (Girardi et al., 2003; 2001; Strid et al., 2008). This was despite there being a replacement epidermal γδ T cell compartment in the $V\gamma5^{-/-}V\delta1^{-/-}$ mice (Strid et al., 2008). Consistent with this tumour surveillance role of DETC, activated DETC were shown *in vitro* and by gene expression studies to express high levels of cytolytic mediators, such as the granzyme molecules; produce

cytokines such as TNFα and IFNγ; and to produce pro-inflammatory chemokines, such as macrophage inflammatory protein (MIP)-lα, MIP-1β, lymphotactin and CCL5 (RANTES) (Boismenu et al., 1996a; Krähenbühl et al., 1992; Matsue et al., 1993; Mohamadzadeh et al., 1996). This has been reproduced following *in vitro* activation of DETC with the recombinant NKG2D ligands H60 and Rae-1, leading to both cytotoxic degranulation and IFNγ production (Ibusuki et al., 2013). Collectively, these data establish the potential for DETC-mediated LSSR in the early immune-surveillance of transformed epithelial cells, and demonstrates the pre-programmed role of DETC that specifically populate the murine epidermis.

However, DETC are functionally pleiotropic and, beyond cancer immune-surveillance, play a role in maintaining skin homeostasis. Both TCRδ$^{-/-}$ and Vγ5$^{-/-}$ Vδ1$^{-/-}$ mice that lack a typical epidermal γδ T cell compartment have impaired epidermal barrier function as measured by increased trans-epidermal water loss (TEWL) and reduced epidermal hydration (Girardi et al., 2006). These mice also showed an augmented contact dermatitis response to topical irritants, and when housed in a "dirty" facility developed a spontaneous dermatitis, with ear swelling and a mixed immune cell infiltrate. Reconstitution of these mice with Vγ5^{+} T cells limited both the irritant and spontaneous dermatitis, further demonstrating the immunoregulatory role of DETC (Girardi et al., 2006; 2002). DETC also have a role in regulating keratinocyte survival, through the production of keratinocyte growth factors. Full-thickness wound closure was reportedly delayed in TCRδ$^{-/-}$ mice, compared with wild type controls (Jameson et al., 2002). *In vitro*, DETC have been reported to be a potent source of keratinocyte growth factors, such as keratinocyte growth factor (KGF)-1/2 and insulin-like growth factor-I (IGF-1), which could promote keratinocyte survival and growth, and promote wound healing *in vitro* (Boismenu and Havran, 1994; Jameson et al., 2002; Sharp et al., 2004).

1.3.2.4. Innate-like activation of DETC can alter systemic immune responses

The innate-like activation of DETC by the acute upregulation NKG2D ligands in the epidermis lead to the rapid reorganisation of the entire epidermal immune compartment, with LC activation and subsequent αβ T cell recruitment (Strid et al., 2008). This indicated a direct cross talk between the γδ T cell compartment and

components of the adaptive immune response in the absence of overt foreign antigen.

Cutaneous exposure to ovalbumin (OVA; a non-microbial antigen) provokes a low-level atopic T_H2 responses in wild-type mice, characterised by low levels of IL-4 and IL-13 and IL-13–dependent induction of ova-specific and total IgG1 and IgE (Strid et al., 2004). Using the tetracycline-dependent BiTg Rae-I inducible mouse, or gentle epidermal abrasion, surprisingly the concomitant up-regulation of Rae-1 in the epidermis during OVA exposure strongly enhanced the atopic response (Strid et al., 2011). This was seen both in the skin and systemically. Strikingly, in this model DETC were the source of T_H2-type and pro-inflammatory cytokines, and mice that lacked either γδ T cells or NKG2D failed to induce local IL-4, IL-13, and IL-25; with IL-33 and IL-1β responses being less durable. In addition, LSSR was important in the induction of non-specific IgE, which was absent in TCR$\delta^{-/-}$ mice and impaired in mice that lacked NKG2D (Klrk1$^{-/-}$). In this system NKG2D-dependent cross talk between dysregulated epithelial cells and tissue-associated γδ T cells was therefore required for normal local and systemic atopic responses to antigen encountered at the epithelial surface. This not only implicates LSSR in the afferent induction of T_H2 responses, but also links atopy with early immune responses to tissue damage and carcinogenesis.

Thus, the innate-like activation of epidermal γδ T cells offers a revised perspective of immune-surveillance within the tissue. LSSR offers an alternative model of immune activation to dysregulated tissues, whereby stresses alter the immunogenicity of epithelial cells to activate tissue-resident ILL, which can directly induce tissue repair but also activate and recruit myeloid cells and αβ T cells (Figure 1.4). This complements conventional innate myeloid responses to microbial products, such as *via* TLRs, to activate adaptive immune responses.

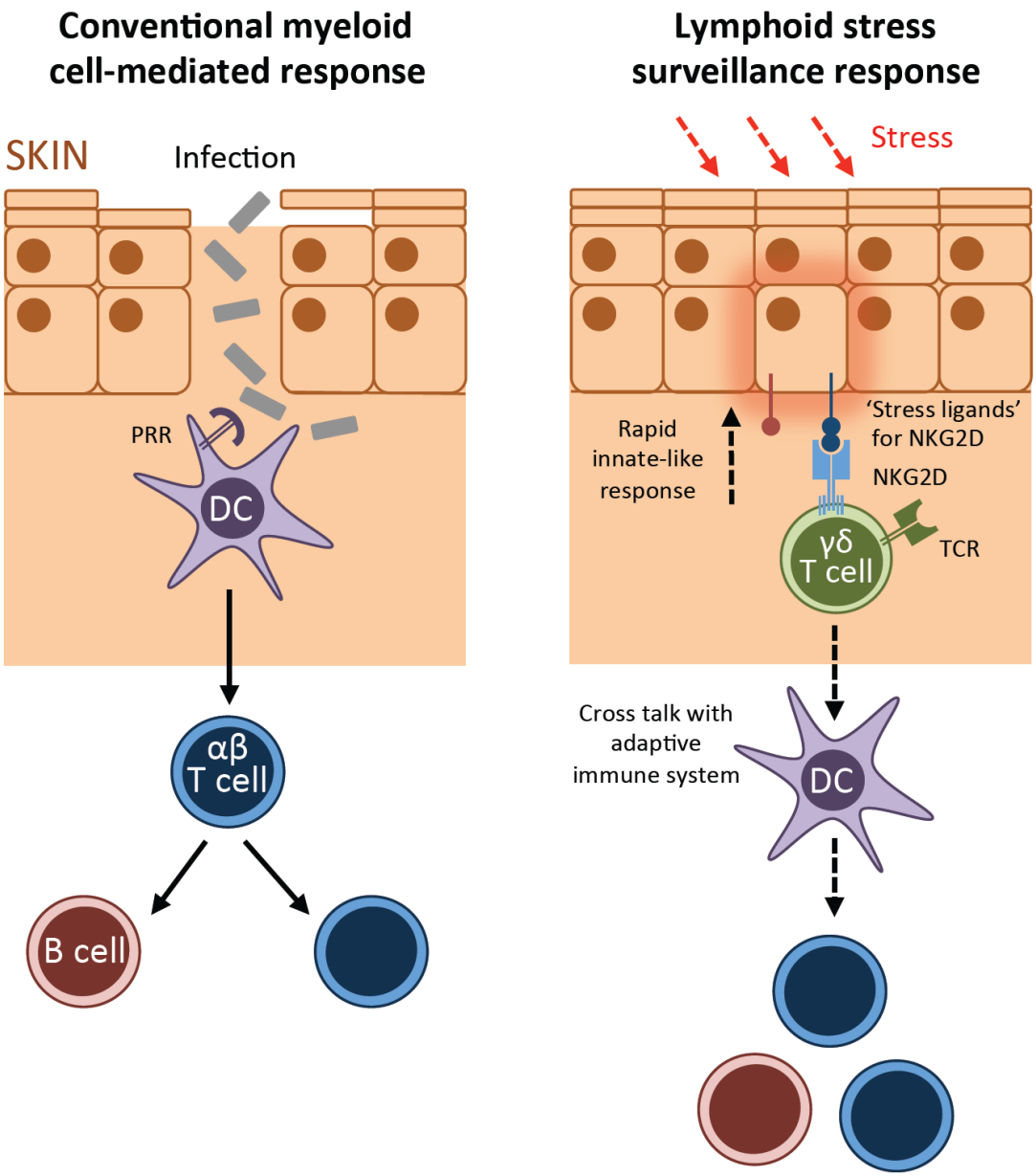

Figure 1.4. The lymphoid stress surveillance response (LSSR) describes the capacity of certain lymphocytes to directly recognise surrounding tissue dysregulation

Activation of myeloid cells by microbial products will initiate a conventional pathogen-specific lymphocyte response. LSSR offers an alternative mode of immune activation whereby tissue stress induces the up-regulation of "stress ligands", such as those for the NKG2D receptor, expressed by stromal cells. Certain $NKG2D^{+}$ lymphocytes, such as γδ T cells in the murine epidermis, have the capacity to directly respond with a rapid innate-like response and influence subsequent myeloid and αβ T cell responses (adapted from Hayday (2009)).

1.3.2.5. Mouse skin contains a population of γδ T cells in the dermis with innate-like potential

γδ T cells make up approximately 50 % of T cells in the murine dermis (Sumaria et al., 2011). These cells express an oligoclonal TCR repertoire, with 30 – 50 % expressing the Vγ4 TCR chain, making them distinct from $V\gamma5^+$ DETC (Sumaria et al., 2011).

Dermal γδ T cells differ from DETC in that they constitutively migrate through the tissue, have a more rounded morphology and seem to require just IL-7 for their development (with no clear dependence on IL-15) (Michel et al., 2012; Sumaria et al., 2011). However, like DETC, they form a long-lived populations capable of peripheral self-renewal (Sumaria et al., 2011). Dermal γδ T cells are central to the immune response to cutaneous Mycobacterium infection (Nakamizo et al., 2015; Sumaria et al., 2011), and can be activated by the topical application of the TLR7 ligand imiquimod, which leads to the development of a psoriasiform dermatitis (Cai et al., 2011; Mabuchi et al., 2011; Pantelyushin et al., 2012; Yoshiki et al., 2014). In such models of inflammation, dermal γδ T cells show some innate-like function – responding directly to stimulation with the cytokines IL-1β and IL-23, which elicit a rapid effector response that is characterised by IL-17 and IL-22 production (Cai et al., 2011; Mabuchi et al., 2011; Martin et al., 2009; Sumaria et al., 2011; Sutton et al., 2009).

In addition to their effector functions in the skin, following activation of $V\gamma4^+$ T cells, with either mycobacterial infection or imiquimod, they become more mobile and migrate to skin-draining lymph nodes where they proliferate (Nakamizo et al., 2015; Ramírez-Valle et al., 2015). Here they can modulate the adaptive immune response, as depletion of $V\gamma4^+$ T cells attenuated $CD8^+$ αβ T cell expansion in the lymph node (Nakamizo et al., 2015). Following their proliferation in skin-draining lymph nodes, $V\gamma4^+$ T cells migrate to both the site of cutaneous sensitization, and to distant sites of un-inflamed skin, where they can reportedly confer some immunological memory on re-challenge (Ramírez-Valle et al., 2015).

Dermal Vγ4$^+$ T cells seem committed to produce IL-17 and IL-22, which are important in the immune response to certain infections and epithelial injury, respectively (Nakamizo et al., 2015; Sumaria et al., 2011; Zheng et al., 2006). However, these cytokines are also central to certain pathological processes, such as the human skin disease psoriasis, with Vγ4$^+$ T cell-mediated dermatitis having distinct psoriasiform features (Cai et al., 2011; Fujita, 2013; Mabuchi et al., 2011; Nestle et al., 2009b; Pantelyushin et al., 2012; Yoshiki et al., 2014; Zheng et al., 2006). The identification of innate-like T cells in the murine dermis that are central to this process is clearly of interest in understanding this complex chronic human disease. Although an attempt has been made to identify human γδ T cells that have an equivalent role in psoriasis, this has remained unresolved (Cai et al., 2011; Laggner et al., 2011).

Thus, dermal γδ T cells have certain features of ILL, such as the enrichment of an oligoclonal Vγ4$^+$ T cell population, and their innate-like response to exogenous cytokine in the context of microbial stress. However, the capacity of Vγ4$^+$ T cells to directly sense "sterile" tissue dysregulation, *via* receptor-ligand interactions such as NKG2D, has not yet been established. In addition, Vγ4$^+$ T cells also show migratory properties that are evocative of αβ T cells, with reports that they may confer immunological memory on subsequent challenges. Vγ4$^+$ T cells are not limited to the dermis, and are found in other epithelial tissues, including the lung and reproductive tract, where they also produce IL-17 in response to infection (Okamoto Yoshida et al., 2010; Rakasz et al., 1998). Therefore, despite their sharing with Vγ5Vδ1$^+$ DETC an innate-like functional responsiveness, dermal γδ T cells also have features suggestive of distinct functional specialisation.

1.4. Human γδ T cells

The study of γδ T cells in mice, and their innate-like properties, has increased our understanding of these cells in humans. Human γδ T cells are similarly defined by their expression of a limited repertoire of TCRs, unconventional modes of MHC-unrestricted activation, and certain subsets are enriched in barrier tissues. The clear role that γδ T cells have in murine LSSR raises the possibility that similar γδ T cell biology may exist in human tissues, such as the skin. Human γδ T cell research has largely focused on the blood compartment, and the tissue-resident γδ T cell compartment remains relatively poorly described.

1.4.1. Human γδ T cell subsets

Human γδ T cells are defined by their expression of heterodimeric TCRs made up of Vγ and Vδ chains. Despite the fact that there seem to be large differences between species regarding the number of γδ T cell subsets, their location and their antigen recognition, human γδ T cells often show highly restricted γδ TCR usage, with distinct populations associated with specific anatomical locations in the periphery (Kalyan and Kabelitz, 2012).

In adult humans, two major subsets of γδ T cells exist that are defined by the usage of either the Vδ2 or Vδ1 TCR chain. γδ T cells in the peripheral blood predominantly express the Vδ2 TCR chain, of which most have a highly restricted pairing with the Vγ9 TCR chain (Vγ9Vδ2$^+$ T cells); whereas, in human tissues Vδ1$^+$ T cells are the predominant γδ T cell subset, with Vδ3$^+$ and Vδ5$^+$ T cells making up minor additional populations (Deusch et al., 1991; Groh et al., 1989; Halary, 2005; Holtmeier et al., 1995; 2001; Toulon et al., 2009). Vδ1$^+$ T cells show a less restricted Vγ chain pairing than blood Vγ9Vδ2$^+$ T cells; however, some preferential pairing with Vγ4 and Vγ5 TCR chains has been reported (Hviid et al., 2000; Kalyan and Kabelitz, 2012). In addition, Vδ1 TCR oligoclonality has been described in both the skin and gut, which was largely distinct from blood Vδ1$^+$ T cells (Chowers et al., 1994; Holtmeier et al., 1995; 2001). "Non-Vδ2" γδ T cells can also be found as rare subsets in the peripheral blood (Halary, 2005).

Human γδ T cell thymic development precedes that of αβ T cells, as in the mouse, with γδ T cells found in the fetal blood and tissues by the eighth week of gestation (Dimova et al., 2015; McVay et al., 1998). In umbilical cord blood the γδ T cell population comprises mainly $V\delta1^+$ cells; however, this composition radically changes during early life and gives way to the predominant $V\gamma9V\delta2^+$ T cell population found in adult peripheral blood (Kalyan and Kabelitz, 2012; Sandberg et al., 2006). This is thought to reflect the migration of naïve "non-Vδ2" γδ T cells from the blood to the tissues; and the expansion of blood $V\gamma9V\delta2^+$ T cells through interactions with their cognate ligand(s), which is reflected in their effector phenotype, primed to readily produce IFNγ and mediate cytolysis on activation (De Rosa et al., 2004; Dimova et al., 2015).

1.4.2. The human lymphoid stress surveillance response

Human γδ T cells do not require priming by myeloid cells for activation, and show the innate-like capacity to be activated in an MHC-unrestricted manner. However, this has largely been characterized in the blood compartment where $V\gamma9V\delta2^+$ T cells have the capacity to recognise and respond *en masse* to specific non-peptide metabolites following cellular dysregulation (Hayday, 2009; Vantourout and Hayday, 2013).

1.4.2.1. $V\gamma9V\delta2^+$ T cells have innate-like properties

During acute systemic infections, such as tuberculosis and malaria, there is often a massive expansion of human peripheral blood $V\gamma9V\delta2^+$ T cells – from less than 10 % of circulating T cells in healthy donors to over 50 % during infection in some individuals (Balbi et al., 1993; Morita et al., 1999; Schwartz et al., 1996). Strikingly, this response is seen to a wide range of bacterial and protozoan pathogens (Eberl et al., 2003; 2004; Morita et al., 1999).

$V\gamma9V\delta2^+$ T cells can rapidly respond to phosphorylated compounds (phosphoantigens) in an MHC-unrestricted manner (Morita et al., 1995; Tanaka et

al., 1995). Phosphoantigens include microbial metabolites synthesised *via* the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. This generates uniquely foreign phosphoantigens, such as (E)-4- hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), which can be isolated from a range of mycobacteria, archaebacteria and protozoa (Eberl et al., 2003; 2004). In mammalian (host) cells, endogenous phosphoantigens of lower potency, such as isopentenyl pyrophosphate (IPP), can be synthesized *via* the mevalonate pathway (Wang et al., 2011). The mevalonate pathway is non-redundant and key to the biosynthesis and prenylation of many other compounds, such as cholesterol and the Ras family of GTPases, respectively. Thus upregulation of this pathway, and of its associated metabolites, has been shown following cellular transformation (Freed-Pastor et al., 2012; Gober et al., 2003) or certain infections (Kistowska et al., 2008). This indicates a mechanism compatible with LSSR, whereby following cellular stress, through either the expression of phosphoantigens or the overexpression of the physiological metabolite IPP, dysregulated cells become immunologically visible to cytotoxic $V\gamma9V\delta2^+$ T cells (Vantourout and Hayday, 2013).

The context of phosphoantigen recognition by the Vγ9Vδ2 TCR remains elusive. Several candidate phosphoantigen presenting-elements have been identified. Most recent work has focused on the butyrophilin molecule BTN3A1, which is thought to either sense or directly present phosphoantigens, however, this remains incompletely understood (Rhodes et al., 2015; Sandstrom et al., 2014; Vavassori et al., 2013; Wang et al., 2013). There is no direct orthologue to BTN3A1 in mice, which may explain why mice do not have an equivalent blood γδ T cell population that reacts to phosphoantigens. However, both human BTN3A1 and murine Skint-1 (the epithelial determinant involved in $V\gamma5V\delta1^+$ DETC development and function [above]) are members of the butyrophilin superfamily and show clear structural homology (Abeler-Dorner et al., 2012). It is striking that the activation of these distinct human and mouse ILL compartments seems to involve a conserved molecular mechanism.

1.4.2.2. Human tissue-resident γδ T cells are less well characterized

As discussed, human tissue-resident γδ T cells predominantly express the Vδ1 TCR chain, with $V\delta3^+$ and $V\delta5^+$ T cells also found. Although less prominent than in

murine tissues, γδ T cells have been estimated to constitute approximately 5 % of the T cell compartment in healthy tissues such as the skin, respiratory tract, reproductive tract and tongue, which is comparable to the blood γδ T cell compartment (Bos et al., 1990; Elbe et al., 1996; Foster et al., 1990; Groh et al., 1989; Vroom et al., 1991). The main exception is the human gut, where γδ T cells are enriched in the IEL compartment, and can account for up to 30 % of resident lymphocytes (Bucy et al., 1989; Deusch et al., 1991; Vroom et al., 1991).

1.4.2.3. Human Vδ1$^+$ γδ T cells have an immunoprotective role

Although Vδ1$^+$ T cells are primarily located within the tissues, functional studies have primarily focused on blood-derived Vδ1$^+$ T cell lines (or Vδ2$^-$ γδ T cell lines), due to the relative ease with which they can be isolated and studied. In such cases, Vδ1$^+$ T cells have been implicated in the immune response to certain infections and cancers.

Although not a common phenomenon, Vδ1$^+$ T cell expansion has also been observed during certain infections; principally with viruses, such as CMV (Alejenef et al., 2014), Epstein Barr virus (EBV) (De Paoli et al., 1990; Orsini et al., 1993), human herpes virus 8 (HHV8) (Barcy et al., 2008), and HIV (Autran et al., 1989; Rossol et al., 1998). Vδ1$^+$ T cells (or Vδ2$^-$ γδ T cells) *in vitro* recognise virally infected cells to mediate cytolytic responses (Alejenef et al., 2014; Barcy et al., 2008; Couzi et al., 2010; Knight et al., 2010; Orsini et al., 1993). Vδ1$^+$ T cells also show some reactivity to certain bacteria, such as *Borrelia burgdorferi,* which causes Lyme disease – Vδ1$^+$ T cells isolated from the synovium of patients with Lyme disease were activated in response to *Borrelia burgdorferi* cell lysates (Glatzel et al., 2002)

A large peripheral expansion of Vδ2$^-$ γδ T cells, including Vδ1$^+$ T cell clones, has also been seen in some patients following haematopoietic stem cell transplantation (HSCT) (Fujishima et al., 2007; Knight et al., 2010). In one study, this expansion was associated with evidence of CMV infection (Knight et al., 2010). As with the studies described above, Vδ1$^+$ T cell lines derived from these BMT patients were cytotoxic towards CMV and EBV infected cells (Fujishima et al., 2007; Knight et al., 2010).

Solid organ transplant recipients that are chronically immunosuppressed are at increased risk of certain cancers, such squamous cell carcinomas (Kasiske et al., 2004; Ramsay et al., 2002). The pathogenesis of transplant-associated malignancies is often associated with viral infections, such as CMV or EBV, with patients that have had pre- or post-transplant CMV infection at increased risk of malignancy (Couzi et al., 2010). However, patients infected with CMV that also have a significant increase in their blood Vδ2$^-$ γδ T cells have been shown to have a reduced risk of developing a cancer (Couzi et al., 2010). Although Vδ2$^-$ γδ T cells seem to be protective against CMV infection (Kaminski et al., 2015), they were also directly cross-reactive towards tumour cell lines in the absence of active CMV infection (Couzi et al., 2010; Halary, 2005). Interestingly, following HSCT with grafts depleted of αβ T cells, it has been reported that those patients that undergo post-treatment Vδ2$^-$ γδ T cell expansion have a significantly improved disease free survival; however, it was not demonstrated if this was due to protection from viral infection or an enhanced graft-*versus*-leukaemia effect (Lamb et al., 1999).

Vδ1$^+$ T cells have also been shown to be a major component of lymphocytes infiltrating certain solid tumours, including malignant melanoma and colorectal carcinomas (Cordova et al., 2012; Maeurer et al., 1996). Blood or tumour-derived Vδ1$^+$ T cell clones that have been activated and expanded *in vitro* were cytotoxic towards melanoma and colonic cancer cells, and a wide range of other allogeneic tumour cells of epithelial origin (Bauer et al., 1999; Cordova et al., 2012; Deniger et al., 2014; Groh et al., 1998; Maeurer et al., 1996). The mechanism of such Vδ1$^+$ T cell-mediated cytotoxicity has not been fully elucidated, but may involve innate receptors (Bauer et al., 1999; Hayday and Vantourout, 2013; Vantourout and Hayday, 2013).

1.4.2.4. The potential reactivity of Vδ1$^+$ γδ T cells

The reactivity of Vδ1$^+$ T cells remains uncertain. The greater diversity of Vδ1$^+$ TCRs implies that they recognise a broader range of antigens than blood Vγ9Vδ2$^+$ T cells, but the nature of such interactions is largely unknown. It remains unclear if Vδ1$^+$ T cell activation depends on the recognition of foreign antigens, PAMPs,

and/or self-encoded MHC-I-like molecules, and if such recognition occurs *via* the TCR or other innate receptors.

The CD1 family of MHC-I-like molecules consists of four isoforms (CD1a–d) that present foreign lipids to T cells, rather than peptides, as is seen with classical MHC molecules. Much work regarding the CD1 family has focused on CD1d, which presents the lipid antigen α-galactosylceramide (α-GalCer) to invariant NKT (iNKT) cells, or non-α-GalCer lipids to NKT subsets that express more variable TCRs (Bendelac, 1995; Van Kaer et al., 2015). There are reports that Vδ2^{-} γδ T cells also recognize CD1 molecules, albeit often rare subsets or isolated clones of these cells (Hayday and Vantourout, 2013). Vδ1^{+} T cells derived from blood or tissue (gut and nasal mucosa) have been shown to be reactive to CD1d-associated with α-GalCer or non-α-GalCer lipids (Agea et al., 2005; Bai et al., 2012; Russano et al., 2007; 2006). This has been subsequently supported by both biochemical and structural modeling data that indicate a direct binding of lipid-loaded CD1d to the Vδ1 TCR (Luoma et al., 2013; Uldrich et al., 2013). In addition, blood-derived Vδ1^{+} T cell clones are also reactive to CD1c, which when activated can induce DC maturation (Leslie et al., 2002; Spada et al., 2000). However, these functional observations are not supported by biochemical data indicating a direct CD1c-Vδ1 TCR interaction. Although the reactivity of Vδ1^{+} T cells to CD1c/d is low, this may be underestimated by these studies have used a limited range of CD1-lipid cargo complexes (Hayday and Vantourout, 2013).

It has also been reported that a Vγ4Vδ5^{+} T cell clone, derived from a CMV-infected transplant patient, could bind to the endothelial protein C receptor (EPCR), which is homologous to CD1 (Willcox et al., 2012). However, the Vγ4Vδ5^{+} T cell clone directly recognised EPCR-expressing CMV-infected and transformed targets, independent of an associated lipid cargo (Willcox et al., 2012).

As previously discussed, human Vδ1^{+} T cell lines are reactive towards both virally infected and transformed cells. Human Vδ1^{+} T cell lines have been shown to be reactive to the MHC-I-like molecule MICA, which is often expressed by infected or transformed cells and can bind the activatory NKG2D receptor. Vδ1^{+} T cell killing of transformed cellular targets could be blocked by monoclonal antibodies against

MICA (Bauer et al., 1999; Groh et al., 1998; 1999). However, there has been some controversy as to whether MICA directly binds the Vδ1 TCR and/or engages the NKG2D receptor to induce $V\delta1^+$ T cell cytotoxicity. MICA could reportedly activate $V\delta1^+$ T cell lines that lacked the NKG2D receptor, and there are both biochemical and structural analyses indicative of direct ligation between MICA and the Vδ1 TCR (Wu et al., 2002; Xu et al., 2011; Zhao et al., 2006). In addition, $V\delta1^+$ T cell killing could be inhibited by anti-TCR monoclonal antibodies, implying a requirement of TCR-engagement for $V\delta1^+$ T cell activation (Groh et al., 1998). However, in similar co-culture experiments with NKG2D ligand-expressing cellular targets, $V\delta1^+$ T cell cytotoxicity could also be impaired by blocking the NKG2D receptor, implying direct activation *via* NKG2D (Bauer et al., 1999; Deniger et al., 2014; Knight et al., 2010; 2012a). In addition, some of the TCRs of γδ T cells that are reportedly specific for MICA were actually found to bind CD1 (Hayday and Vantourout, 2013).

It is important, however, to emphasise that these functional studies have been undertaken using $V\delta1^+$ T cell lines that have been derived using protocols that involve strong pre-activation. They have received repeated and prolonged activation *in vitro* with a combination of MICA/MICB ligands, anti-γδ TCR antibodies that would cross-link the γδ TCR, and/or mitogens (such as phytohaemagglutinin) (Bauer et al., 1999; Groh et al., 1998; 1999); or they have been isolated as expanded clones from patients with viral infections (Knight et al., 2010; 2012a). These atypical conditions may significantly alter the subsequent responsiveness of these cells to innate stimuli, and account for differences between studies. Similarly, $V\delta2^+$ T cell lines and $CD8^+$ αβ T cell lines that have been generated under similar conditions show some NKG2D-dependent cytotoxic responses (Lanca et al., 2010; Maccalli et al., 2007; Meresse et al., 2004; Rincon-Orozco et al., 2005; Shafi et al., 2011; Siegers et al., 2011; Verneris et al., 2004); whereas, under different conditions NKG2D will act only as a co-stimulatory signal for TCR-mediated activation of these cells (Groh et al., 2001; Nedellec et al., 2010) [see this thesis].

1.5. Could LSSR play a role in human skin health and disease?

LSSR offers an alternative mode of immune activation whereby tissue-resident lymphocytes have the capacity to rapidly and directly respond to surrounding tissue dysregulation (Hayday, 2009). In addition to the predominant αβ T_{RM} population, healthy human skin also contains subsets of "unconventional" lymphocytes that are also predominantly located in the dermis, which include γδ T cells, NK cells and innate lymphoid cells (Bos et al., 1990; Clark et al., 2006a; Ebert et al., 2006; Kim et al., 2013; Salimi et al., 2013; Teunissen et al., 2014a). Rare populations of NKT and MAIT cells have been described in human skin but only under certain disease states (Gober et al., 2007; Teunissen et al., 2014b).

In murine skin, γδTCR$^+$ DETC are prototypic effector cells of the LSSR, and their development is determined by the epithelial butyrophilin-like molecule Skint-1 (Barbee et al., 2011; Boyden et al., 2008; Lewis et al., 2006). Interestingly, in humans and most other non-human primates there is a common inactivating mutation in the *SKINT-1 like* gene, which may explain the lack of epidermal γδ T cell population homologous to DETC (Mohamed et al., 2015). Despite this, a population of dermal γδ T cells has been described in healthy skin, which predominantly express the Vδ1 TCR chain (Ebert et al., 2006; Groh et al., 1989; Toulon et al., 2009). In addition, γδ T cells have been reported in the context certain skin diseases, such as malignant melanoma, where in some cases both Vδ1$^+$ and Vδ2$^+$ T cells reportedly make up a significant component of tumour-infiltrating lymphocytes (Cordova et al., 2012). γδ T cells have also been associated with certain chronic inflammatory states within the skin, which include acute wounds (Toulon et al., 2009), tissue fibrosis (Giacomelli et al., 1998), and psoriasis (Cai et al., 2011; Laggner et al., 2011). However, to date, human skin-resident γδ T cells have not been comprehensively analysed – especially their potential functional responsiveness to conditions of tissue dysregulation.

In murine studies, the NKG2D axis serves as a model by which LSSR can be mediated. The upregulation of NKG2D ligands is under tight control, limited to states of tissue dysregulation. This can clearly provoke an effective immune response in humans, as under this selective pressure, a wide range of viruses have

evolved mechanisms to evade such immune-surveillance – for example, human cytomegalovirus (CMV) produces the protein UL16, which can bind and retain NKG2D ligands intracellularly, reducing their cell surface expression (Bennett et al., 2010; Cosman et al., 2001; Dunn et al., 2003; Rolle et al., 2003). Similarly, human tumours can also undergo a process of immunoediting, whereby they shed NKG2D ligands. This reduces their cell surface expression of these immunogenic self-peptides, and releases soluble forms of the ligand ectodomains that may down-regulate NKG2D-mediated lymphocyte responses – although the effect of this *in vivo* remains controversial (Paschen et al., 2009).

Although the functional responses of human skin-resident lymphocytes to ligands for the NKG2D receptor have not been reported upon, components of the NKG2D-axis have been implicated in human skin disease. These include both cancer and auto-inflammatory processes.

Malignant melanoma cells, from both primary lesions and immortalised cell lines, show variably increased NKG2D ligand expression (Maccalli et al., 2007; Paschen et al., 2009; Pende et al., 2002). In patients with melanoma, soluble NKG2D ligands have also been detected in the blood serum, with increasing titres of soluble MICA and ULBP2 associated with advanced disease, and ULBP2 levels correlating with poor outcome (Paschen et al., 2009). Although the effect of chronic exposure to soluble NKG2D ligands *in vivo* is not fully understood, the association of NKG2D ligands with advanced melanoma may indicate potential cancer immunoediting, or it may also just reflect the greater disease burden seen in patients with more advanced disease.

In chronic plaque psoriasis, both linkage and genome wide association studies (GWAS) have identified that variation within the MHC-I region on chromosome 6 has the greatest effect on disease susceptibility (G.A.O.P.C.T.W.T.C.C.C. 2, 2010). This is primarily thought due to variation within the HLA-Cw*0602 allele; however, further conditional analysis of the MHC-I region has identified additional independent risk loci that associate with *MICA* and *MICB* genes (Knight et al., 2012b). In addition, functional polymorphism within the *MICA* gene and certain MICA isoforms is reportedly associated with psoriasis susceptibility, independent of

both HLA-B and HLA-C-associated risk (Pollock et al., 2013; 2011; Song et al., 2013).

In alopecia areata (AA) – an inflammatory disease of the hair follicle – GWAS data has identified disease susceptibility loci associated with both the *MICA* gene (6p21.32) and *ULBP3/6* gene cluster (6q25.1) (Petukhova et al., 2010). Consistent with this, in immunohistological studies both MICA and ULBP3 are up-regulated at the hair follicle outer-root sheath during active disease; which was also associated with increased IL-15 expression, and an infiltration of $NKG2D^+$ lymphocytes that expressed CD8 and/or CD56 (Ito et al., 2007; Petukhova et al., 2010). AA has also been studied using the C3H/HeJ mouse model, which spontaneously develops alopecia that mimics many histopathological features of human AA (McElwee et al., 1998). In this model, NKG2D ligands (H60 and Rae-1) are also upregulated at the hair follicle epithelium during active disease, with an associated $NKG2D^+$ $CD8^+$ T cell infiltrate (Xing et al., 2014). Transfer of $NKG2D^+$ $CD8^+$ T_{EM} cells from skin draining lymph nodes of affected mice can transfer disease, and alopecia was reversed through the systemic blockade of IFNγ and IL-15 signalling with monoclonal antibodies or by oral/topical janus kinase (JAK) inhibitors (Xing et al., 2014). Similarly, preliminary reports of the use of oral JAK1/2 and JAK3 inhibitors in patients with AA describe rapid near-complete hair regrowth and disease remission (Craiglow and King, 2014; Jabbari et al., 2015; Xing et al., 2014). These studies clearly implicate both the NKG2D axis and $NKG2D^+$ T_{EM} cells in AA disease pathology; however, they overlook a potential role of other $NKG2D^+$ lymphocyte subsets, especially during the early initiation of disease when innate-like responses may be paramount.

* * * * *

In conclusion, a model of adaptive immunity has largely shaped the current view of skin immunobiology; whereby tissues become progressively populated with a large number of diverse αβ T_{RM} cells, following successive microbial challenges. However, additional subsets of $\gamma\delta TCR^+$ lymphocytes preferentially populate barrier tissues, such as the skin, where they can mediate early innate-like responses to tissue stress, such as *via* the activatory NKG2D receptor. Thus, murine skin is dominated

by T_{RM} cells and ILL, which share in the fact that both seem to have been previously activated – the former by antigen priming, and latter as part of a developmental programme.

Despite no conspicuous homologue to DETC in human skin, this suggests an evolutionary niche within the tissue that may be filled by a functionally equivalent lymphocyte population. Human γδ T cells show features of ILL, however, their capacity to mediate immune responses compatible with LSSR in the skin, or other tissues, has not been fully characterised. Alternatively, this functional niche may be filled by another lymphocyte subset(s). Characterising such innate-like responses in human skin is fundamental to our understanding of both human lymphocyte biology and early immune surveillance in the tissue, which may influence both local and systemic immunity in health and disease.

Aims of thesis

The aim of this thesis is to delineate the distinct T cell subset composition of human skin, and to investigate whether any such subsets have the capacity to respond to tissue stress according to the model of LSSR, and to understand the nature of such lymphocyte responses.

To test this hypothesis, my primary objectives are to:

- Establish protocols to isolate and characterise skin-resident lymphocytes, including rare subsets of unconventional lymphocytes.
- Characterize the functional responsiveness of skin-resident lymphocytes to innate-like stimuli, focusing on the NKG2D axis.
- Understand mechanistic factors that may influence such innate-like function.

Chapter Two: Characterisation of the skin-resident lymphocyte compartment in healthy human skin

In order to delineate the tissue-resident lymphocyte compartment found in human skin, we needed to establish a system for T cell isolation that was reproducible and robust; and which yielded sufficient numbers of cells that were representative of the T cell compartment *in vivo*. Thereupon, we wanted to describe the phenotypes of the different lymphocyte subsets isolated; in particular, establishing if they show receptors indicative of prior activation – especially as this seems a shared property of murine T_{RM} cells and innate-like T cells – and/or stable skin-residence. Next we sought to ascertain which receptors they express that might mediate the cells' regulation, such as CD28 and NKG2D.

By this means, we confirmed the presence of a large and complex T cell compartment in the skin, which nonetheless displayed reproducible characteristics across a large number of donors. In particular, we were able to consistently isolate substantial numbers of cells that may have the functional properties of T_{RM} cells and innate-like T cells.

2.1. Isolation of skin-resident lymphocytes by tissue disaggregation

Tissue-resident lymphocytes have conventionally been studied *ex vivo* following enzymatic digestion and/or mechanical disaggregation of the tissue. Using this approach with healthy adult human skin, distinct lymphocyte populations were isolated, with a clear $CD45^+$ leukocyte population identified on flow cytometry (Figure 2.1). Subsequent gating demonstrated NK cells ($CD3^-CD56^+$) and within the $CD3^+$ population, γδ T cells, and $CD4^+$ and $CD8^+$ αβ T cells (γδ TCR^-). Across different donors reproducible lymphocyte subsets were isolated; expressed as a percentage of $CD45^+$ singlet events there were 46.1 % (± 15.2) $CD4^+$ T cells, 20.2 % (± 14.8) $CD8^+$ T cells, 1.6 % (± 1.0) γδ T cells and 6.2 % (± 5.0) NK cells (mean values ± standard deviation (SD); n = 7) (Table 2.1; Figure 2.2). However, *ex vivo* assays were limited by a poor overall viability of isolated cells, and low cell yield of

just a few thousand $CD45^+$ events per donor. This was particularly limiting for the more rare "unconventional" lymphocyte subsets.

2.2. Establishing a skin explant culture system to optimise the isolation of unconventional lymphocytes

To increase the number and quality of skin-resident lymphocytes isolated, we adapted an organotypic culture protocol established by Clark and colleagues for this purpose (Clark et al., 2006a) In brief, the mechanical/enzymatic digestion of tissue is avoided, in place of which skin explants were cultured *in vitro* on three-dimensional tantalum-carbon matrices that were coated with collagen. During the 21-day culture period, dermal fibroblasts grew out onto both the matrices and tissue culture plate. Skin-resident lymphocytes subsequently crawled out of the tissue and remained in close proximity to the fibroblasts, which was observed on inverted light microscopy (Figure 2.3A).

Using this approach a large number of skin-resident lymphocytes could be isolated from the tissue. Reproducible lymphocyte subsets were identified across multiple donors, comparable to *ex vivo* studies, using different culture conditions (culture media with/without supplemented IL-2 and IL-15) (Figures 2.2 and 2.4). However, the explant lead to the isolation of many more lymphocytes, whose viability and subsequent expansion could be promoted by supplementing the cultures with IL-2 and IL-15 (median 270 000 lymphocytes/matrix at day 21; range 180 000 – 600 000; n = 8). These conditions mimic both the cells' own production of IL-2 as a necessary growth factor, and the constitutive production of IL-15 by epithelial and mesenchymal cells. Notably, with IL-2 and IL-15 there was also a further enrichment in the number of γδ T cells isolated, compared with both *ex vivo* and plain media explant protocols (3.4% of lymphocytes, compared with 1.6 % and 1.0%, respectively; p = 0.0005 – 0.0076). There was an apparent loss of NK cells using the plain media for the explant protocol (although this did not reach statistical significance), which was not seen in the presence of supplemented IL-2 and IL-15 (p = 0.0025).

The culture media used was described as either un-supplemented or supplemented with IL-2 and IL-15; however, it is known that skin explants also condition the media with additional chemokines and cytokines, which facilitate the migration of the tissue-resident lymphocytes out of the skin (Clark et al., 2006a). In fully characterising this system, analyses of explant-conditioned media demonstrated multiple additional chemokines and cytokines to be present (Figure 2.5). This was undertaken using a 34-plex ProcartaPlex^{TM} Luminex assay at day 21 of culture. As with published data, high levels of the T cell chemoattractants IL-8, IFNγ-inducible protein 10 (IP-10) and monocyte chemoattractant protein 1 (MCP-1), and the anti-apoptotic cytokine IL-6 (Clark et al., 2006a). Additional chemoattractants that were also identified included stromal cell-derived factor 1α (SDF-1α), growth related oncogene α (GROα), macrophage inflammatory protein 1α/β (MIP-1α/β), granulocyte macrophage colony stimulating factor (GM-CSF) and regulated on activation, normal T cell expressed and secreted (RANTES). Strikingly, high levels of IL-1 receptor anatgonist (IL-1RA) were also detected, which is the natural inhibitor of the pro-inflammatory effects of IL1β (Galkowska et al., 1995).

Although many of the chemokines and cytokines measured in the explant-conditioned media are thought to be primarily derived from skin stromal cells in response to injury, such as keratinocytes and fibroblasts, they may also be derived from activated skin-resident immune cells present *in vitro*, such as dendritic cells or lymphocytes. Additional cytokines detected in the media that may be primarily produced by tissue-resident immune cells included low levels of IFNγ, IL-13, IL-22 and IL-9. These analyses were performed on media that had been supplemented with IL-2 and IL-15, which are considered key growth factors for lymphocyte survival and proliferation (Ma et al., 2006; Sprent, 2008). This may indirectly account for some of the cytokines and chemokines measured in the explant-conditioned media at 3 weeks. Analysis of un-supplemented media did not detect significant amounts of soluble IL-2 or IL-15 (Figure 2.6), which therefore justified their addition, as this lead to an increase in the cells isolated from the skin, with an enrichment in “unconventional” lymphocytes.

As the explant culture system using media supplemented with IL-2 and IL-15 yielded the greatest number of skin-resident “unconventional” lymphocytes, this was used as the primary method for subsequent lymphocyte isolation.

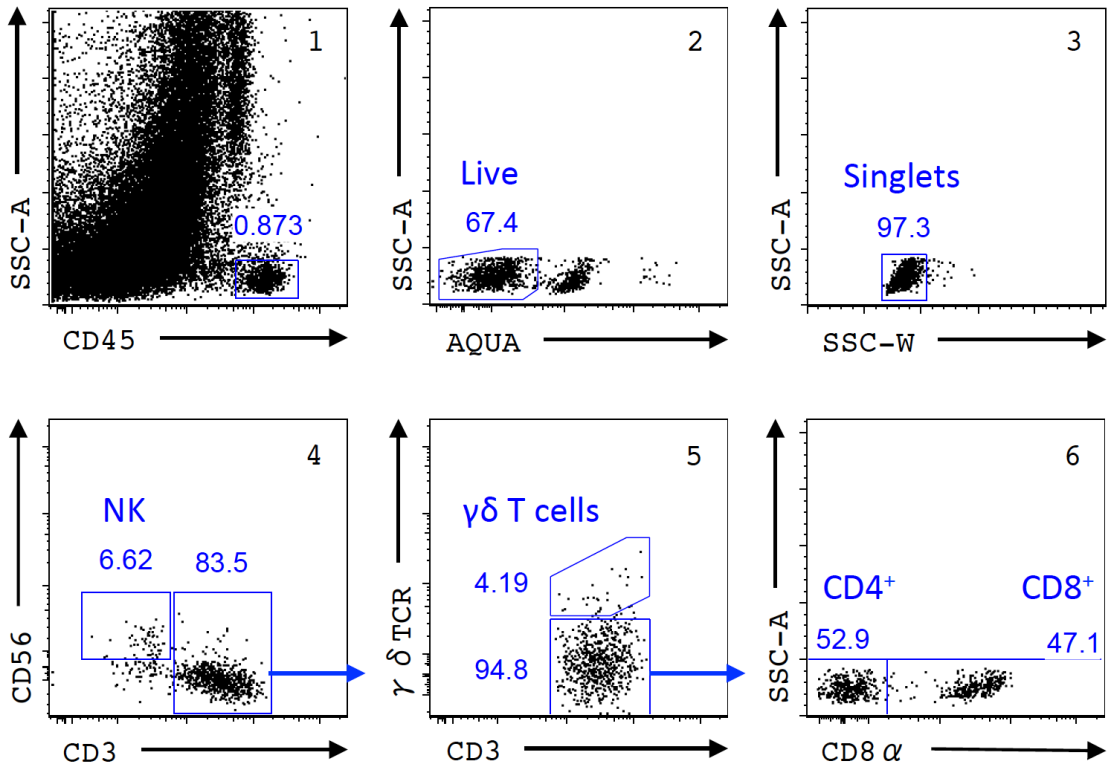

Figure 2.1. Consistent lymphocyte populations were isolated from human skin following enzymatic and mechanical disaggregation of tissue

Representative flow cytometry analysis showing gating strategy for major lymphocyte populations identified *ex vivo* following tissue disaggregation.

A

	Percentage of CD45$^+$ events (%)			
	CD4$^+$	**CD8$^+$**	**γδ T cells**	**NK**
Ex vivo	46.1 (± 15.2)	20.2 (± 14.8)	1.6 (± 1.0)	6.2 (± 5.0)
Explant *(Media only)*	64.3 (± 10.3)	19.5 (± 6.8)	1.1 (± 1.1)	2.8 (± 3.9)
Explant *(IL2 + IL15)*	62.3 (± 7.6)	18.5 (± 3.9)	3.4 (± 1.0)	8.1 (± 3.5)

B

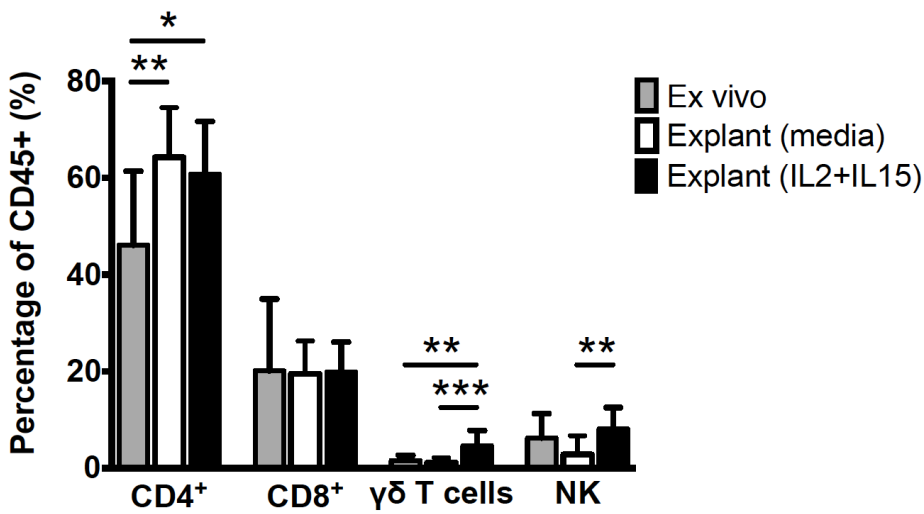

Figure 2.2. Consistent lymphocyte subsets were isolated from human skin following *ex vivo* and explant protocols

(A) Table and (B) graph summarising the different lymphocyte subsets isolated by either *ex vivo* or explant protocols; e*x vivo* lymphocyte subsets expressed as percentage of CD45$^+$ events, and explant lymphocyte subsets expressed as percentage of FSC-A/SSC-A lymphocyte gate (n = 7 – 14; mean with SD).

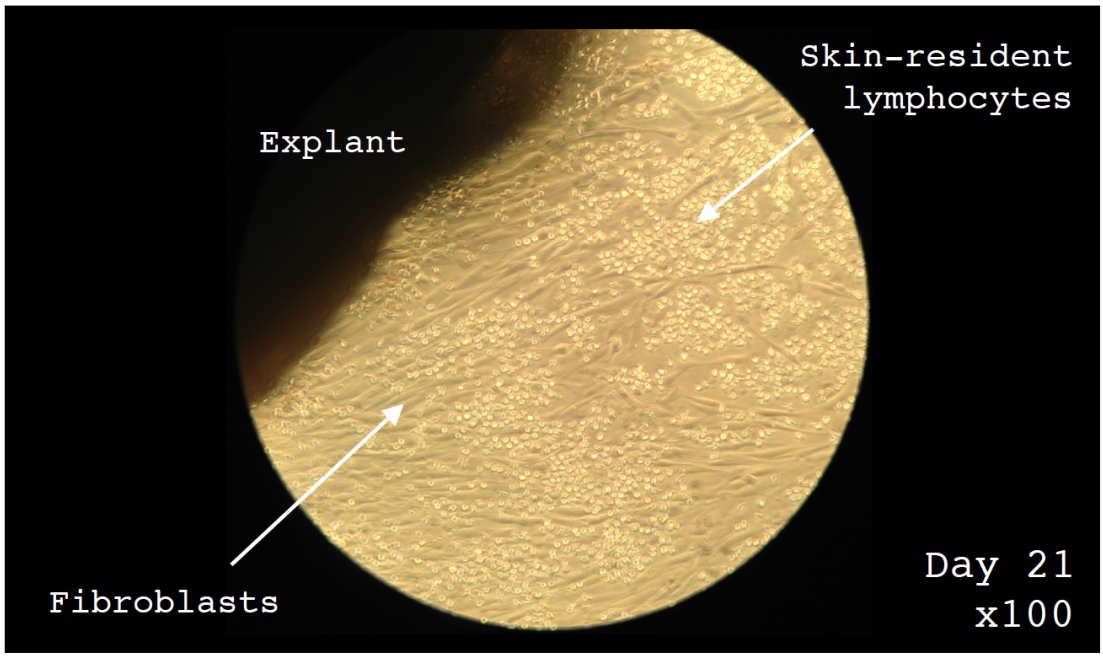

Figure 2.3. Skin explant culture allowed the isolation of skin-resident lymphocytes

Inverted light microscopy photograph of a skin explant following 21 days in culture with media supplemented with IL-2 (100 IU/ml) and (IL-15 10 ng/ml) (magnification x 100).

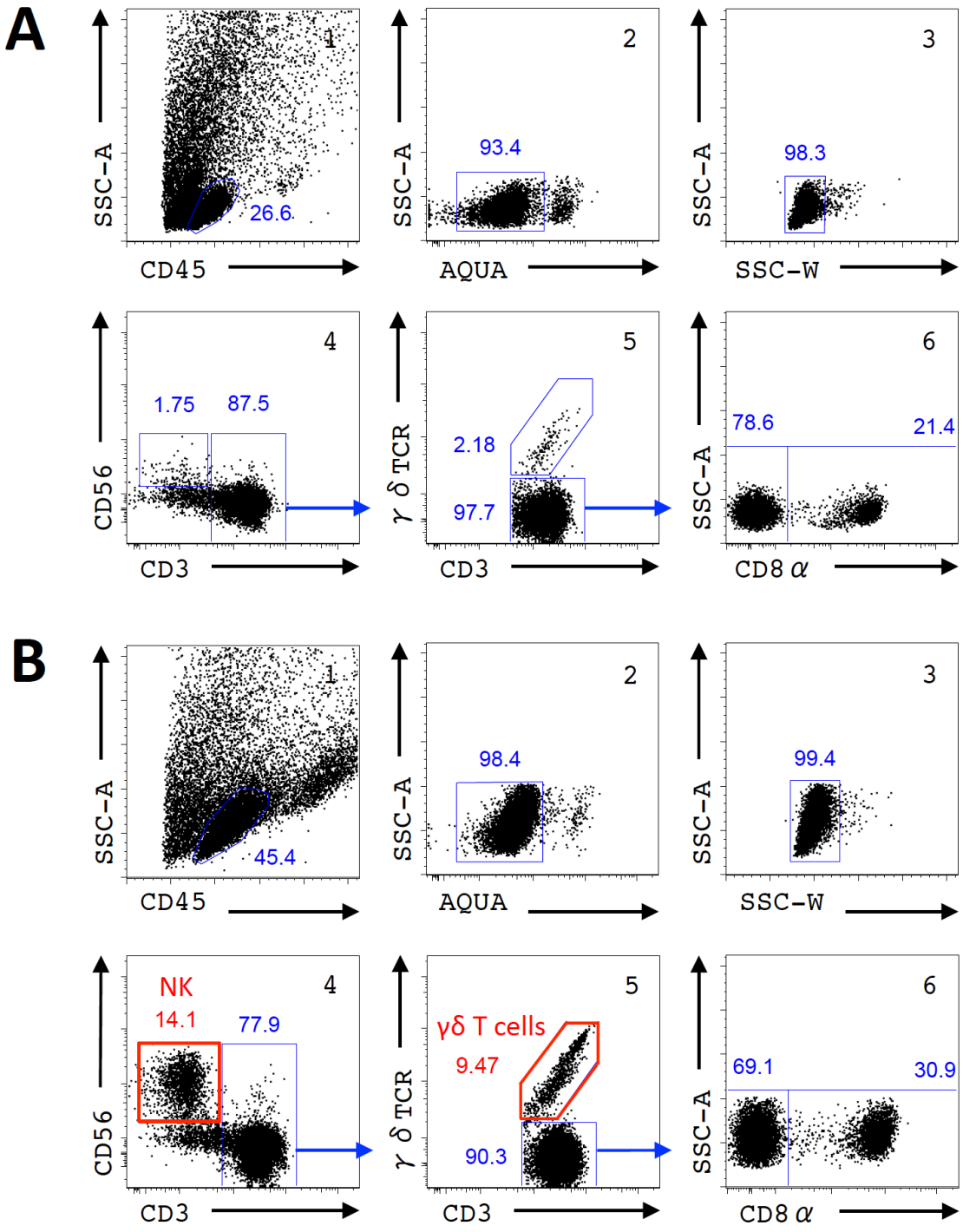

Figure 2.4. Skin-resident lymphocytes could be isolated by explant culture, with an enrichment of unconventional lymphocytes following the addition of IL-2 and IL-15

Representative flow cytometry analysis showing gating strategy for major lymphocyte populations following explant culture for 21 days in (A) plain media or (B) media supplemented with IL2 (100 IU/ml) and IL15 (10 ng/ml).

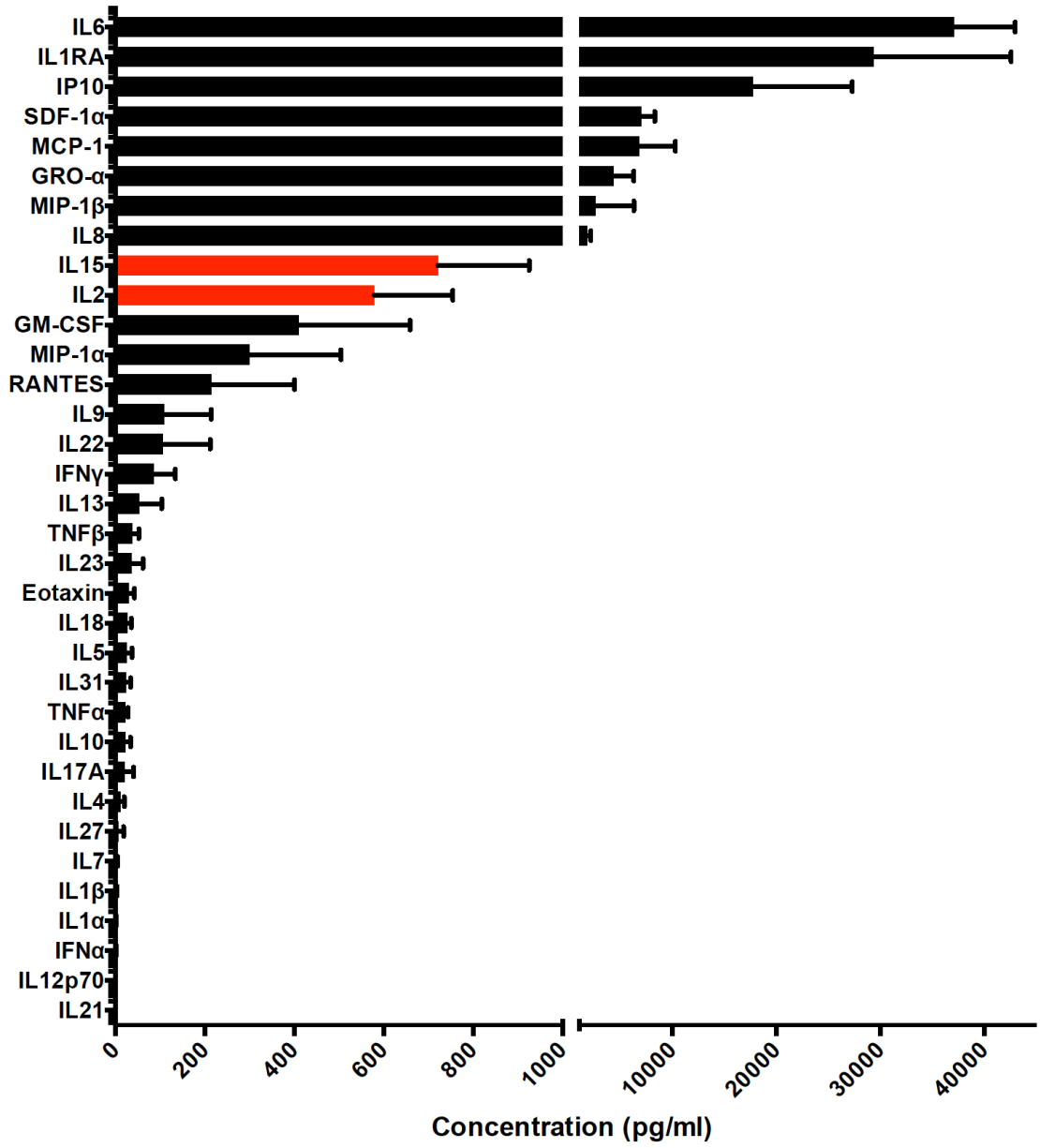

Figure 2.5. Skin explants conditioned the cell culture media with multiple chemokines and cytokines

ProcartaPlex™ Luminex assay of soluble cytokines and chemokines in explant supernatants at day 21 of culture. IL2 (100 IU/ml) and IL15 (10 ng/ml) had been added to the culture media (indicated in red) (n = 4; mean with SD).

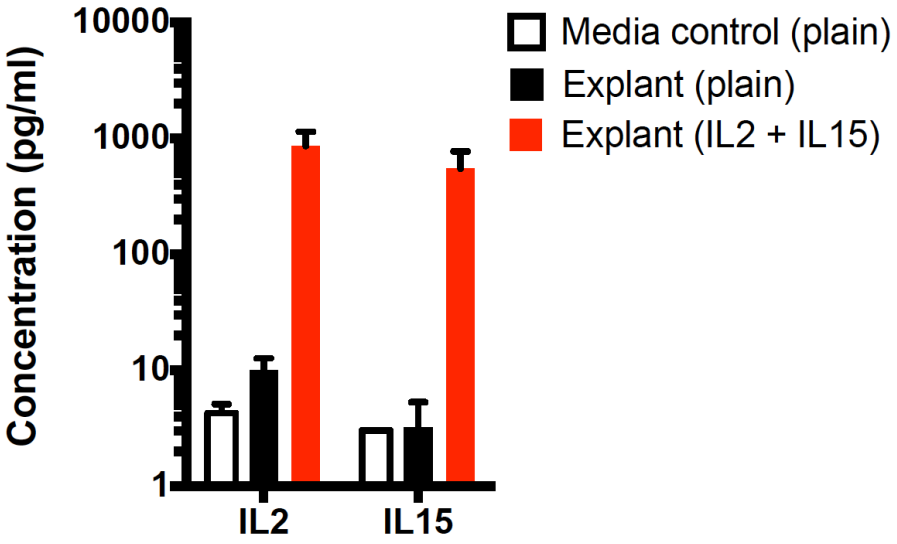

Figure 2.6. Soluble IL-2 and IL-15 were not readily detected in un-supplemented explant-conditioned media

Analysis of soluble IL-2 and Il-15 measured in explant-conditioned media at day 3 of culture using ProcartaPlex™ Luminex assay. Plain media had no cytokine added, whereas supplemented media contained additional IL-2 (100 IU/ml) and IL-15 (10 ng/ml). Unused plain media shown as background control for assay (n = 2; mean with SD).

2.3. Characterisation of the T_{RM} phenotype of skin-resident lymphocytes

Isolated skin-resident αβ T cells had a memory phenotype. This was based on CD45RA and CCR7 co-staining, where skin-resident $CD4^+$ αβ T cells had a predominant T_{EM} ($CD45RA^-CCR7^-$) and T_{CM} ($CD45RA^-CCR7^+$) phenotype; and $CD8^+$ αβ T cells a T_{EM} ($CD45RA^-CCR7^-$) and T_{EMRA} ($CD45RA^+CCR7^-$) phenotype (Figure 2.7) (Sallusto et al., 1999). Few naïve cells ($CD45RA^+CCR7^+$) were isolated using in this system. Using this classification, γδ T cells resembled T_{EM}/T_{EMRA} cells, essentially being $CCR7^-$ and with variable CD45RA expression. Consistent with a T_{RM} phenotype, most skin-resident T cells did not express the secondary lymphoid homing marker CD62L (L-selectin) (range 85.6 – 87.7 % $CD62L^-$; n = 6) (Figure 2.8). Skin-resident NK cells also did not express CCR7 or CD62L (Figure 2.8).

Skin-resident lymphocytes isolated using the explant system, including γδ T cells, retained markers indicative of skin-residency. They expressed the skin-associated E-selectin receptor CLA, with skin-resident T cells also expressing CCR4, which is associated with lymphocyte homing to the dermis (Figure 2.9) (Picker et al., 1990). Skin-resident NK cells did not obviously express CCR4. Skin-resident lymphocytes also strongly expressed CD69, which is associated with lymphocyte retention in the tissue and is considered a marker of T_{RM} cells (Mackay et al., 2015) (Figure 2.10). Skin-resident T cells also showed some constitutive expression of programmed cell death protein 1 (PD-1), a negative regulator of T cell function and marker of T cell exhaustion (mean 43 – 52% $PD\text{-}1^+$; n = 9 – 10) (Figure 2.10) (Barber et al., 2005).

DETC localisation and maintenance in the epidermal compartment depends on expression of the integrin CD103 (αEβ7) which can engage E-cadherin expressed by epithelial cells (Schön et al., 2002). Although CD103 expression was reportedly enriched within the human epidermal T cell compartment, it is neither ubiquitously nor exclusively expressed by these cells in human skin (Watanabe et al., 2015). Human skin-resident lymphocytes isolated from whole skin explants were largely $CD103^-$ (Figure 2.11). The limited CD103 expression seen was uniform across different lymphocyte subsets, including γδ T and NK cells (mean 25.0 – 32.7 % $CD103^+$; n = 9).

To confirm that explant-isolated lymphocytes had a representative skin-resident phenotype, skin-resident lymphocytes isolated by tissue digestion were also analysed for skin-homing and T_{RM} markers. *Ex vivo* T cells also mostly expressed the skin-homing markers CLA and CCR4, although expression was more variable on γδ T cells (Figures 2.12). T cells *ex vivo* also expressed both the activation marker CD69 and the inhibitory receptor PD-1 (Figures 2.12), which was consistent with explant-isolated T cells. NK cells *ex vivo* were largely negative for the markers tested; fewer NK cells expressed CLA and CD69 when compared with explant-isolated NK cells (17.7 % CLA^+ *ex vivo* compared with 57.4 %, $p = 0.003$; 21.3 % $CD69^+$ *ex vivo* compared with 94.8 % respectively, $p < 0.0001$); while NK cells *ex vivo* were $CCR4^-$ and $PD\text{-}1^-$, consistent with their explant-isolated counterparts.

For skin-resident T cells, the overall consistency of data between the isolation protocols, coupled with the greatly increased cell yields validated the three-dimensional explant protocol as a platform for the cells' further characterisation.

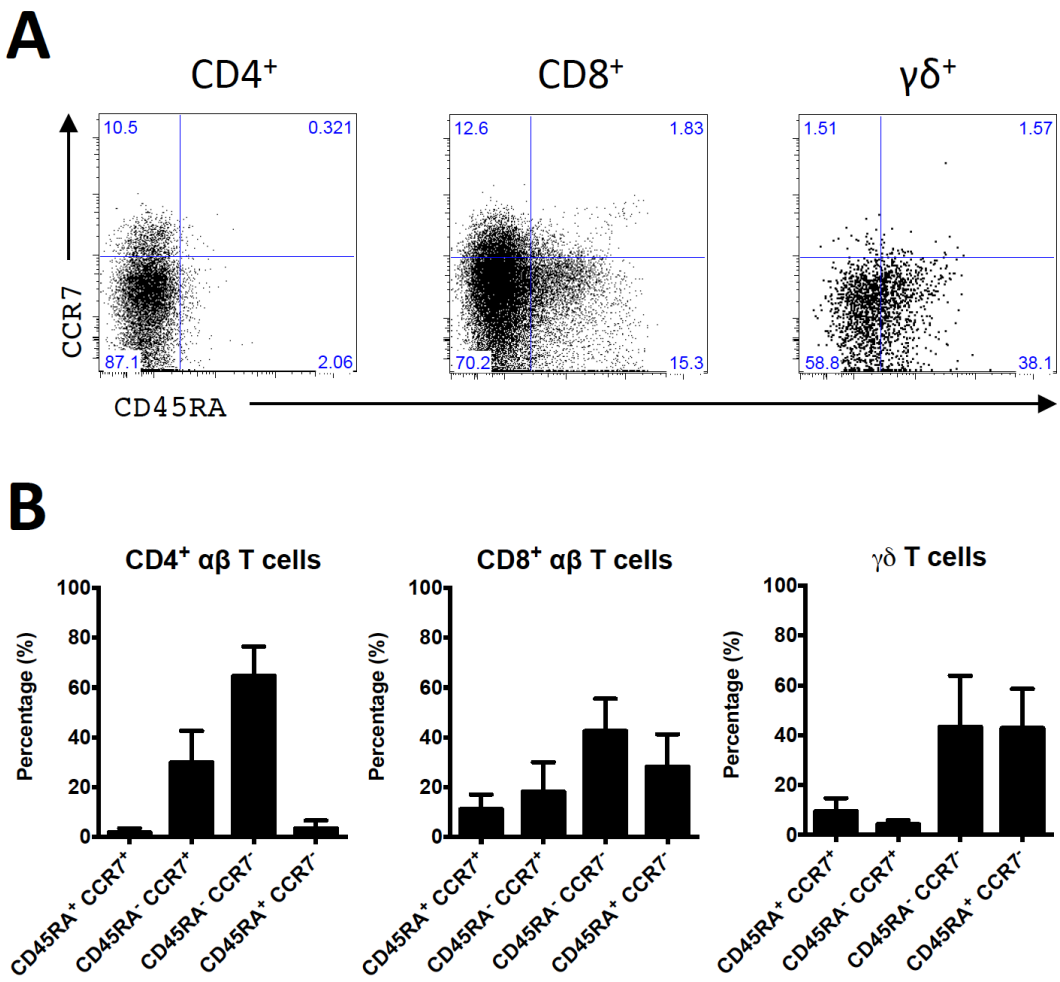

Figure 2.7. Explant-isolated skin T cells had a memory phenotype

(A) Representative flow cytometry analysis and (B) summary data showing CD45RA/CCR7 co-staining of skin-resident T cells isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml) (n = 8; mean with SD).

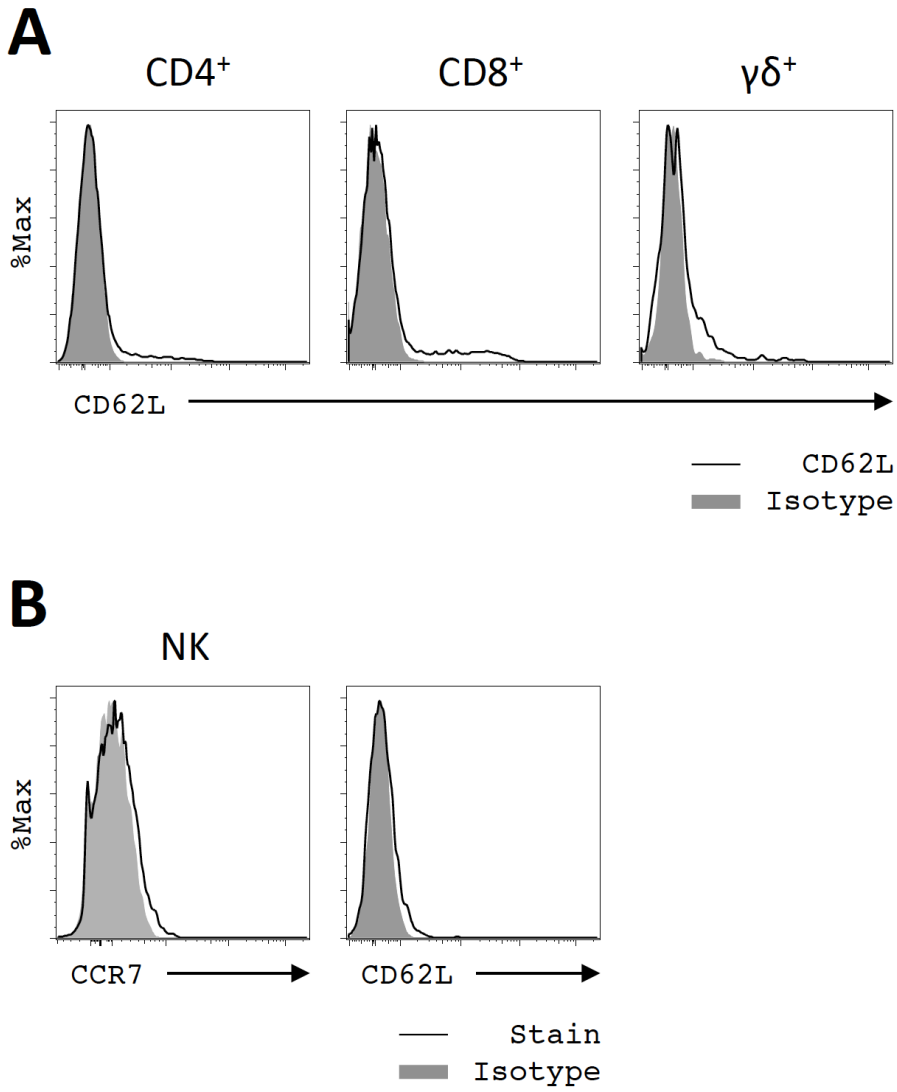

Figure 2.8. Explant-isolated skin lymphocytes lacked secondary lymphoid-homing markers

(A) Representative flow cytometry analysis showing CD62L (L-selectin) staining of skin-resident T cells. (B) Representative flow cytometry analysis showing CCR7 and CD62L staining of skin-resident NK cells. Cells isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml) (representative of 6 – 8 donors).

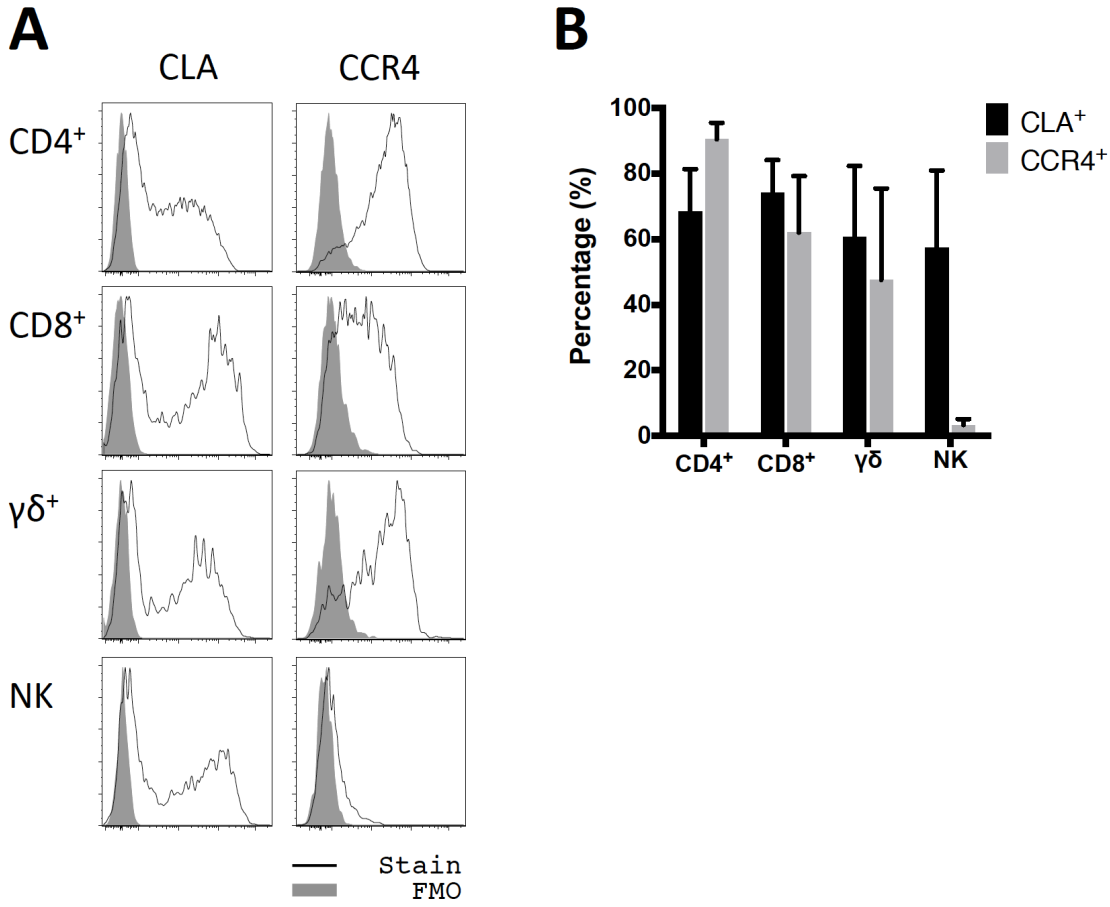

Figure 2.9. Explant-isolated skin lymphocytes had a skin-homing T_{RM} phenotype

(A) Representative flow cytometry analysis and (B) summary data of CLA and CCR4 expression by skin-resident lymphocytes isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml) (n = 7 – 9; mean with SD; gated against FMO).

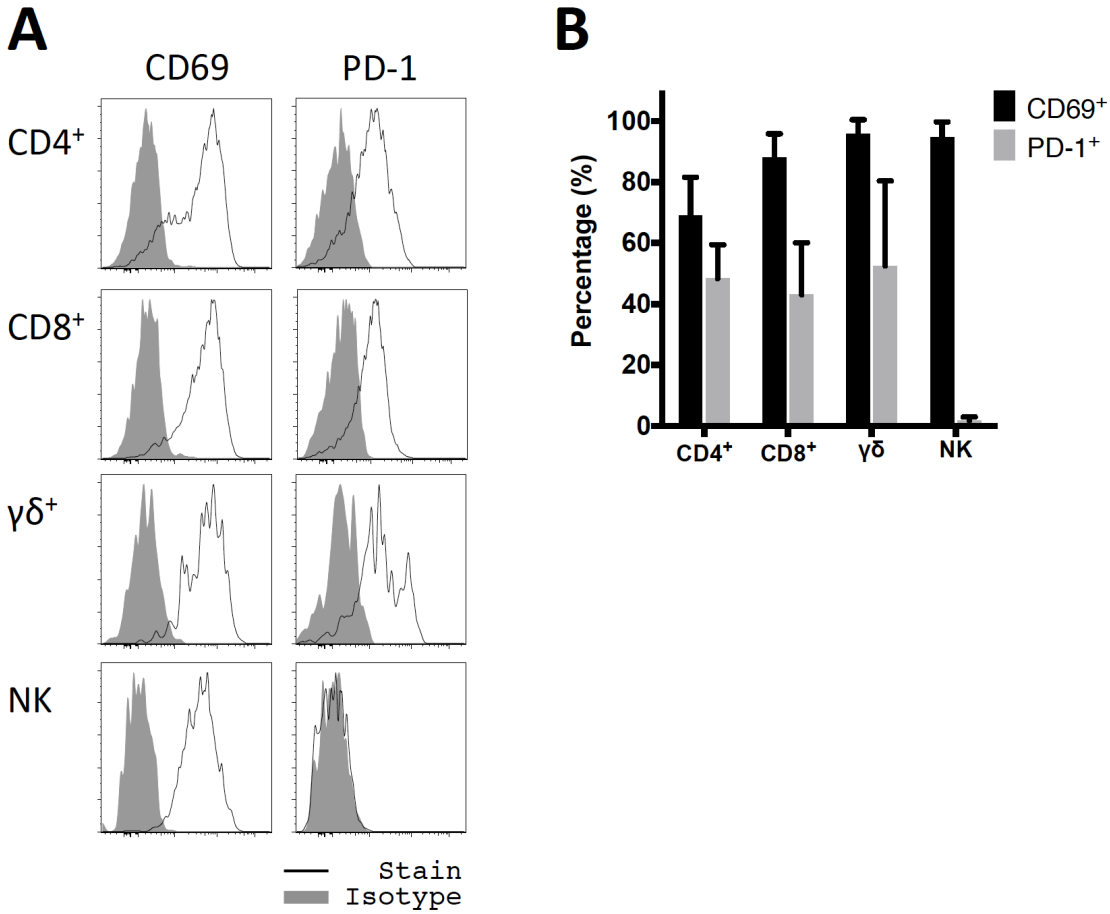

Figure 2.10. Explant-isolated skin lymphocytes display a skin-homing T_{RM} phenotype

(A) Representative flow cytometry analysis and (B) summary data of CD69 and PD-1 expression by skin-resident lymphocytes isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml) (n = 9 – 10; mean with SD; gated against isotype controls).

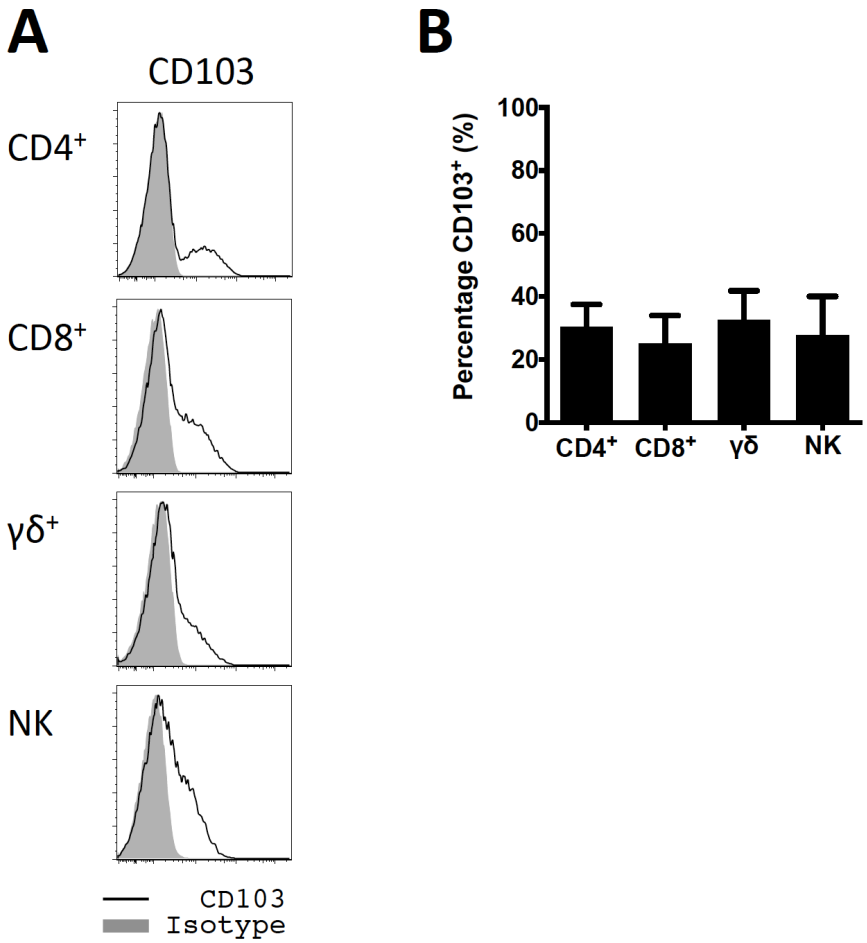

Figure 2.11. Explant-isolated skin lymphocytes from whole skin were predominantly CD103^{-}

(A) Representative flow cytometry analysis and (B) summary data showing CD103 expression by skin-resident lymphocytes isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml) (n = 10; mean with SD; gated against isotype controls).

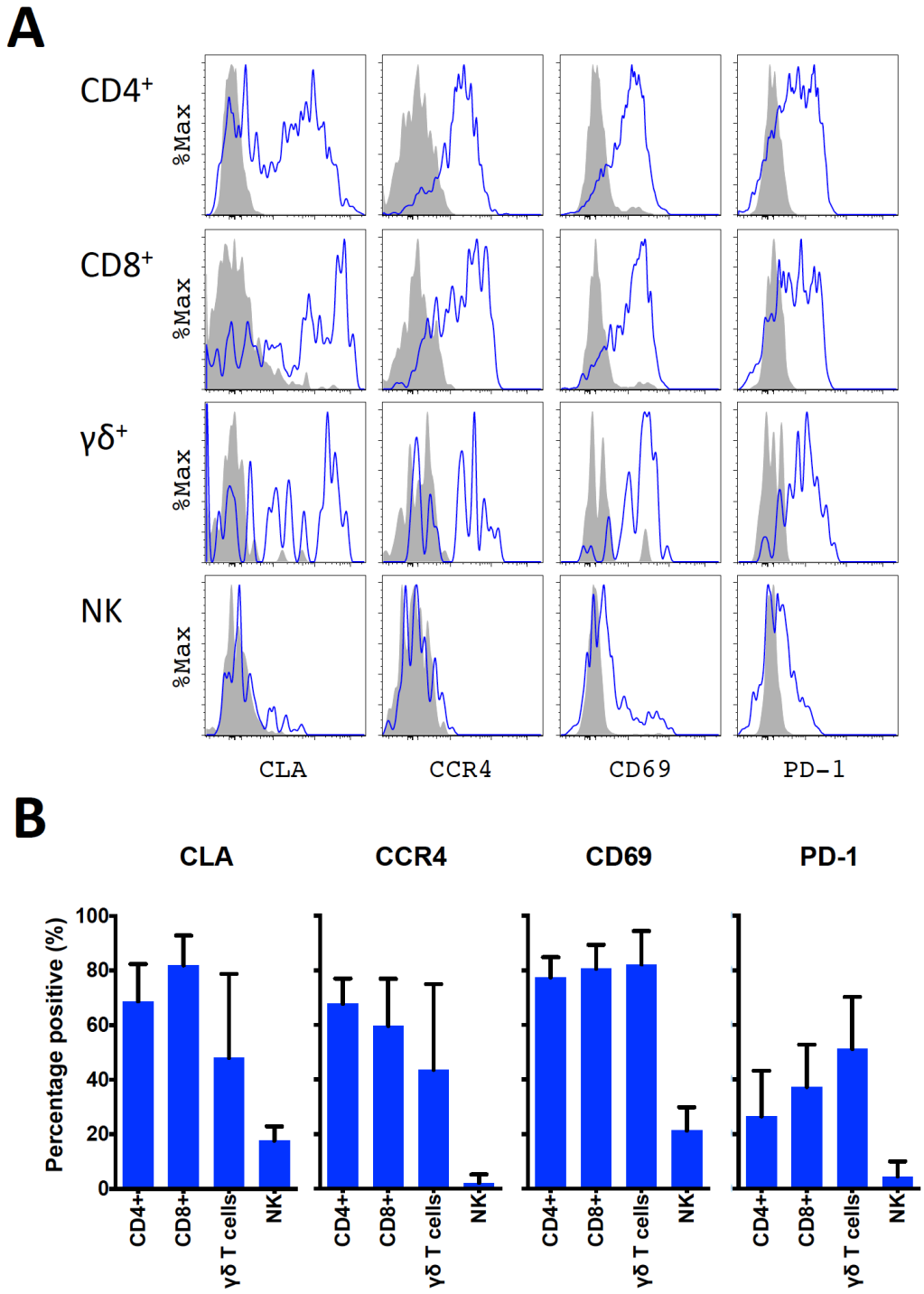

Figure 2.12. Skin-resident lymphocytes isolated *ex vivo* by tissue disaggregation largely had the same CLA, CCR4, CD69 and PD-1 expression profile as those isolated following explant culture

(A) Representative flow cytometry analysis and (B) summary data of *ex vivo* skin-resident lymphocyte CLA, CCR4, CD69 and PD-1 expression. Grey = FMO or isotype control. (n = 5; mean with SD; gated against FMO or isotype controls).

2.4. The skin-resident NKG2D$^+$ compartment

The innate-like function of murine skin-resident γδ T cells has been described in their response to MHC-I-like molecules *via* the NKG2D receptor. In characterising the potential of human skin-resident T cells to mediate such responses, we established which cells express this innate activatory receptor. Skin-resident CD8$^+$ αβ T cells, γδ T cells and NK cells were all found to express high levels of NKG2D using both *ex vivo* and explant protocols (Figure 2.13), whereas CD4$^+$ αβ T cells were NKG2D$^-$. This was consistent across a large number of individuals, and comparable to NKG2D$^+$ lymphocyte subsets found in human blood (Bauer et al., 1999; Sutherland et al., 2002).

Expression of the classical co-stimulatory molecule CD28, which interacts with CD80/86 expressed by APCs during MHC-restricted activation, was variable amongst skin-resident T cells (Figure 2.14). γδ T cells were largely CD28$^-$ but CD8$^+$ αβ T cells displayed variable CD28 expression, with distinct CD28$^+$ and CD28$^-$ subsets seen. Skin-resident CD4$^+$ αβ T cells were largely CD28$^+$. Further analysis of these subsets revealed that NKG2D expression was higher in CD28$^-$ populations (Figure 2.15). Loss of CD28 expression has been described in association with the development of memory CD8$^+$ αβ T cells; thus, the increase in NKG2D expression in this subset may indicate a transition in the activatory or co-stimulatory requirement(s) of these cells (Azuma et al., 1993).

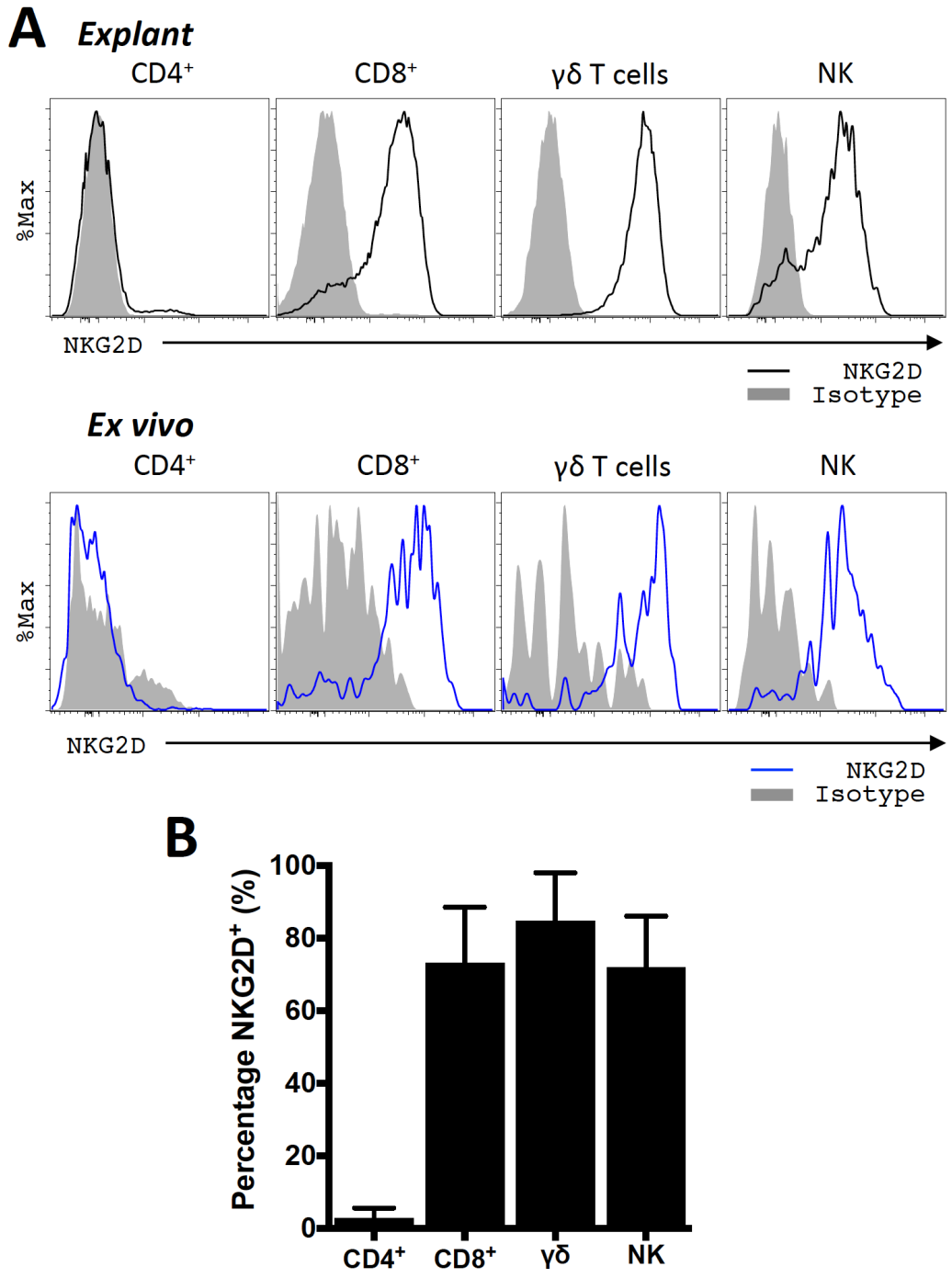

Figure 2.13. Skin-resident γδ T cells, CD8$^+$ αβ T cells and NK cells expressed the activating NKG2D receptor

(A) Representative flow cytometry analysis and (B) summary data of skin-resident lymphocyte NKG2D expression, isolated by *ex vivo* or explant (with IL2 and IL15) protocols (n = 22; mean with SD).

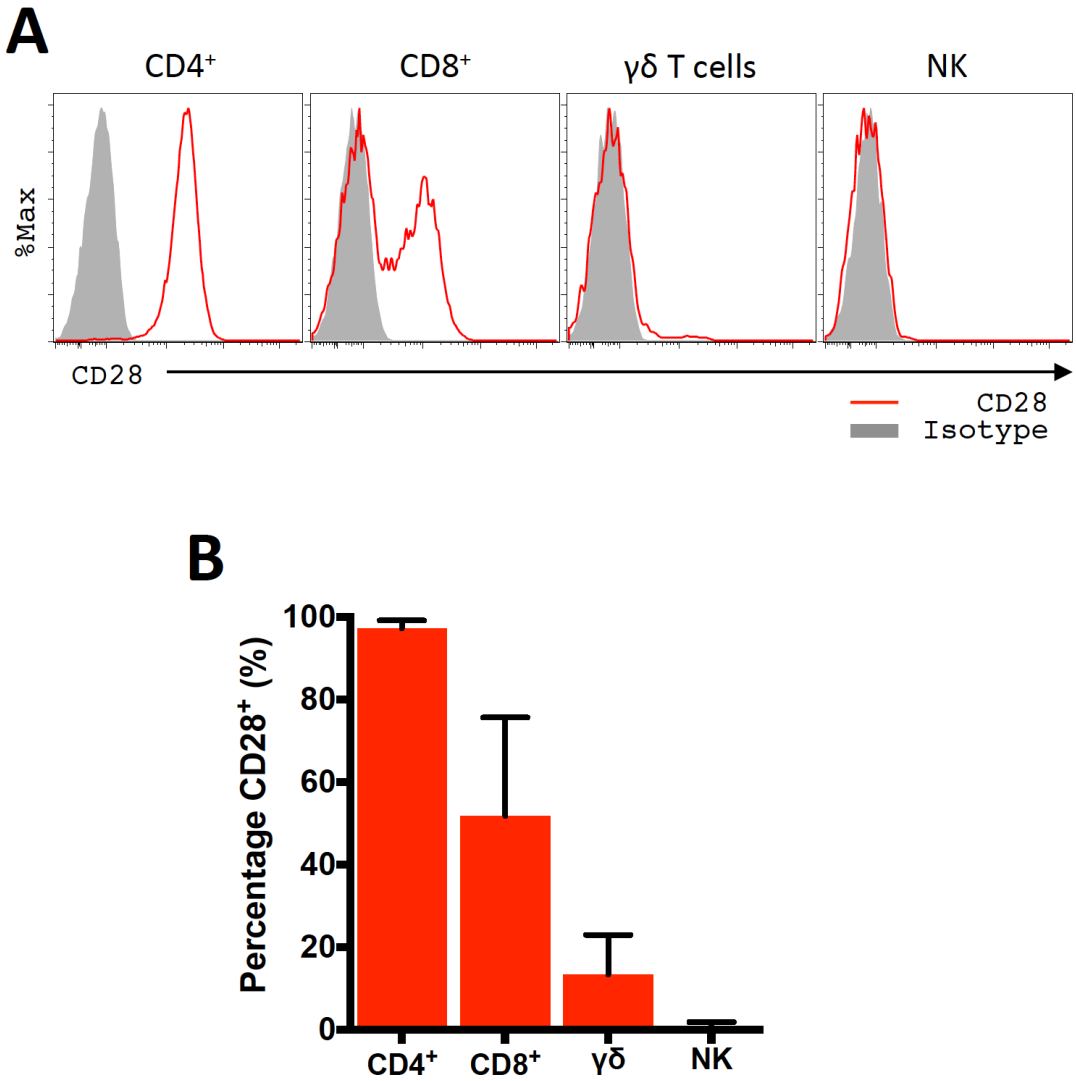

Figure 2.14. Skin-resident T cells showed variable CD28 expression, with γδ T cells being largely CD28^{-}

(A) Representative flow cytometry analysis and (B) summary data showing CD28 expression by skin-resident lymphocytes isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml) (n = 14; mean with SD).

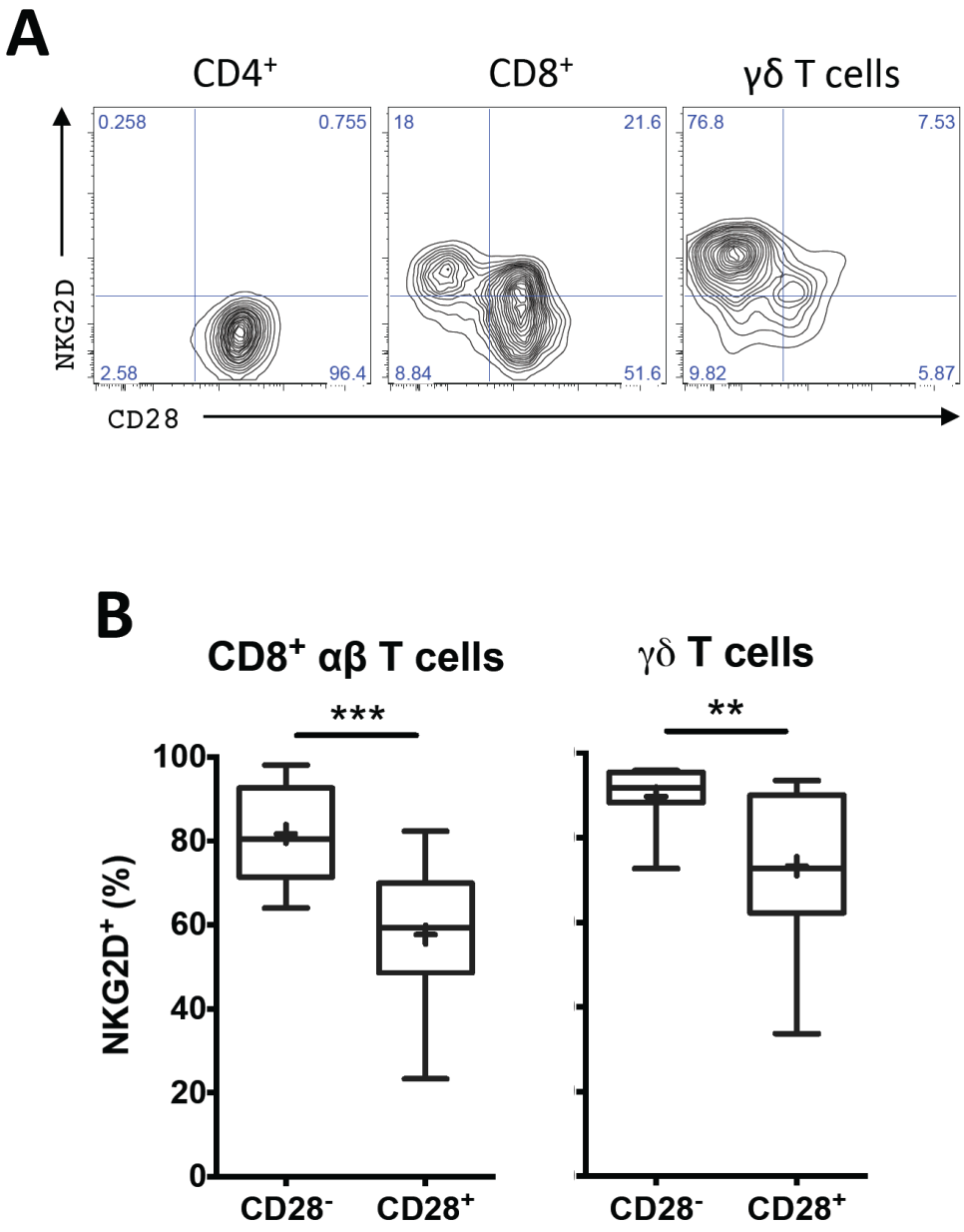

Figure 2.15. NKG2D expression was higher on CD28⁻ skin-resident T cells

(A) Representative flow cytometry analysis and (B) summary data of CD28/NKG2D co-staining by skin-resident T cells isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml) (n = 12; Box with mean [+], median and interquartile range, whiskers indicate min to max range; test: *t* test).

2.5. Characterisation of the skin-resident γδ T cell compartment

Further analysis of the skin-resident γδ T cells revealed that they predominantly expressed the Vδ1 TCR chain (Figures 2.16A and B). $V\delta1^+$ cells made up 4.82 % (± 4.0) of the T cells that were isolated, compared with just 0.28 % (± 0.39) for $V\delta2^+$ T cells (Figure 2.17B). Therefore, $V\delta1^+$ T cells outnumbered $V\delta2^+$ T cells by a mean ratio of 33.4 to 1 (± 33.3). This was in contrast to the peripheral blood γδ T cell compartment, which is defined by the predominant $V\gamma9^+V\delta2^+$ T cell population. In a cohort of unrelated healthy adult donors 4.28 % (± 2.67) of peripheral blood $CD3^+$ T cells were $V\delta2^+$, compared with 0.46 % (± 0.18) for $V\delta1^+$ cells (Figures 2.16C and B). Therefore, in this cohort blood $V\delta2^+$ T cells outnumbered $V\delta1^+$ T cells with a mean ratio of 10.6 to 1 (± 7.8). Thus, skin-resident γδ T cells are highly distinct from blood γδ T cells, predominantly expressing the Vδ1 TCR chain.

$V\delta1^+$ T cells, and the less frequent $V\delta2^+$ T cells, expressed variable levels of the co-stimulatory molecule CD27, which, in combination with CD45RA co-staining, was consistent with a phenotype associated with non-terminally differentiated γδ T cells (Figure 2.17) (Dieli et al., 2003). $V\delta1^+$ T cells also expressed higher levels of NKG2D than $V\delta2^+$ cells (Figure 2.18A). $V\delta1^+$ T cells were largely $CD28^-$, with the $V\delta2^+$ subset accounting for the small number of $CD28^+$ γδ T cells seen (Figure 2.18B).

In addition to the NKG2D receptor, additional activatory natural killer-type receptors have been described, which have been implicated in the function of $V\delta1^+$ T cells derived from the blood (Correia et al., 2011; Lanier, 2005). In our system, skin-resident $V\delta1^+$ T cells only expressed low levels of the natural cytotoxicity receptors (NCRs) NKp30 and NKp44, and were largely negative for NKp46 (mean 21.6 % [± 16.6]; 21.2 % [± 16.9]; 9.9 % [± 7.2], respectively; n = 6 – 8) (Figure 2.19A). The other isolated lymphocyte subsets, including the few $V\delta2^+$ T cells found, also did not express significant levels of the NCRs, except for skin-resident NK cells – mean NKp30 expression 70.0 % (± 16.1); NKp44 65.0 % (± 19.0); NKp46 55.6 % (± 23.9) (n = 6 – 8) (Figure 2.19B). Skin-resident lymphocytes were also largely negative for other members of the NKG2/CD94 receptor family (Lanier, 2005), including the inhibitory NKG2A receptor and the activatory NKG2C receptor (Figures 2.19C and

D); just 2.57 % (± 1.8) of Vδ1^{+} T cells expressed NKG2A and 0.77 % (± 0.28) NKG2C. The exception were NK cells that showed low levels of NKG2A expression (mean 13.9 % (± 6.8) NKG2A^{+}) (Figure 2.19D).

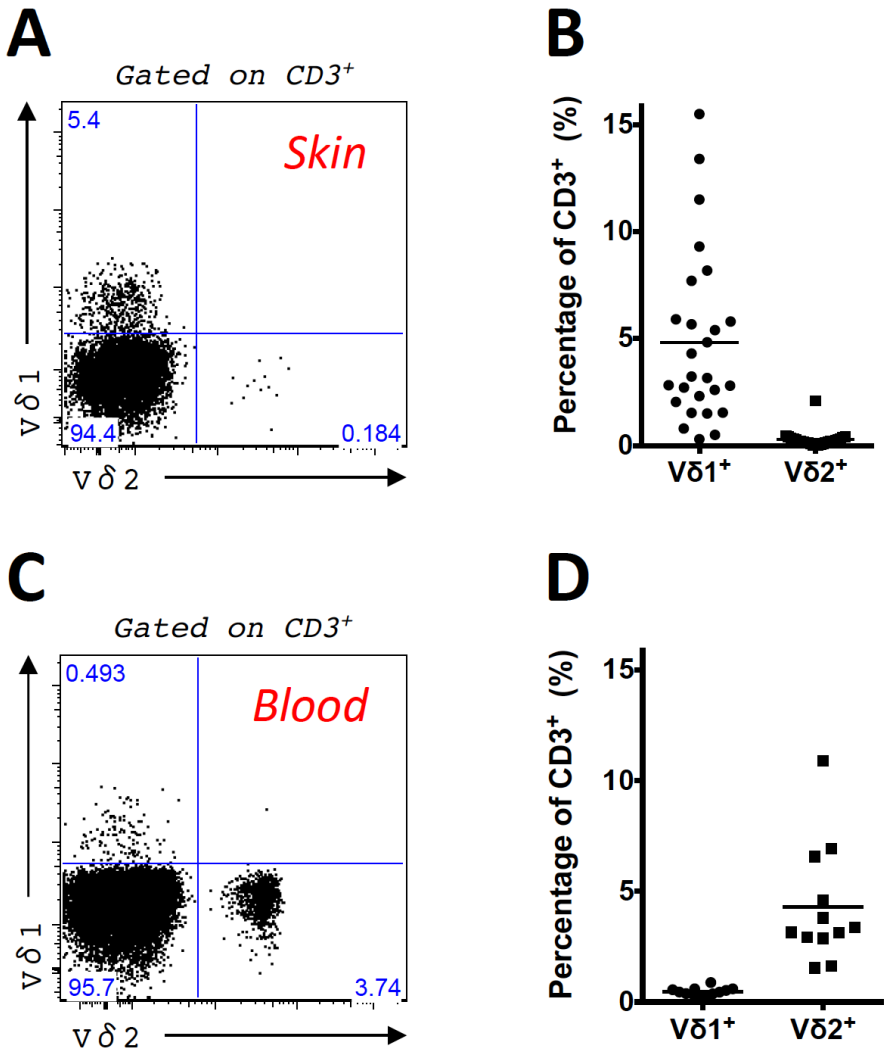

Figure 2.16. Skin-resident γδ T cells predominantly expressed the Vδ1 TCR chain, making them distinct from the blood γδ T cell compartment

(A) Representative flow cytometry analysis and (B) summary data of Vδ TCR chain usage by skin-resident T cells isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml) (n = 26; mean indicated; lymphocytes isolated after 21 – 28 days of explant culture). (C) Representative flow cytometry analysis and (D) summary data of Vδ TCR chain usage by peripheral blood T cells (n = 12; unmatched blood donors; mean indicated).

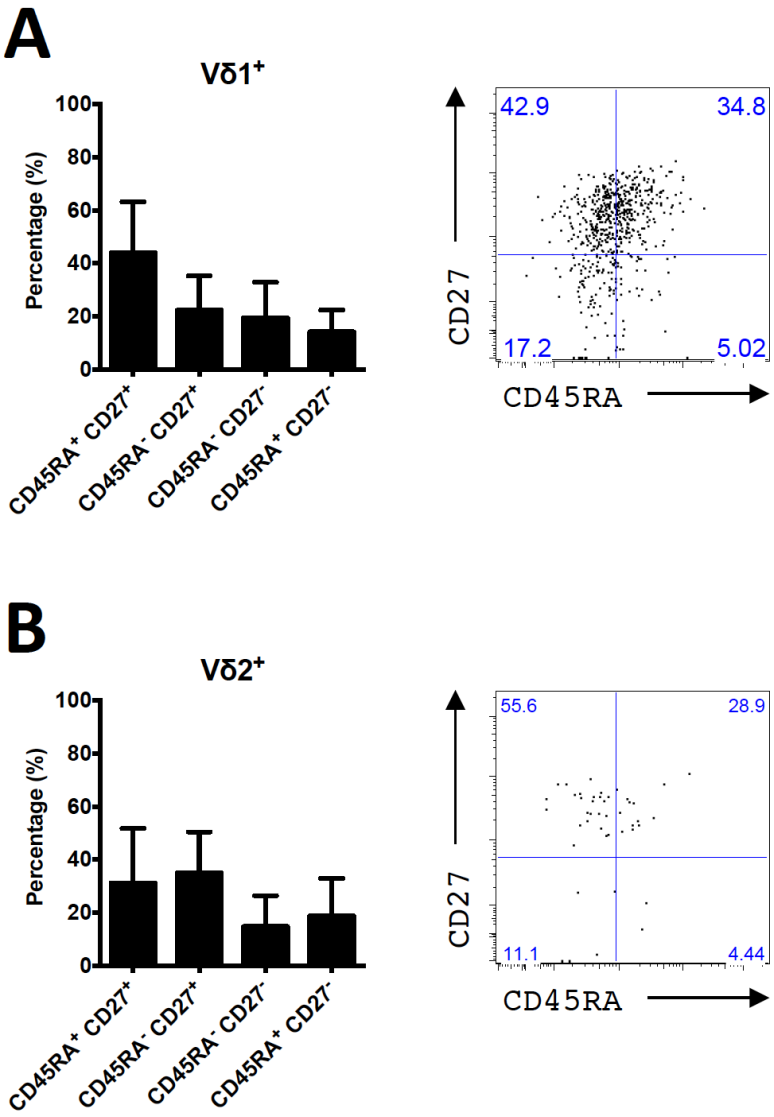

Figure 2.17. Explant-isolated skin Vδ1$^+$ and Vδ2$^+$ T cells had predominantly a non-terminally differentiated phenotype

(A) Summary data and (B) representative flow cytometry analysis showing CD45RA/CD27 co-staining of skin-resident Vδ1$^+$ and Vδ2$^+$ T cells isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml) (n = 8; gated against istotype matched control; mean with SD).

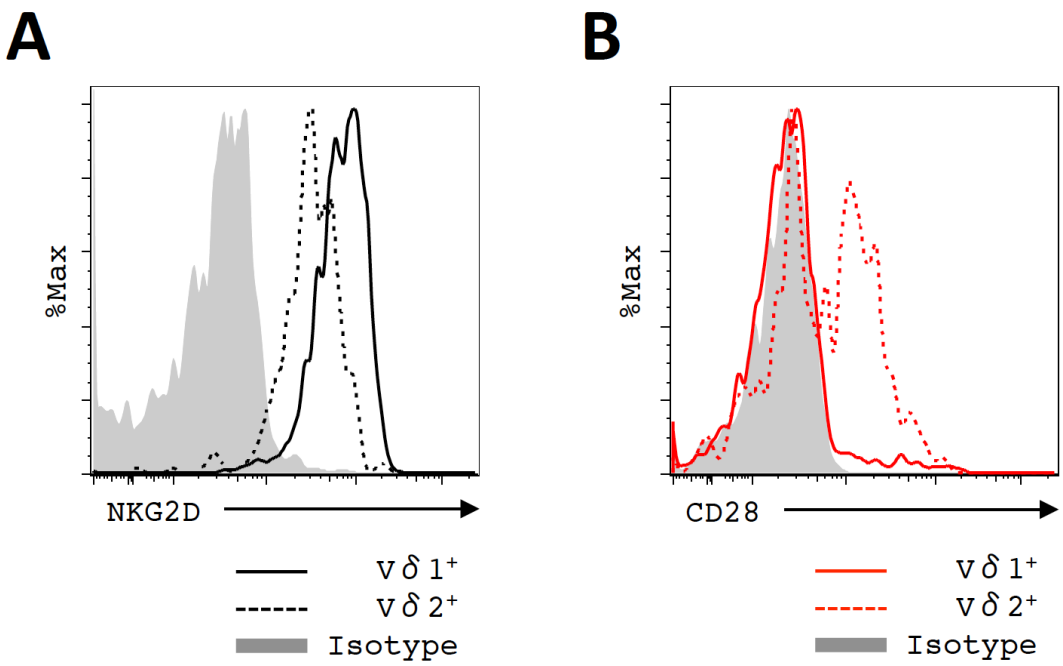

Figure 2.18. Skin-resident Vδ1$^+$ T cells expressed higher levels of NKG2D than Vδ2$^+$ T cells, and were CD28$^-$

Representative flow cytometry analysis of the relative expression of (A) NKG2D and (B) CD28 by skin-resident Vδ1$^+$ and Vδ2$^+$ T cells isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml) (Representative of 9 or 5 donors, respectively).

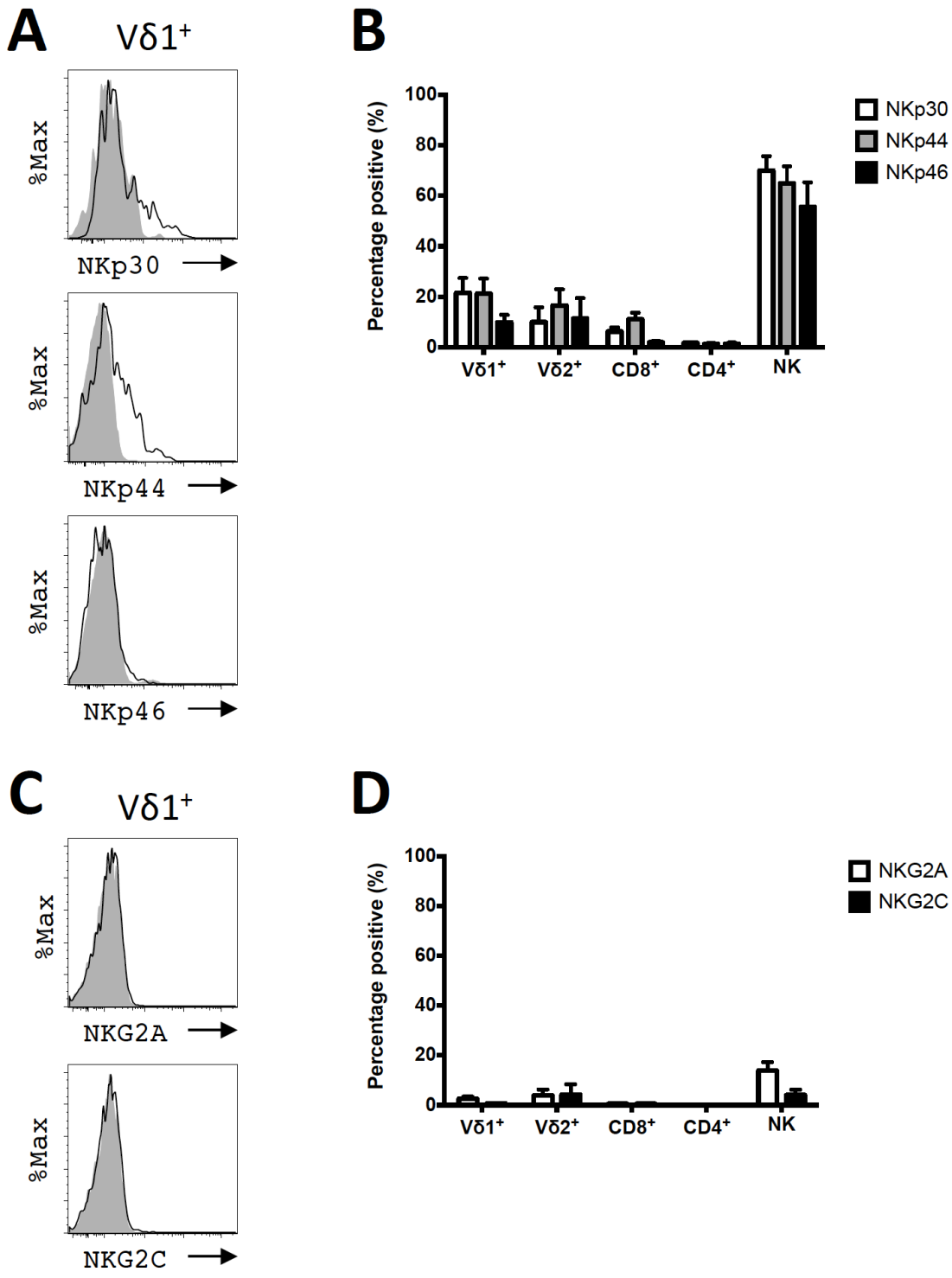

Figure 2.19. Skin-resident Vδ1$^+$ T cells expressed low levels of other natural killer-type receptors

A) Representative flow cytometry analysis of Vδ1$^+$ T cells and (B) summary data for skin-resident lymphocytes showing NKp30, NKp44 and NKp46 expression following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml). Grey = isotype

control (n = 6 – 8; mean with SD). (C) Representative flow cytometry analysis of $V\delta1^+$ T cells and (B) summary data for skin-resident lymphocytes showing NKG2A and NKG2C expression following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml). Grey = isotype control (n = 4; mean with SD).

2.6. Conclusion

We were able to demonstrate that skin-resident lymphocytes could be isolated from healthy tissue by both tissue disaggregation and novel explant protocols. The explant protocol supplemented with the cytokines IL-2 and IL15, which provokes the egress of immune cells from the tissue, allowed the isolation of large numbers of both αβ T_{RM} cells and γδ T cells. This facilitated their further study. Skin-resident γδ T cells predominantly expressed the Vδ1 TCR chain, confirming their distinctiveness relative to circulating γδ T cells found in the blood. Skin-resident γδ T cells and $CD8^+$ αβ T_{RM} were $NKG2D^+$, but showed variable expression of CD28 and lacked expression of other NCRs. Skin-resident NK cells were $NKG2D^+$ and expressed a range of NCRs. This system therefore provided a platform to further characterise the functional potentials of human skin-resident lymphocytes, and their capacity to make innate-like responses *via* the NKG2D receptor.

Chapter Three: Characterising the functional potentials of human skin-resident lymphocytes, and their capacity to make innate-like responses

Having established an explant system that enabled the consistent isolation of distinct skin-resident lymphocyte subsets, systems were developed to assess the functional potentials of these cells.

3.1. Skin-resident lymphocytes isolated by explant protocol were readily activated *in vitro* to reveal their functional potential

In order to ascertain the functional potential and differentiation bias of skin-resident lymphocyte subsets, they were activated *in vitro* with Phorbol 12-myristate 13-acetate (PMA) and ionomycin and stained intracellularly for the cytokines IFNγ, TNFα, IL-4, IL-13, IL-17A and IL-22, respectively, since these are well established to differentiate effector function bias among conventional, adaptive αβ T cells. Thus, IFNγ is primarily produced by T_H1 or CTLs, programmed by the transcription factor, Tbx21; IL-4 is primarily produced by T_H2 cells, programmed by the transcription factor, GATA3; IL-17 is primarily produced by T_H17 cells, programmed by the transcription factor, RORC; and IL-22 is primarily produced by T_H22 cells, regulated by AHR and the transcription factor, RORC (Iwasaki and Medzhitov, 2015; Trifari et al., 2009).

Upon activation all skin-resident lymphocyte subsets readily produced cytokines. γδ T cells predominantly displayed a T_H1-like phenotype, producing IFNγ and TNFα (56.5 % ± 20.6 $IFN\gamma^+$ and 82.4 % ± 20.6 $TNF\alpha^+$ on intracellular cytokine staining; n = 10 – 18) (Figure 3.1A). Although there was inter-individual variation in the frequency of IFNγ-producing γδ T cells (range 13.0 – 90.8 %), IFNγ was largely co-produced by $TNF\alpha^+$ cells (Figure 3.1B). In some individuals there appeared to be low-level production of IL-4, IL-13 and IL-17A by γδ T cells (2.4 % ± 2.4, 3.3 % ± 3.3, and 2.9 % ± 2.6, respectively). Although this was not a consistent observation across donors in this cohort, for some individuals, γδ T cell T_H2-type responses were more pronounced with upto 9.8 % and 14.1 % staining for IL-4 and IL-13, respectively.

Skin-resident αβ T cells showed a wide range of cytokine production, which were in contrast to the γδ T cells isolated under the same conditions (Figure 3.2). TNFα production was also a dominant response following $CD4^+$ αβ T cell activation (92.7 % ± 4.0 and 89.6 % ± 6.5, respectively; n = 19). Skin-resident $CD4^+$ αβ T cells were also a source of the cytokines IFNγ, IL-4, IL-13, IL-17A and IL-22 (21.6 % ± 8.6, 17.6 % ± 7.8, 28.6 % ± 13.5, 11.0 % ± 8.4 and 15.1 % ± 9.6, respectively; n = 17 – 19). IFNγ was largely polarised from IL-4 or IL-13 production (Figure 3.3A), with IL-13 being the predominant type 2 cytokine detected (Figure 3.3B). In addition, IL-17A- and IL-22-producing $CD4^+$ αβ T cells were also largely polarised (Figure 3.3C). These data were consistent with the constitutive presence of skin-resident T_H1, T_H2, T_H17 and T_H22 populations within normal healthy tissue, as patients with known inflammatory skin disease were excluded from this study.

Skin-resident $CD8^+$ αβ T cells, in addition to TNFα, predominantly produced IFNγ (55.1% ± 15.5; n = 19), which was consistent with a T_C1 phenotype (Figure 3.2). However, skin-resident $CD8^+$ T cells showed some additional functional diversity, and were a source of IL-4, IL-13, IL-17A and IL-22 – albeit less than the $CD4^+$ αβ T cells (13.2 % ± 10.2, 13.5 % ± 9.8, 5.9 % ± 4.8 and 6.0 % ± 7.7, respectively; n = 17 – 19). $CD8^+$ T cell IFNγ production was also polarised from IL-4/IL-13 production; and IL-4 and IL-13 were largely co-produced by the same cells (Figure 3.3D and E). IL-17A- and IL-22-producing $CD8^+$ T cells were also polarised (Figure 3.3F). Therefore, in addition to a diverse population of $CD4^+$ αβ T cells, healthy skin contained resident T_C1, T_C2, T_C17 and T_C22 cells.

The cytokine profile of skin-resident NK cells was comparable to that of the γδ T cell compartment. They predominantly stained positive for IFNγ and TNFα (37.7 % ± 14.8 and 74.8 % ± 12.0, respectively; n = 16) (Figure 3.2). NK cells did not seem to produce the cytokines IL-4, IL-13, IL-17A or IL-22 under the conditions employed.

To investigate cells' responses to more physiological stimuli, plate-bound anti-CD3 antibody was used to cross-link the TCR for 6 hours and mimic activation *via* the TCR complex. Skin-resident T cells again displayed distinct cytokine-producing profiles similar to those seen following activation with PMA and ionomycin (Figure

3.4). Predictably however, the extent of cytokine production was less than that seen with the PMA and ionomycin. All T cell subsets produced TNF-α (range 44.7 – 50.9 %) (Figure 3.4). Similarly, all T cell subsets produced IFN-γ upon activation, with a greater proportion of $CD8^+$ αβ T cells and γδ T cells being IFN-γ^+ (28.5 % ± 8.6 and 26.2 % ± 11.7, respectively), compared with $CD4^+$ αβ T cells (8.6 % ± 3.9) (Figure 3.4). As with PMA and ionomycin assays, both $CD4^+$ and $CD8^+$ αβ T cells were also a source of IL-4, IL-13 and IL-17, and $CD4^+$ αβ T cells a source of IL-22. As expected, NK cells were not directly activated by plate-bound anti-CD3 antibody (Figure 3.4).

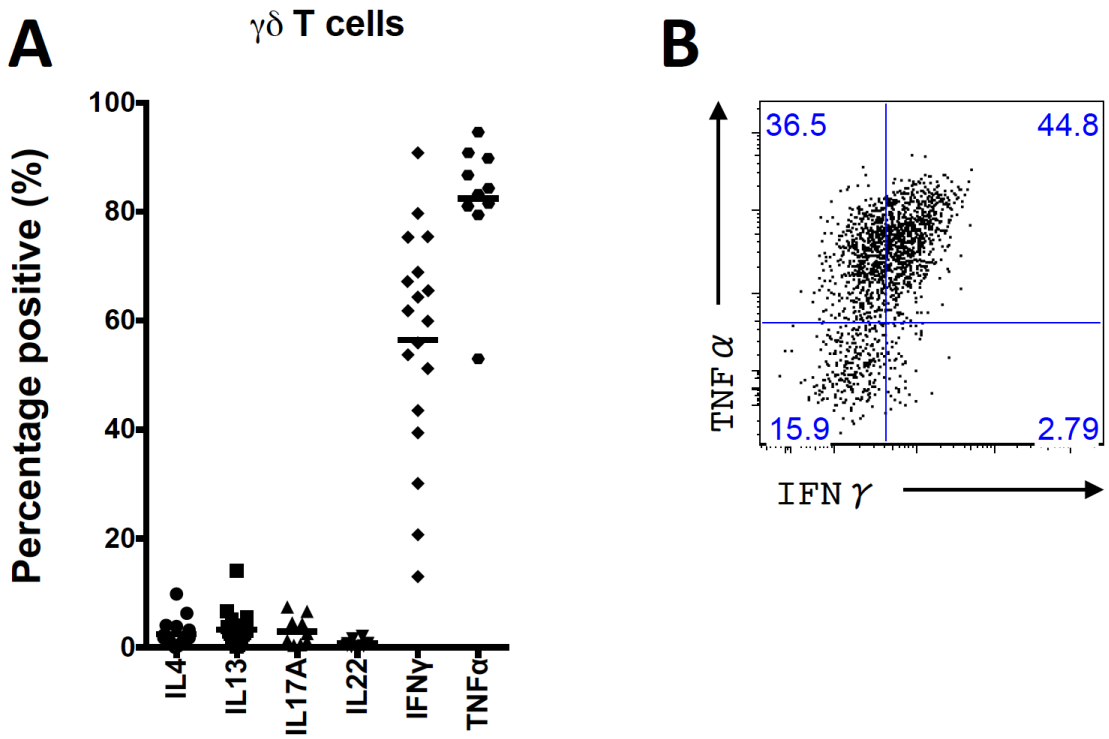

Figure 3.1. Upon activation *in vitro*, skin-resident γδ T cells had a predominantly T_H1-like phenotype

(A) Summary data showing intracellular cytokine staining of skin-resident γδ T cells, isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml), following *in vitro* activation with PMA (10 ng/ml) and ionomycin (1 μg/ml) [5h] with BFA (20 μg/ml) [4h] (gated against un-stimulated controls; mean indicated; n = 10 – 18). (B) Representative flow cytometry analysis showing intracellular co-staining for TNFα and IFNγ in skin-resident γδ T cells isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml), following *in vitro* activation with PMA (10 ng/ml) and ionomycin (1 μg/ml) [5h] with BFA (20 μg/ml) [4h]. Gated against un-stimulated controls.

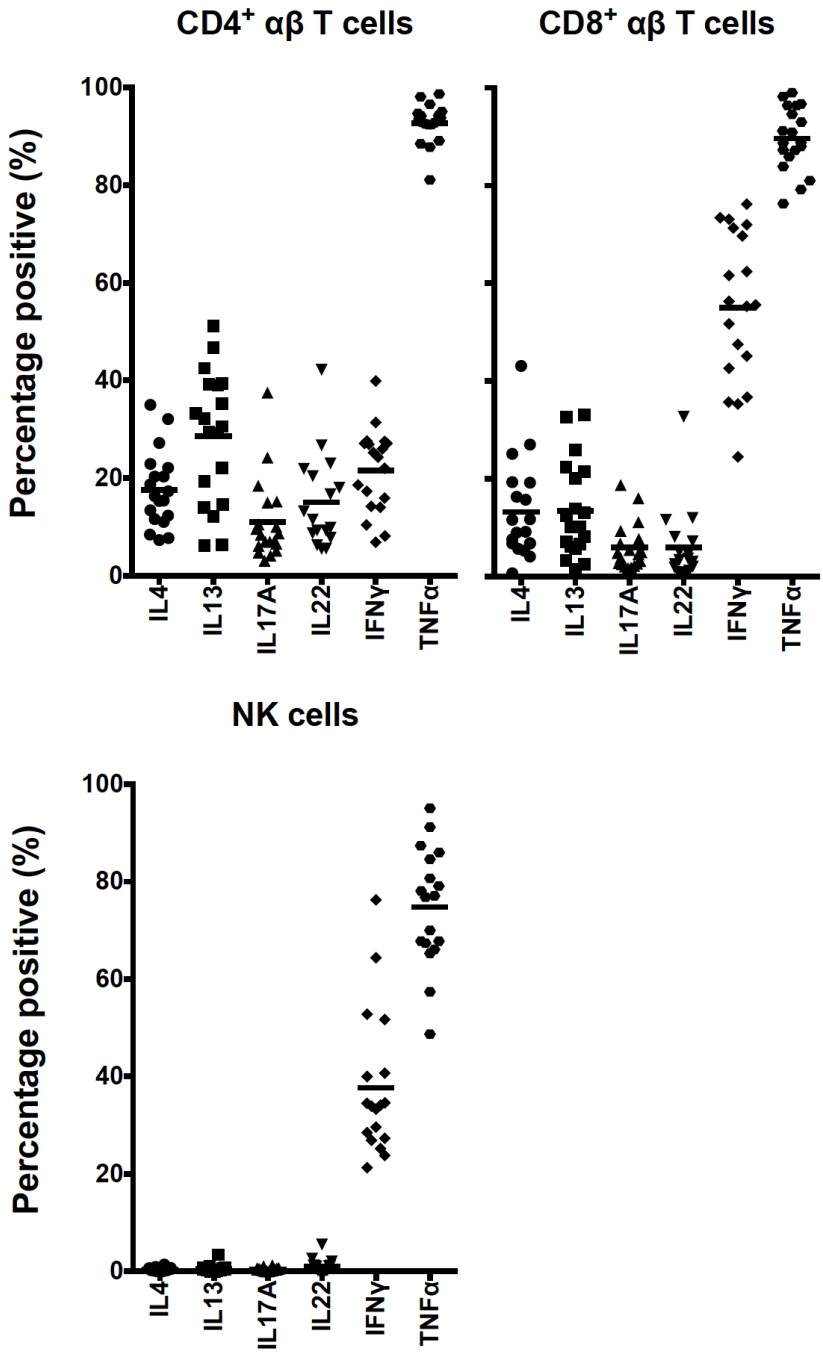

Figure 3.2. Skin-resident αβ T cell subsets and NK cells had distinct but diverse cytokine-producing profiles

Intracellular cytokine staining of skin-resident αβ T cells and NK cells, isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml), following *in vitro* activation with PMA (10 ng/ml) and ionomycin (1 μg/ml) [5h] with BFA (20 μg/ml) [4h] (gated against un-stimulated controls; mean indicated; n = 16 – 19).

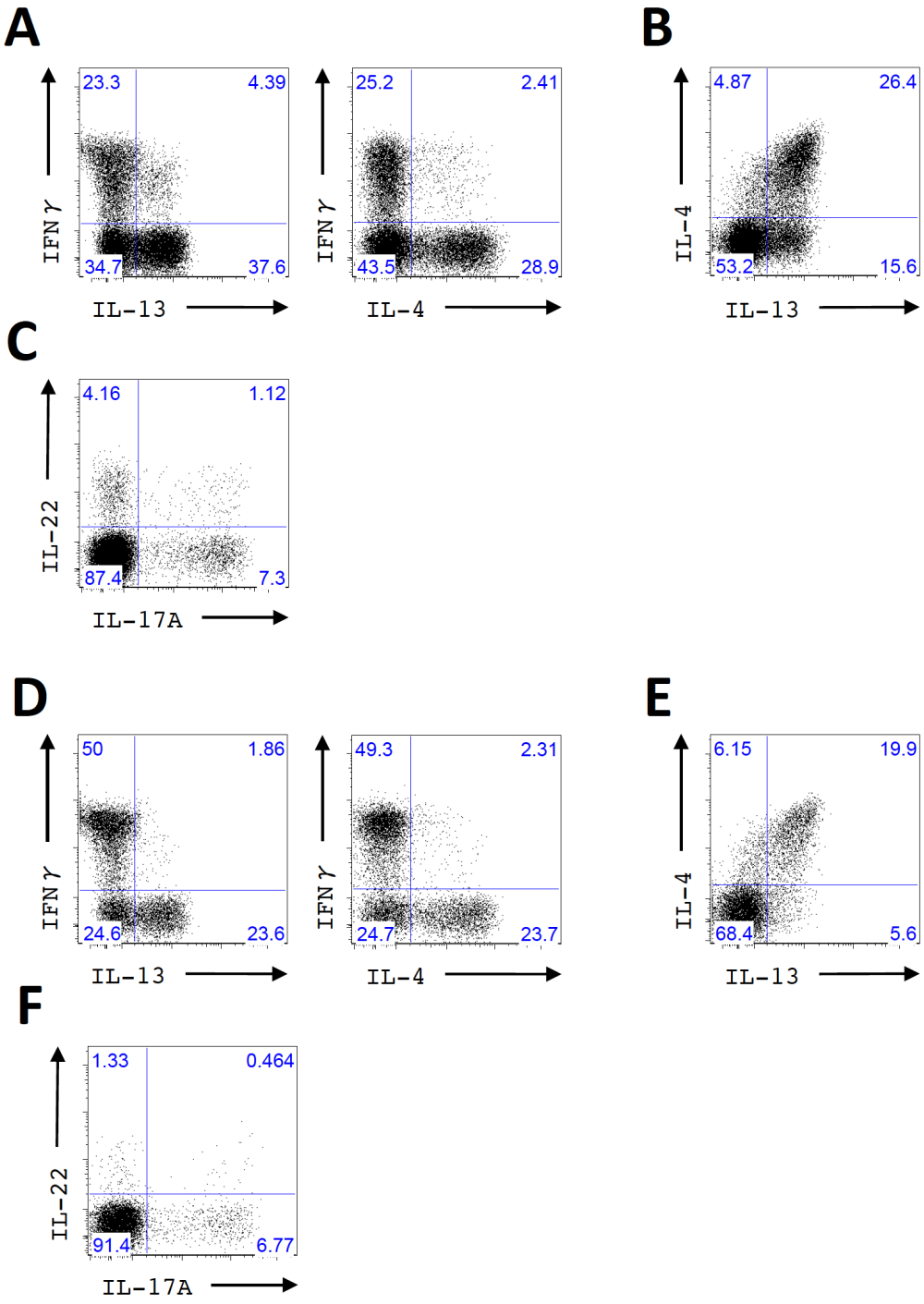

Figure 3.3. Healthy skin contained distinct αβ T cell populations of multiple lineages

Representative flow cytometry analysis showing intracellular cytokine co-staining of skin-resident (A–C) $CD4^+$ or (D–F) $CD8^+$ αβ T cells, isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml), following *in vitro* activation with PMA (10 ng/ml) and ionomycin (1 μg/ml) [5h] with BFA (20 μg/ml) [4h]. Gated against un-stimulated controls.

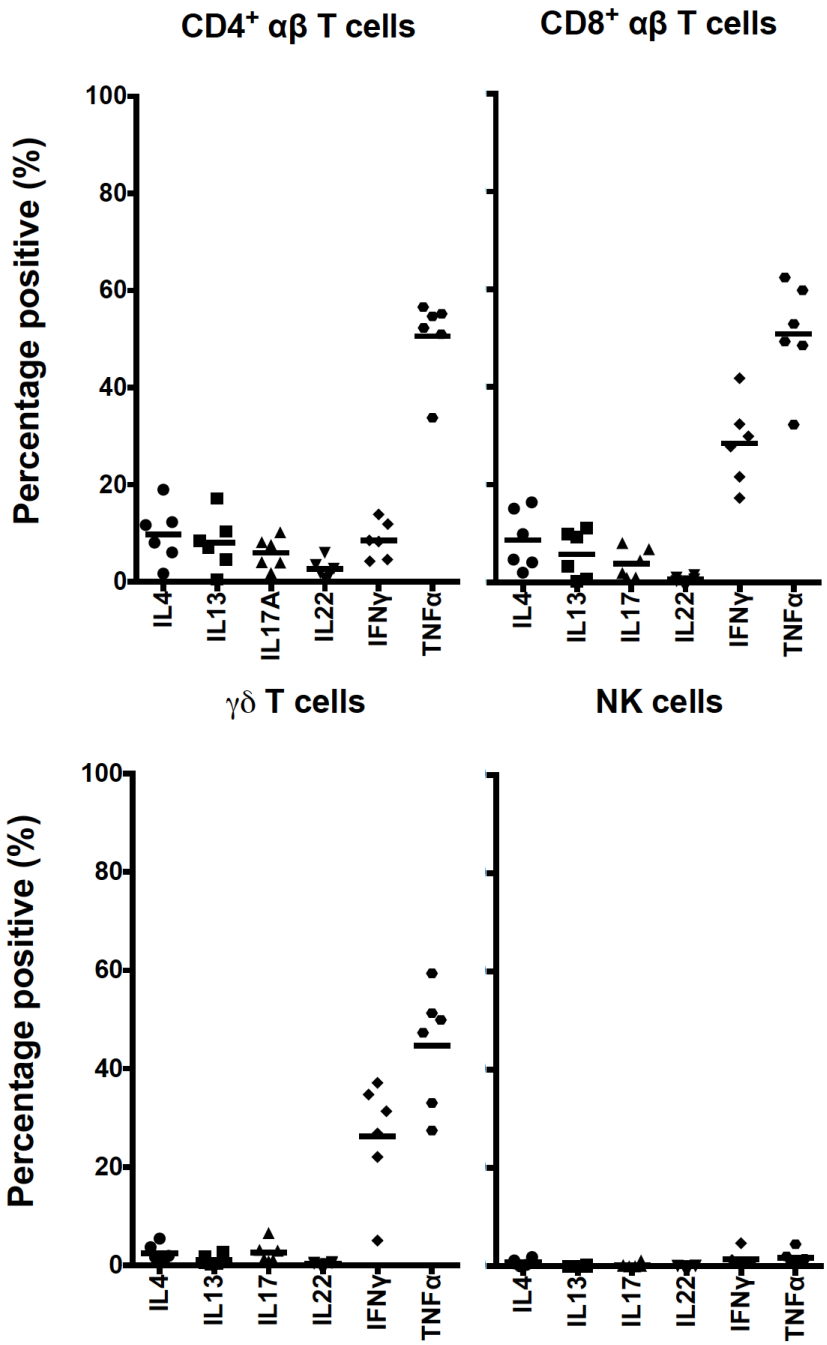

Figure 3.4. Upon TCR-mediated activation *in vitro* skin-resident T cells readily produced cytokines with distinct cytokine-producing profiles

Intracellular cytokine staining of skin-resident lmphocytes, isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml), following *in vitro* activation with plate-bound anti-CD3 antibody [6h] with BFA (20 μg/ml) [5h] (gated against un-stimulated controls; mean indicated; n = 6).

3.2. Skin-resident Vδ1$^+$ T cells showed innate-like responsiveness to the NKG2D ligand MICA

Having established the functional potentials of the skin-resident lymphocytes, an *in vitro* assay was establish to investigate their responsiveness to NKG2D ligands.

Strikingly, upon engagement with plate-bound recombinant MICA, skin-resident Vδ1$^+$ T cells showed rapid innate-like responsiveness, producing TNFα within 6 hours of activation (Figure 3.5). This innate-like activation was directly mediated *via* the NKG2D receptor as pre-treating the cells with a monoclonal antibody specific for NKG2D blocked it. This innate-like activation was unique to the Vδ1$^+$ subset of T cells, whereas NKG2D$^+$ CD8$^+$ T_{RM} cells and the much rarer Vδ2$^+$ T cells failed to be activated by MICA alone (Figures 3.5 and 3.9). Skin-resident NK cells also showed an innate response to MICA, and, as with the Vδ1$^+$ T cells, this was blocked by the anti-NKG2D antibody (Figure 3.5).

Following *in vitro* activation with plate-bound MICA, Vδ1$^+$ T cells showed diverse effector responses, with both TNFα (mean 15.3 % ± standard error of the mean (SEM) 5.1) and IFNγ (12.9 % ± 2.0) production, but also mobilisation of cytotoxic granules as assessed by cell surface levels of the lytic-associated membrane protein CD107a (12.6 % ± 2.5) (n = 8 – 11; Figure 3.6). As a positive control, PMA and ionomycin also induced cytotoxic degranulation (44.9 % ± 6.6% CD107a$^+$). Upon activation with either MICA or PMA/Ionomycin, both TNFα and IFNγ were largely co-produced by the same cells (Figure 3.7A). In contrast, especially following MICA-mediated activation, TNFα or IFNγ did not consistently co-stain with the cytotoxicity marker CD107a, implying that there may be some functional sub-specialisation within the Vδ1$^+$ T cell population for either cytokine production or cytotoxic responses (Figure 3.7B). Collectively these data demonstrated that Vδ1$^+$ T cells could initiate a robust cytotoxic response and effector cytokine production following innate-like activation *via* the NKG2D receptor, whereas this was not obviously the case for CD8+ T_{RM} cells.

Mixed populations of skin-resident lymphocytes were activated with plate-bound MICA to characterise additional potential effector responses, and the supernatants

analysed by Luminex array. This identified the chemokine CCL4 (MIP-1β) and growth factor GM-CSF as potential effector molecules produced under these conditions. Flow cytometry analysis following intracellular staining of lymphocyte subsets confirmed that MICA-mediated activation lead to 29.9 % (± 3.8) of $V\delta1^+$ T cells being $CCL4^+$, and, in keeping with a T_H1-like phenotype, 6.7 % ± (2.1) were $GM\text{-}CSF^+$ (Figure 3.8). Both CCL4 and GM-CSF were also produced following activation with PMA and ionomycin (80.8 % ± 6.7 and 67.5 % ± 6.7, respectively) (Figure 3.8B).

Strikingly, although skin-resident NK cells were activated by MICA, leading to TNFα and IFNγ production (3.9 % ± 0.8 and 4.0 % ± 1.0, respectively), this was much less than that seen in the $V\delta1^+$ subset (Figure 3.9). NKG2D engagement primarily lead to NK cell cytotoxic degranulation ($CD107a^+$ 17.3 % ± 3.7) and CCL4 production (24.1 ± 6.2), which were also provoked by activation with PMA and ionomycin (66.5 % ± 3.5 and 48.2 % ± 7.8, respectively) (Figures 3.9 and 3.10). Although NK cells produced GM-CSF following PMA and ionomycin (64.1 % ± 8.9), this failed to reach significance following NKG2D-mediated activation. The differential production by NK cells of cytokines *versus* cytolytic effectors is a well-reported phenomenon that can reflect the cells' development and the strength of signal to which they are exposed (Caligiuri, 2008; Chan et al., 2007; Cooper et al., 2001).

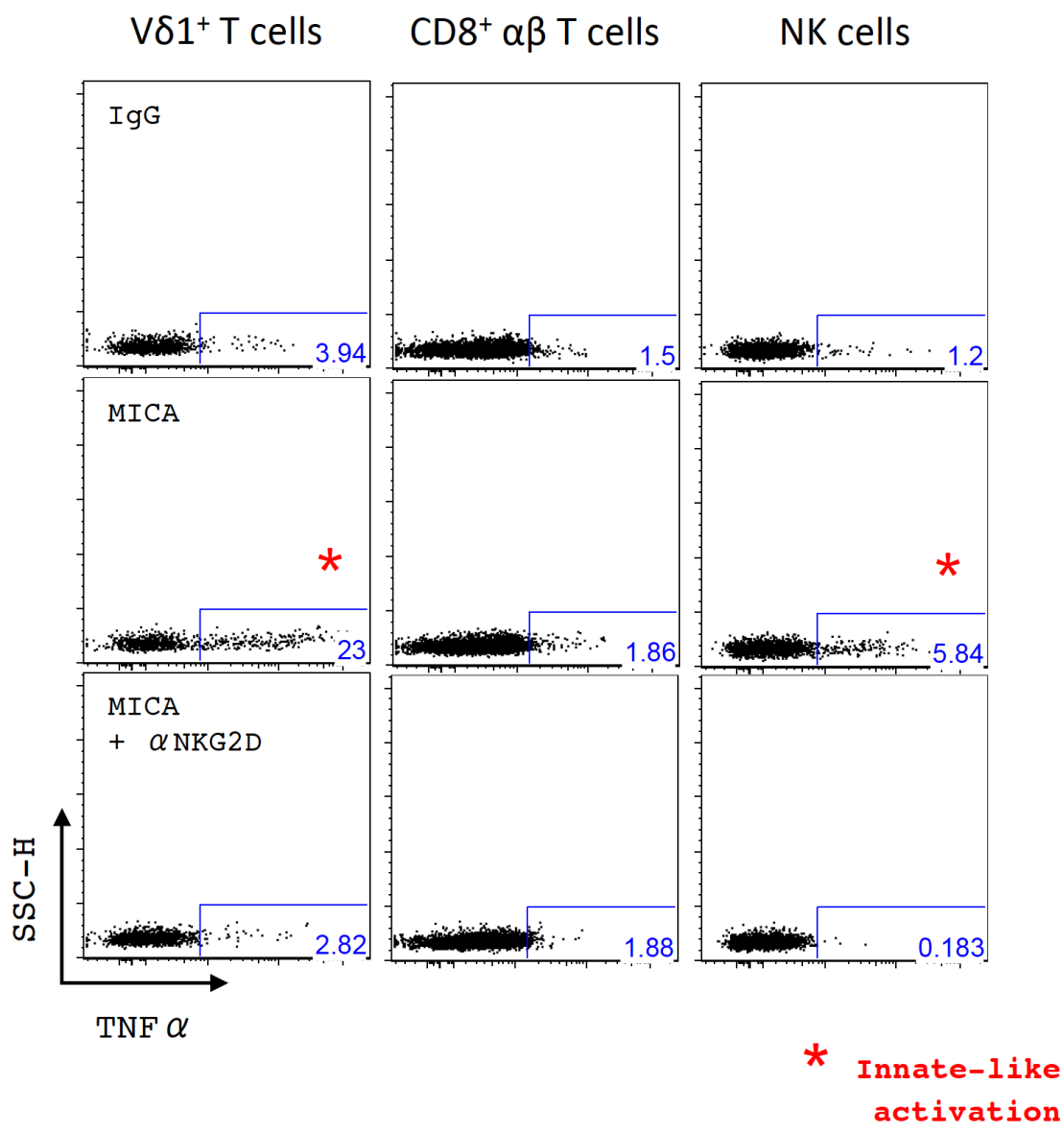

Figure 3.5. Skin-resident Vδ1^{+} T cells showed innate-like responsiveness to the NKG2D ligand MICA

Representative intracellular staining for TNFα following *in vitro* activation of skin-resident NKG2D^{+} lymphocytes with plate-bound recombinant MICA (10 µg/ml) or human IgG control (10 µg/ml). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). MICA stimulated cells pre-treated with either anti-human NKG2D antibody (10 µg/ml) or isotype-matched control (10 µg/ml). 6-hour activation with BFA (20 µg/ml) and IL-2 (100 IU/ml). NK cells = CD3$^{(-)}$ gate, which may include other ILC populations (Representative donor of 8; gated against un-stimulated controls).

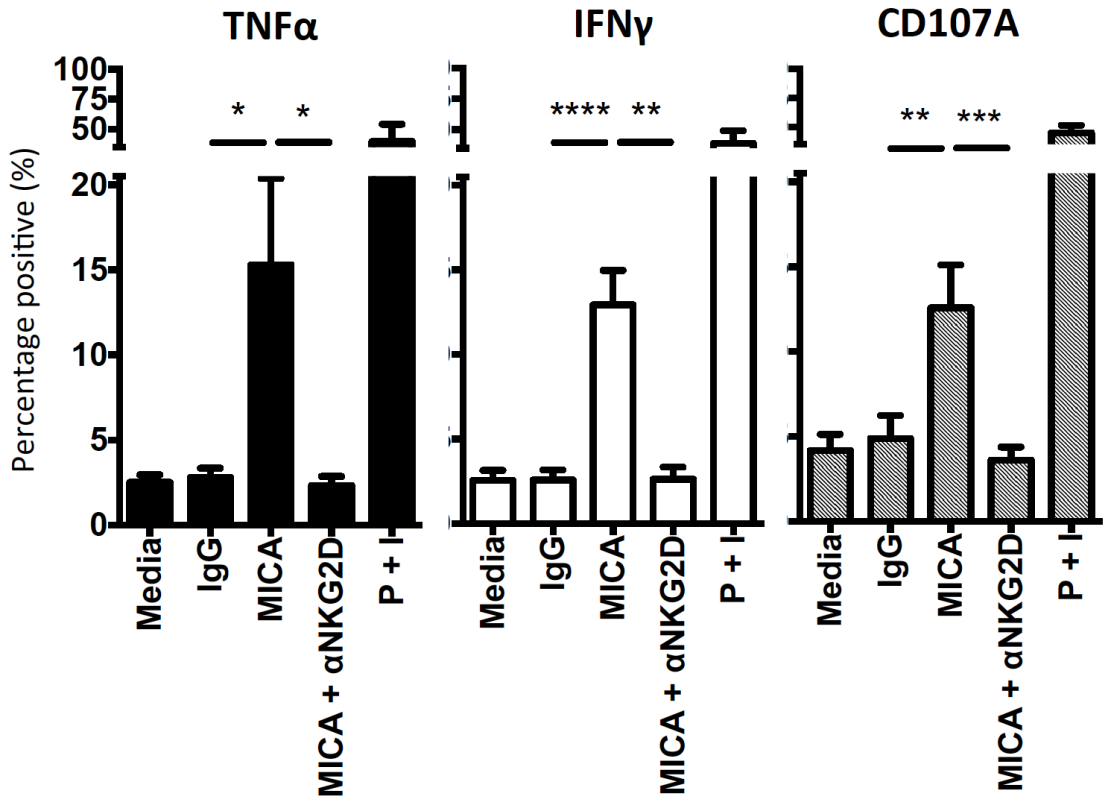

Figure 3.6. Skin-resident Vδ1$^+$ T cell activation by MICA lead to cytokine production and cytotoxic degranulation

Summary data of intracellular staining for TNFα and IFNγ, and cell surface CD107a staining, following *in vitro* activation of skin-resident Vδ1$^+$ T cells with plate-bound recombinant MICA (10 μg/ml) or human IgG control (10 μg/ml). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). MICA stimulated cells pre-treated with anti-human NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml). 6-hour activation with BFA (20 μg/ml) and IL-2 (100 IU/ml) (experiments performed in duplicate for each donor; gated against un-stimulated controls; mean with SEM; n = 8 – 11).

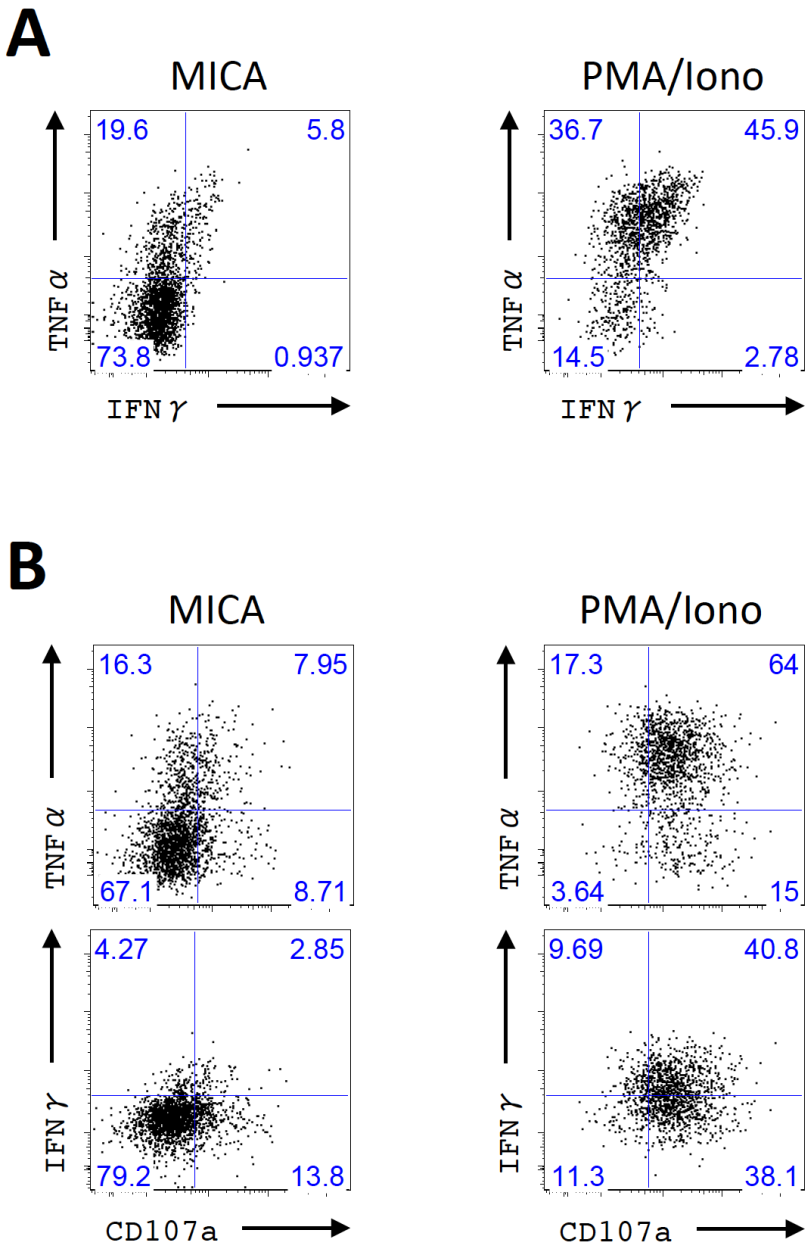

Figure 3.7. Skin-resident Vδ1$^+$ T cell co-production of cytokines and/or cytolytic degranulation upon activation

(A and B) Summary data of TNFα, IFNγ and CD107a co-staining following *in vitro* activation of skin-resident Vδ1$^+$ T cells with plate-bound recombinant MICA (10 μg/ml) or PMA (10 ng/ml) and ionomycin (1 μg/ml). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). 6-hour activation with BFA (20 μg/ml) and IL-2 (100 IU/ml). Gated against un-stimulated controls.

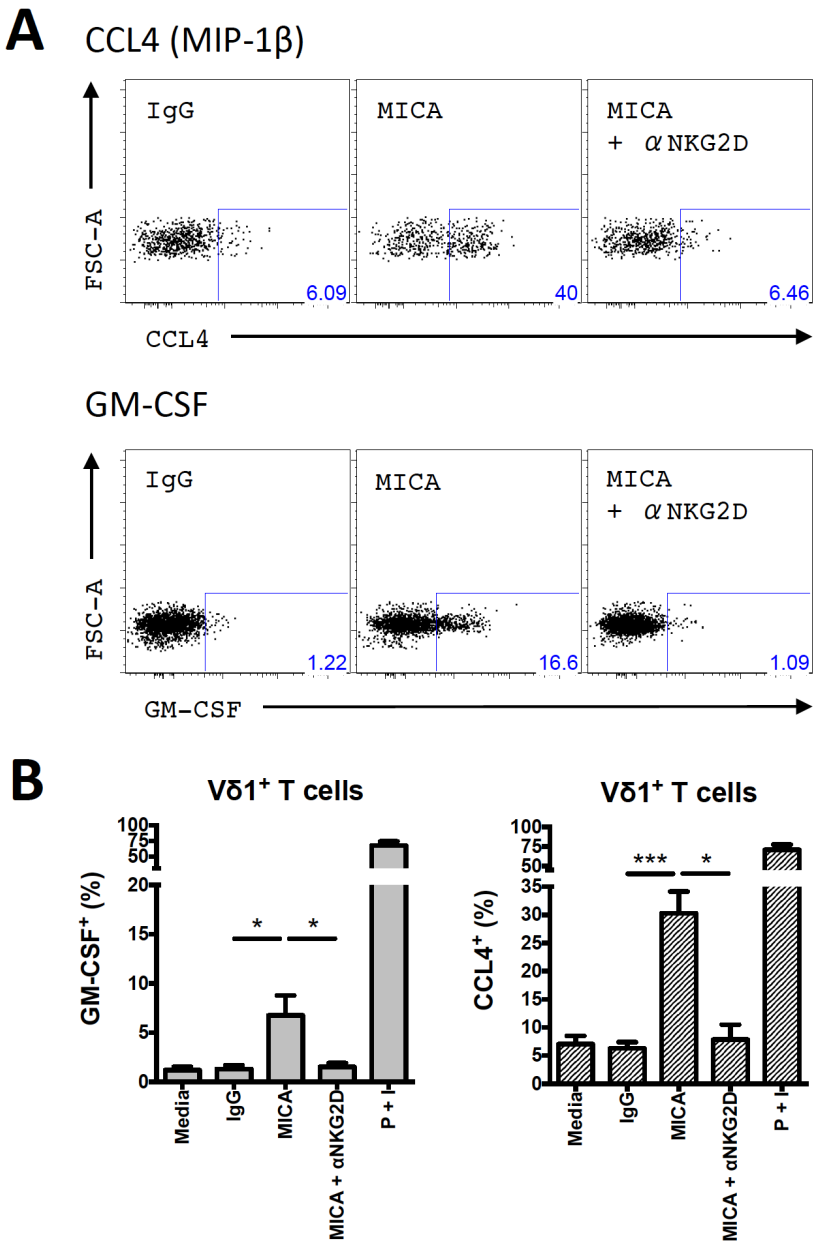

Figure 3.8. Skin-resident Vδ1$^+$ T cell activation by MICA lead to chemokine and growth factor production

(A) Representative intracellular staining and (B) summary data for CCL4 and GM-CSF following *in vitro* activation of Vδ1$^+$ T cells with plate-bound recombinant MICA (10 μg/ml) or human IgG control (10 μg/ml). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). MICA stimulated cells pre-treated with anti-human NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml). 6-hour activation with BFA (20 μg/ml) and IL-2 (100 IU/ml) (experiments performed in duplicate for each donor; gated against un-stimulated controls; mean with SEM; n = 4 – 7).

3.3. MICA co-stimulated TCR-mediated activation of NKG2D$^+$ skin-resident T cells

Although MICA alone failed to activate other NKG2D$^+$ skin-resident T cells, it provided a strong co-stimulatory signal in the context of low-dose TCR-mediated activation. This was seen in both the Vδ2$^+$ and CD8$^+$ αβ T cell subsets, and was blocked by pre-treating the cells with anti-NKG2D antibody (Figures 3.9 and 3.10). NKG2D-mediated co-stimulation of the TCR induced a range of functional responses from these cells, including the production of TNFα, IFNγ, GM-CSF and cytotoxic degranulation.

Compared to either MICA- or low-dose anti-CD3-mediated activation alone, the activation of Vδ1$^+$ T cells was greater in the presence of both MICA and anti-CD3. However, it was unclear if this was an additive effect of separate modes of activation or a true co-stimulatory effect of NKG2D on TCR-mediated activation of the Vδ1$^+$ T cells (Figure 3.11).

A signatory response of T cells following the engagement of the TCR is a rapid and transient increase in cytosolic calcium concentration, which is released from intracellular calcium stores and imported calcium *via* plasma-membrane channels (Weiss et al., 1984). This leads to the activation of certain transcription factors, such as the nuclear factor of activated T cells (NFAT) family, which are central to T cell activation (Rusnak and Mertz, 2000). Using a flow cytometry assay to measure dynamic changes following TCR-activation, we could detect in all skin-resident T cells a rapid and transient rise in cytosolic calcium that subsided over the course of a few minutes (Figure 3.12). The amplitude of the calcium flux was less in CD8$^+$ and Vδ1$^+$ T cell subsets, than for CD4$^+$ T cells. Cross-linking the NKG2D receptor alone, using a biotinylated anti-NKG2D monoclonal antibody, failed to induce a measurable change in cytosolic calcium in NKG2D$^+$ lymphocytes, including NK cells. However, the concomitant cross-linking of NKG2D and the TCR lead to a quantitative difference in intracellular calcium flux, with an increase in the amplitude of calcium flux seen in the NKG2D$^+$ CD8$^+$ and Vδ1$^+$ T cells. NKG2D cross-linking had no effect on the CD4$^+$ αβ T cells that lack the NKG2D receptor. Therefore, in addition to NKG2D engagement causing the innate-like activation of

skin-resident Vδ1^{+} T cells and NK cells, this indicates that NKG2D engagement could also quantitatively enhance intracellular processes in NKG2D^{+} T cells downstream of the TCR-signalling complex.

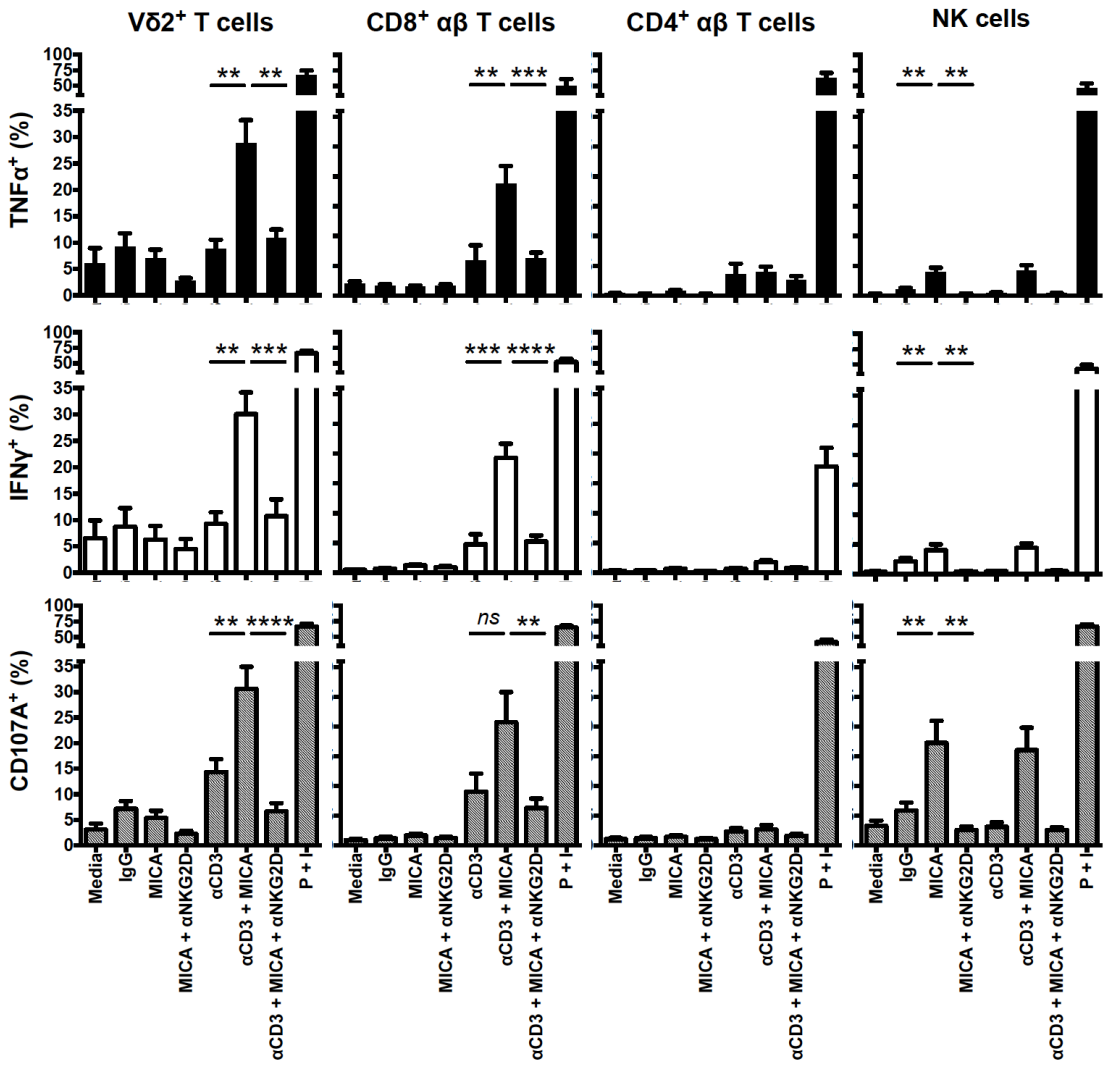

Figure 3.9. MICA provided a strong co-stimulatory signal to TCR-mediated activation, *via* NKG2D, for skin-resident Vδ2$^+$ T cells and CD8$^+$ αβ T cells. Skin-resident NK cells showed an innate response to MICA alone

Summary data of intracellular staining for TNFα and IFNγ, and cell surface CD107a, following *in vitro* activation of skin-resident lymphocytes with plate-bound recombinant MICA (10 μg/ml) or human IgG control (10 μg/ml) with/without low-dose plate-bound anti-CD3 antibody (50 ng/ml). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). MICA stimulated cells pre-treated with anti-human NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml). 6-hour activation with BFA (20 μg/ml) and IL-2 (100 IU/ml). NK cells = CD3$^{(-)}$ gate (experiments performed in duplicate for each donor; gated against un-stimulated controls; mean with SEM; n = 8 – 11).

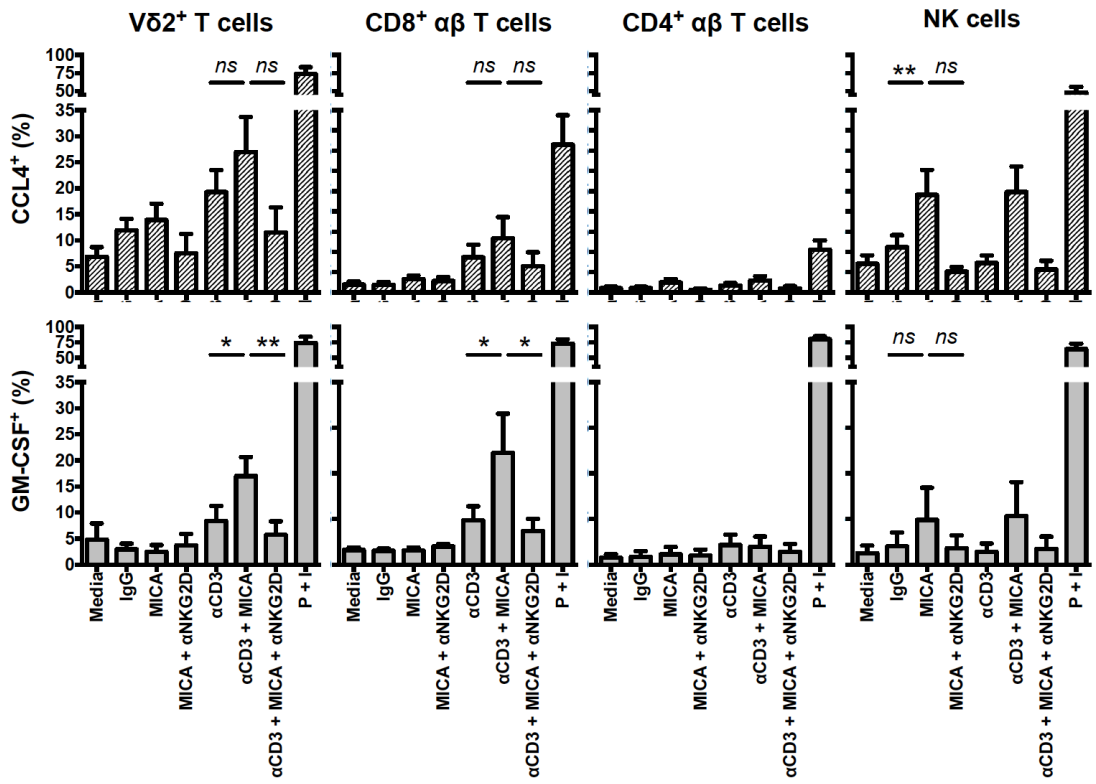

Figure 3.10. MICA provided a strong co-stimulatory signal to TCR-mediated activation, *via* NKG2D, for skin-resident Vδ2$^+$ T cells and CD8$^+$ αβ T cells. Skin-resident NK cells showed an innate response to MICA alone

Summary data of intracellular staining for CCL4 (MIP-1β) and GM-CSF, following *in vitro* activation of skin-resident lymphocytes with plate-bound recombinant MICA (10 μg/ml) or human IgG control (10 μg/ml) with/without low-dose plate-bound anti-CD3 antibody (50 ng/ml). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). MICA stimulated cells pre-treated with anti-human NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml). 6-hour activation with BFA (20 μg/ml) and IL-2 (100 IU/ml). NK cells = CD3$^{(-)}$ gate (experiments performed in duplicate for each donor; gated against un-stimulated controls; mean with SEM; n = 8 – 11).

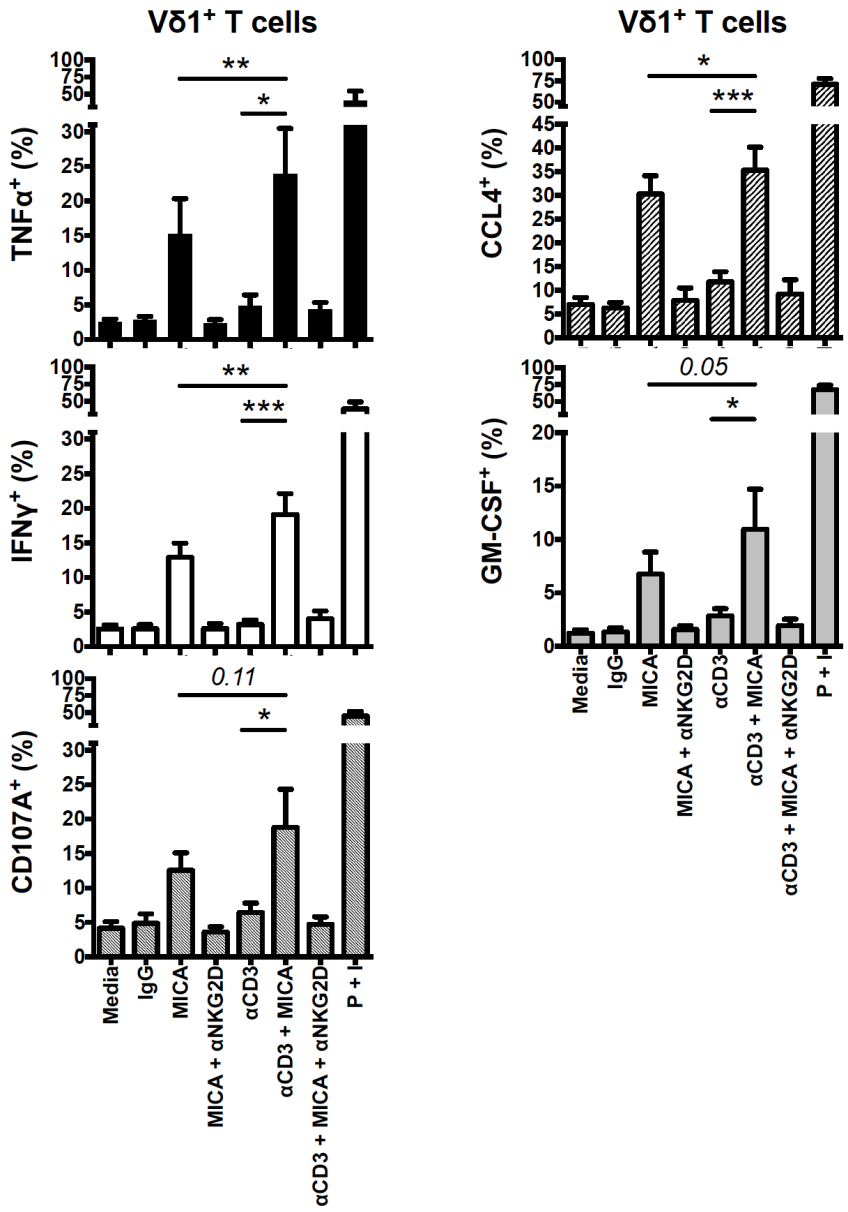

Figure 3.11. Activation of skin-resident Vδ1$^+$ T cells was greater in the presence of MICA and TCR-mediated activation

Summary data of intracellular staining for TNFα and IFNγ, and cell surface CD107a staining, following *in vitro* activation of skin-resident Vδ1$^+$ T cells with plate-bound recombinant MICA (10 μg/ml) or human IgG control (10 μg/ml) with/without low-dose plate-bound anti-CD3 antibody (50 ng/ml). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). MICA stimulated cells pre-treated with anti-human NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml). 6-hour activation with BFA (20 μg/ml) and IL-2 (100 IU/ml) (experiments performed in duplicate for each donor; gated against un-stimulated controls; mean with SEM; n = 8 – 11).

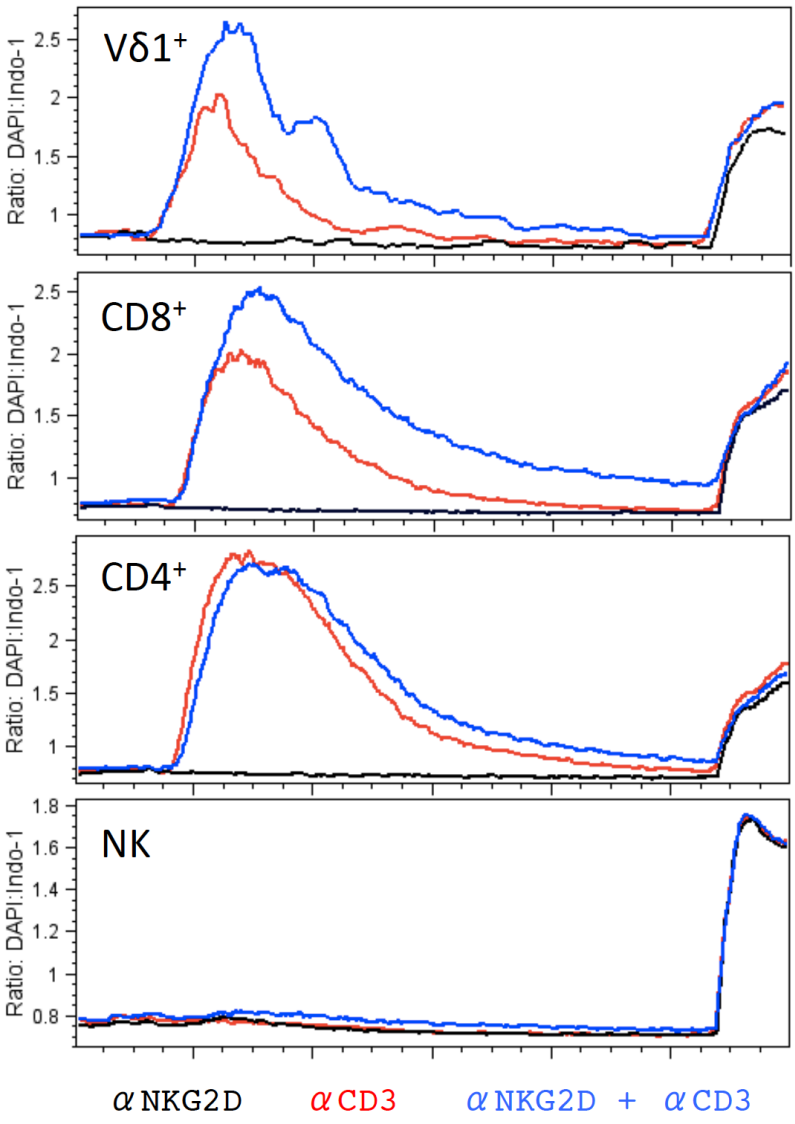

Figure 3.12. Skin-resident lymphocyte cytosolic calcium flux following TCR engagement was increased in NKG2D$^+$ T cells following concomitant cross-linking of NKG2D

Intracellular calcium mobilization in skin-resident lymphocytes, isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml), stimulated with biotinylated anti-CD3ε (10 μg/ml) and/or biotinylated anti-NKG2D (10 μg/ml) followed by cross-linkage with streptavidin (20 μg/ml) and then assayed over 10 minutes. Ionomycin (2 μg/ml) was added after 9 minutes as a positive control (representative of 2 donors).

3.4. NKG2D$^+$ skin-resident T cells responded to multiple ligands for the NKG2D receptor

One feature of NKG2D-dependent lymphocyte activation that remains unresolved is the existence of multiple ligands for a single activatory NKG2D receptor, such as in humans MICA/B and the ULBP family, which seem to have a degree of non-redundant functionality (Lanca et al., 2010; Raulet et al., 2013). This may indicate either the signaling of different forms of cellular stress, the differential regulation of downstream immune responses *via* NKG2D, or a selective pressure exerted by the multiple immune-evasion strategies of pathogens that target NKG2D ligands, driving the evolution of multiple ligands (Eagle and Trowsdale, 2007; Raulet et al., 2013). Consistent with such promiscuity of the NKG2D receptor, skin resident Vδ1$^+$ T cells were activated by multiple stress ligands. Both ULBP2 and ULBP3 could induce the innate-like activation of Vδ1$^+$ T cells, which also co-stimulated TCR-mediated activation of both Vδ1$^+$ and CD8$^+$ αβ T cell populations (Figure 3.13). Under these conditions, both ULBP2 and ULBP3 did not seem as effective in activating NKG2D$^+$ cells as recombinant MICA; however, this may reflect different binding affinities for these ligands, a different quality of recombinant protein production, or inter-individual variation (Mistry and O'Callaghan, 2007; Raulet et al., 2013; Shafi et al., 2011).

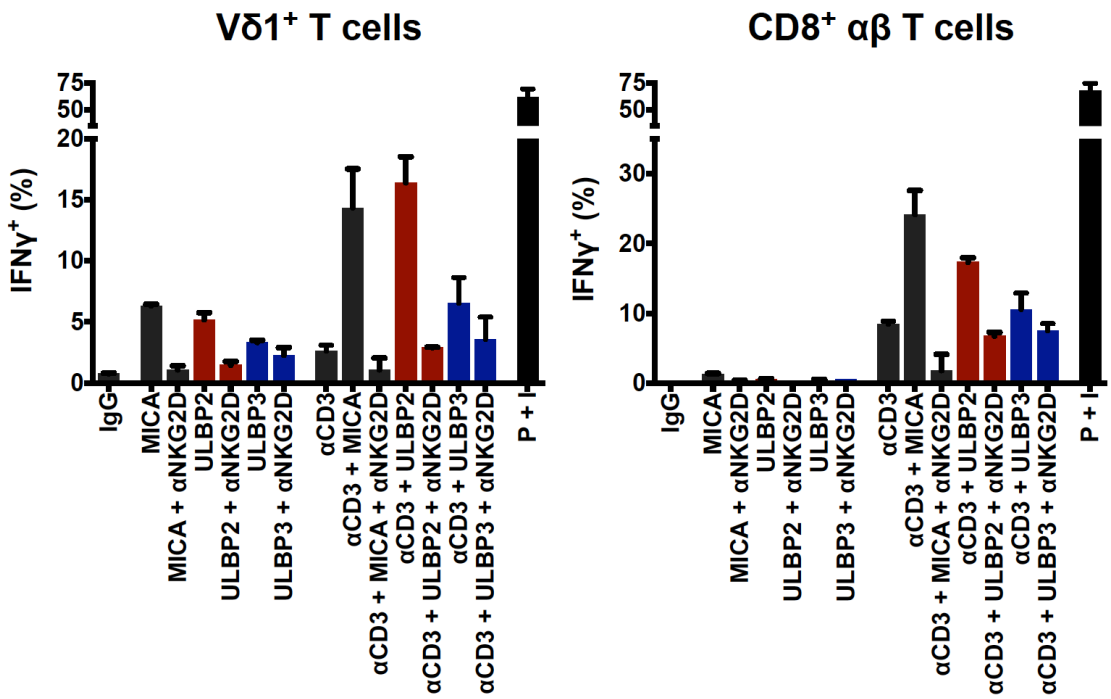

Figure 3.13. NKG2D$^+$ skin-resident T cells responded to multiple ligands for the NKG2D receptor

Summary data of intracellular cytokine staining for IFNγ following *in vitro* activation of skin-resident lymphocytes with plate-bound human IgG control (10 μg/ml) or recombinant MICA, ULBP2 or ULBP3 (all at 10 μg/ml), with/without low-dose plate-bound anti-CD3 antibody (50 ng/ml). MICA, ULBP2 or ULBP3 stimulated cells pre-treated with anti-human either anti-NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml). 6-hour activation with BFA (20 μg/ml) and IL-2 (100 IU/ml) (experiments performed in duplicate for each donor; gated against un-stimulated controls; mean with SD; representative donor of 3).

3.5. Activation *via* NKG2D in some individuals lead to IL-13 production by both skin-resident Vδ1$^+$ T cells and CD8$^+$ αβ T cells

The responses of DETC in the murine epidermis are biased toward cytolysis, and the production of IFNγ and IL-13, which characterise cutaneous LSSR (Girardi et al., 2001; Strid et al., 2011; 2008). Although human skin-resident γδ T cells had predominantly a T_H1-like bias, in some individuals they were also a source of T_H2-type cytokines (Figures 3.1 and 3.16). This was most clearly seen following activation with PMA and ionomycin (3.3 % ± 3.3 IL-13$^+$ for panTCRγδ$^+$ cells; 5.8 % ± 6.7 IL-13$^+$ for Vδ1$^+$ T cells). However, there were certain individuals, where Vδ1$^+$ T cells also produced IL-13 following their innate-like activation with MICA (Figure 3.14). For these donors, Vδ1$^+$ T cell IL-13 production following PMA and ionomycin was even more robust (Figures 3.14A and B). These individuals had no record of atopic skin disease.

In addition, in the context of TCR-mediated activation, when CD8$^+$ αβ T cells were co-stimulated with MICA, in some individuals they were also a potential source of IL-13 (Figure 3.14). This was largely seen in the same individuals where Vδ1$^+$ T cells were also IL-13 producers (Figure 3.14B). Thus, there was some evidence that NKG2D-mediated activation of skin-resident T cells was not limited to a cytolytic T_H1-like response, but also leads to the production of IL-13 under certain conditions.

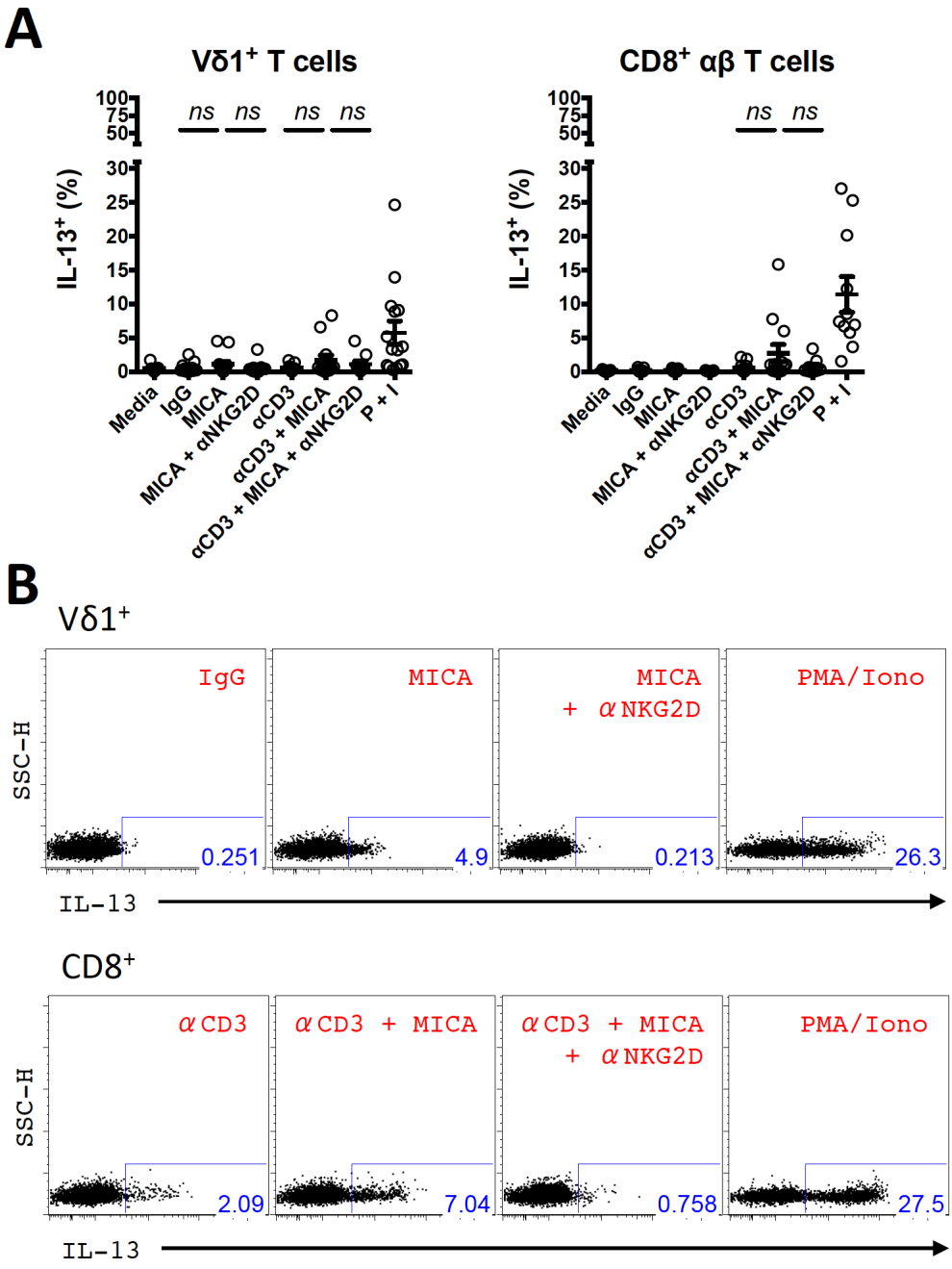

Figure 3.14. Activation *via* NKG2D in some individuals lead to IL-13 production by both skin-resident Vδ1$^+$ T cells and CD8$^+$ αβ T cells

(A) Summary data for intracellular IL-13 staining following *in vitro* activation of skin-resident Vδ1$^+$ T cells or CD8$^+$ αβ T cells with plate-bound recombinant MICA (10 μg/ml) or human IgG control (10 μg/ml) with/without low-dose plate-bound anti-CD3 antibody (50 ng/ml). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). MICA stimulated cells pre-treated with anti-human

NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml). 6-hour activation with BFA (20 μg/ml) and IL-2 (100 IU/ml). (experiments performed in duplicate for each donor; gated against un-stimulated controls; mean with SEM; n = 9 – 15). (B) Flow cytometry analysis of donor demonstrating IL-13 staining following *in vitro* activation of skin-resident $V\delta1^+$ T cells with plate-bound recombinant MICA (10 μg/ml) or human IgG control (10 μg/ml); or $CD8^+$ αβ T cells with plate-bound anti-CD3 antibody (50 ng/ml) with/without plate-bound recombinant MICA (10 μg/ml). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). MICA stimulated cells pre-treated with anti-human NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). PMA/Iono = PMA (10 ng/ml) and ionomycin (1 μg/ml). 6-hour activation with BFA (20 μg/ml) and IL-2 (100 IU/ml). Gated against un-stimulated controls.

3.6. Skin-resident γδ T cells showed *in vitro* cytotoxic activity against transformed epithelial cells, in part mediated *via* NKG2D

Given the T_H1-like/cytolytic potentials of skin-resident γδ T cells, to test directly the response of skin-resident T cells to cancer cells, short-term co-cultures with transformed epithelial cell lines were set up. The cervical carcinoma-derived Hela cell line expresses high levels of cell surface MICA and low levels of the ULBPs (Figure 3.15A). Negatively sorted skin-associated γδ T cells (αβ TCR^-) and αβ T cells (γδ TCR^-) from the same donor were mixed with Hela cells at increasing effector-to-target (E:T) ratios. Negative sorting was undertaken to prevent cross-linking of either γδ or αβ TCRs, which may alter the subsequent responsiveness of the T cells.

Following 24 hours in co-culture, skin-derived γδ T cells showed robust cytotoxicity towards Hela cells, as measured by an ELISA for the epithelia-derived apoptotic protein caspase-cleaved cytokeratin 18 (Figure 3.15B). In addition, γδ T cell killing was partially blocked by pre-treating the lymphocytes with anti-NKG2D antibody, indicating that the cytolytic response was in part dependent on NKG2D-mediated innate-like activation. This effect was reproducible across donors, with consistent inhibition of γδ T cell-mediated Hela killing following NKG2D blockade (26.7 – 29.1 % inhibition; n = 4) (Table 3.1). In many donors γδ T cell killing was far more effective than the cytolytic capacity of matched skin-derived αβ T cells (Figure 3.15B). In addition, NKG2D blockade did not have a significant effect on αβ T cell killing.

Using this assay, the cytolytic potential of skin-associated γδ T cells was evaluated against other transformed epithelial cell lines. These included the breast carcinoma-derived cell line HCC1954, and the colorectal carcinoma-derived cell line Caco-2, which both expressed high levels of MICA, MICB and/or ULBPs (Figure 3.16A). γδ T cells were cytolytic towards these cancer cell targets, in a similar dose-dependent fashion. Often epithelial cell killing was higher than for the staurosporin (a cytotoxic agent) control, to which these immortalised cell lines had some resistance. In most donors, cytotoxicity was also in part mediated *via* NKG2D, with

some impairment in killing seen through NKG2D blockade (12.1 – 15.3 % inhibition of HCC1954 killing; 18.0 – 27.6 % inhibition of Caco-2 killing) (Table 3.1). However, such impaired cytotoxicity was less consistent across donors (than in Hela cell assays), and despite an apparent trend towards reduced killing some experiments did not reach statistical significance.

Thus, skin-associated γδ T cells showed a profound capacity to induce cell death of a range of cancer cell lines *in vitro*, without prior TCR-mediated activation, as isolated effector cells had not been consciously manipulated *via* the TCR during their retrieval and/or culture. This indicates that these responses were innate-like, in part mediated *via* NKG2D. However, the failure to achieve full preservation of the target cells indicates the involvement of other innate pathways besides NKG2D.

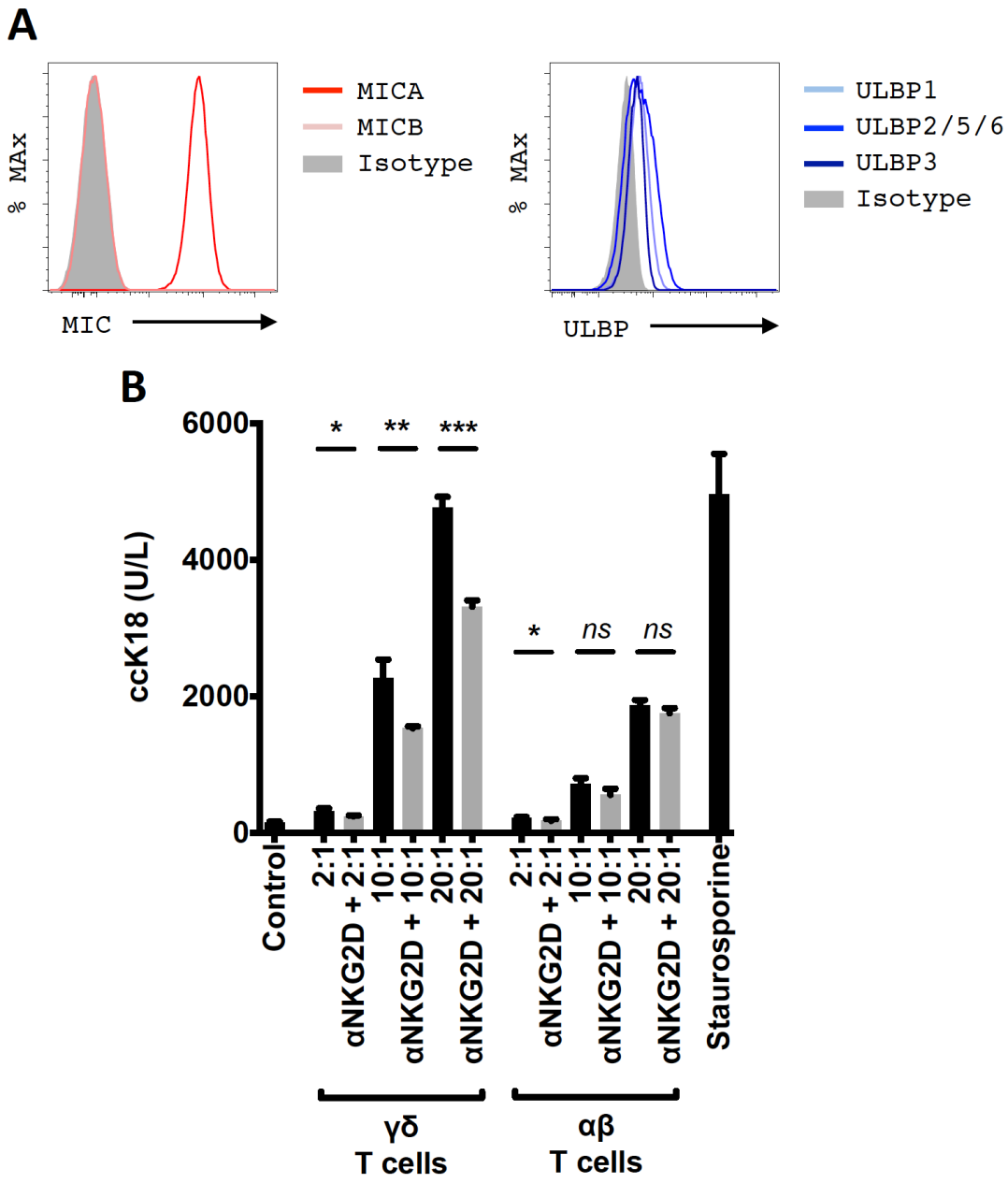

Figure 3.15. Skin-resident γδ T cells showed cytotoxic activity *in vitro* against MICA$^+$ transformed epithelial cells that was in part mediated *via* NKG2D

(A) Flow cytometry analysis of MICA/B and ULBP expression by HELA cells. (B) Cytotoxicity ELISA assay measuring the epithelia-derived apoptotic protein caspase-cleaved cytokeratin 18 (ccK18) following 24 hour co-culture of negatively sorted skin-derived γδ T cells or αβ T cells (Effectors) with HELA cells (targets). Effectors pre-treated with anti-NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). Assay performed in presence of IL-2 (100 IU/ml). Staurosporine (50 μM) positive control (representative of 4 donors; experiment in triplicate; mean with SD).

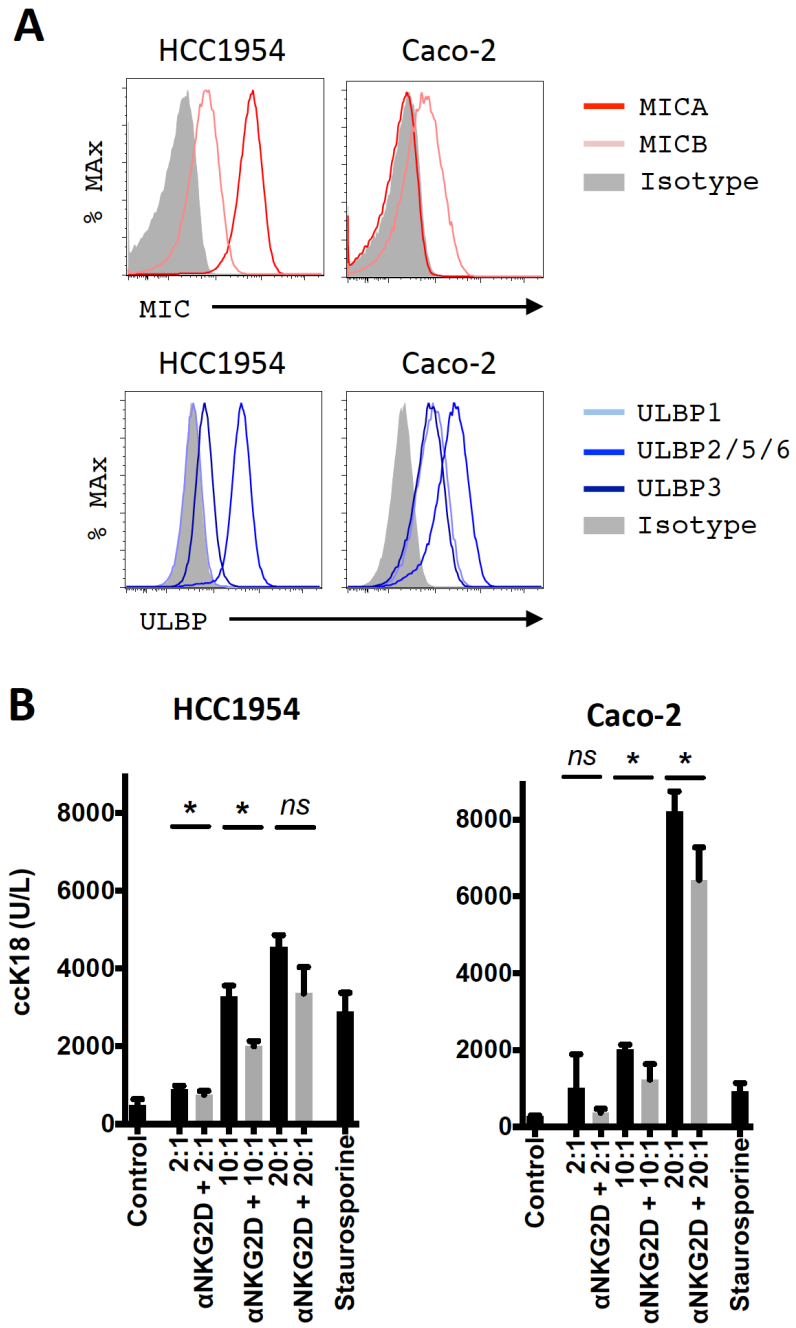

Figure 3.16. Skin-resident γδ T cells showed cytotoxic activity *in vitro* against transformed epithelial cells that was in part mediated *via* NKG2D

(A) Flow cytometry analysis of MICA/B and ULBP expression by HCC1954 and Caco-2 cells. (B) Cytotoxicity ELISA assay measuring caspase-cleaved cytokeratin 18 (ccK18) following 24 hour co-culture of negatively sorted skin-derived γδ T cells (Effectors) with HCC1954 or Caco-2 cell (targets). Effectors pre-treated with anti-NKG2D antibody (10 µg/ml) or isotype-matched control (10 µg/ml). Assay performed in presence of IL-2 (100 IU/ml). Staurosporine (50 µM) positive control (representative of 3 – 4 donors; experiment in triplicate; mean with SD).

	Mean percentage reduction in killing following NKG2D blockade (± SEM)		
E:T	**Hela** *n = 4*	**HCC1954** *n = 3*	**Caco-2** *n = 4*
2:1	26.7 (± 3.8)	12.1 (± 5.3)	27.6 (± 17.6)
10:1	29.1 (± 5.1)	15.3 (± 9.9)	25.4 (± 7.3)
20:1	27.8 (± 1.4)	12.2 (± 6.3)	18.0 (± 5.1)

Table 3.1. Skin-resident γδ T cells showed cytotoxic activity *in vitro* against MICA$^+$ transformed epithelial cells that was in part mediated *via* NKG2D

Summary data showing the mean (with SEM) percentage reduction in γδ T cell-mediated killing of Hela, HCC1954 and Caco-2 cells, as measured by caspase-cleaved cytokeratin 18 (ccK18) ELISA, at different effector to target (E:T) ratios following pre-treatment with anti-NKG2D antibody (10 μg/ml) (experiments performed in duplicate/triplicate for each donor; mean with SEM; n = 3 – 4).

3.7. Skin-resident lymphocytes showed innate responses to exogenous cytokines

Human skin-resident γδ T cells were analogous to murine DETC, having the capacity to be directly activated by MHC-I-like molecules *via* the NKG2D receptor. Murine tissue-resident γδ T cells have additional innate-like properties that include their response to combinations of IL-1 family and STAT-activating cytokines. In such experiments, IFNγ-producing γδ T cells, such as DETC, respond simply to exposure to exogenous IL-18 and IL-12 to produce IFNγ (Sugaya et al., 1999); whereas IL-17-producing γδ T cells respond to IL-1β and IL-23 to produce IL-17 (Cai et al., 2011; Mabuchi et al., 2011; Martin et al., 2009; Sumaria et al., 2011; Sutton et al., 2009; Wencker et al., 2013). To determine if human skin-resident $V\delta1^+$ T cells had such functional potential, given their T_H1-like bias, we assayed their response to short-term culture with IL-12 and IL-18. After 24 hours in culture, $V\delta1^+$ T cells were seen to produce IFNγ, with a significant number of cells staining for intracellular cytokine, which was most robust following their exposure to IL-12 and IL-18 in combination (mean 37.6 % ± 6.1 [SEM] $IFN\gamma^+$) (Figure 3.17).

This innate response to exogenous cytokines was, however, not unique to $V\delta1^+$ T cells. The rare skin-resident $V\delta2^+$ T cell population showed the greatest response to IL-12 and IL-18 (53.8 % ± 7.9 $IFN\gamma^+$), and skin-resident NK cells responded similarly to $V\delta1^+$ T cells (43.0 % ± 5.1) (Figure 3.18). $CD8^+$ αβ T_{RM} also responded to IL-12 and IL-18, but a lower proportion of these cells stained for IFNγ when compared with the "unconventional" lymphocyte subsets (15.7 % ± 3.2 of $CD8^+$ T_{RM} cells $IFN\gamma^+$) (Figure 3.18). $CD4^+$ αβ T cells did not obviously produce IFNγ under these conditions.

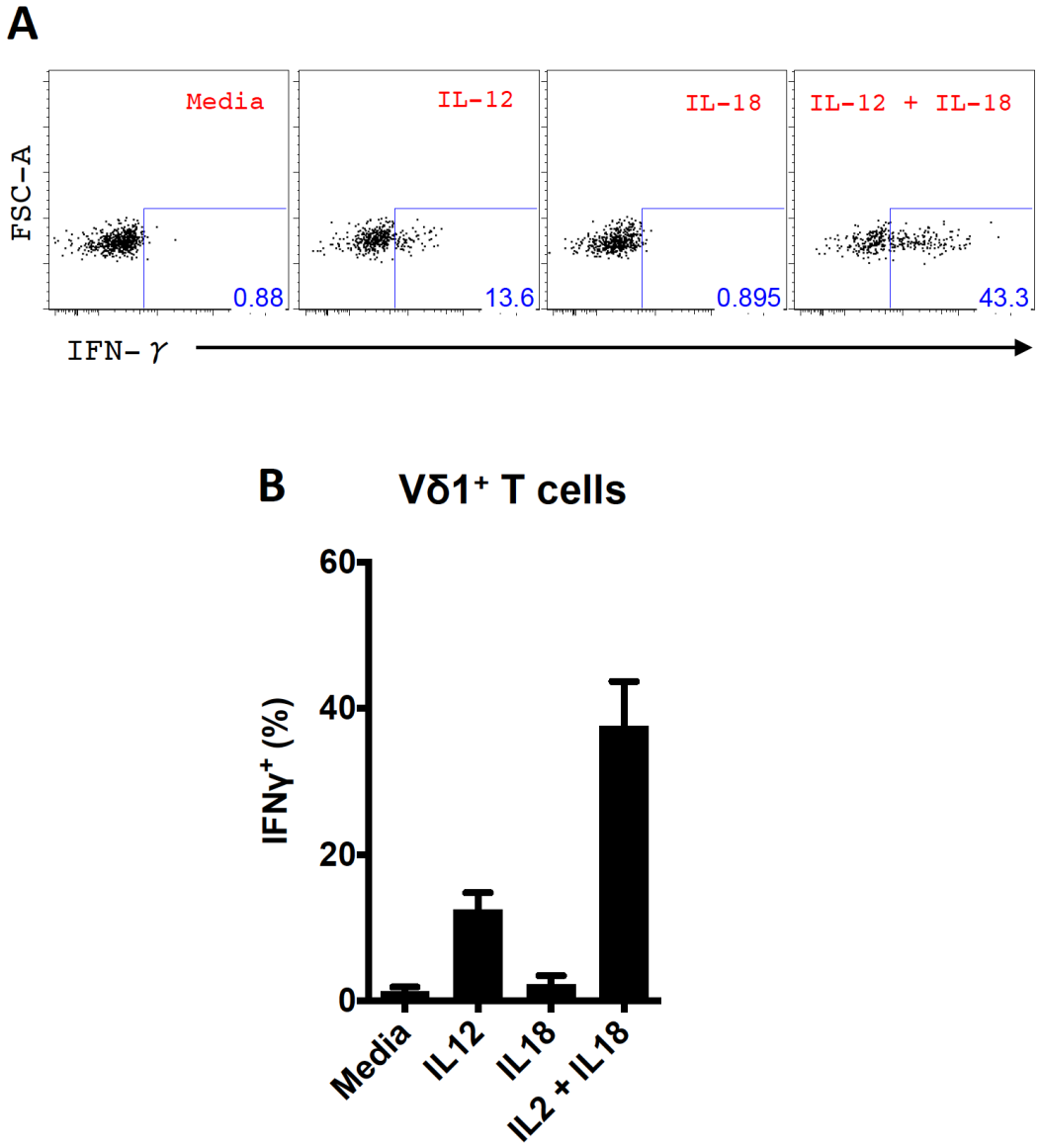

Figure 3.17. Skin-resident Vδ1$^+$ T cells showed innate responses to exogenous cytokine(s)

(A) Representative intracellular cytokine staining and (B) summary data for IFNγ following *in vitro* activation of skin-resident Vδ1$^+$ T cells with IL-12 (100 ng/ml) and/or IL-18 (100 ng/ml). 24-hour activation with BFA (20 μg/ml) for last 4 hours. Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml) (experiments performed in duplicate for each donor; gated against un-stimulated controls; mean with SEM; n = 3 – 8).

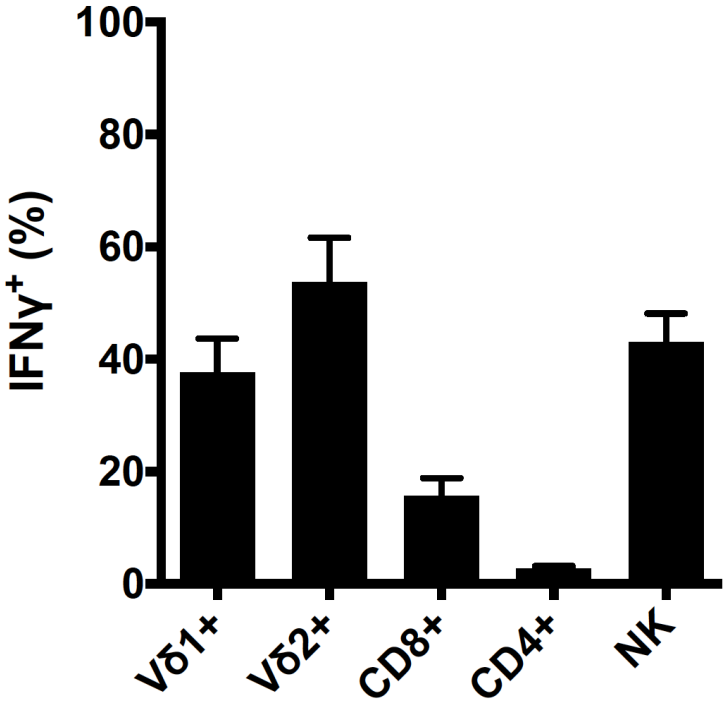

Figure 3.18. Certain other skin-resident lymphocyte subsets had the capacity to respond to exogenous cytokines

Summary data showing intracellular IFNγ staining following *in vitro* activation of skin-resident lymphocytes with IL-12 (100 ng/ml) and IL-18 (100 ng/ml). 24-hour activation with BFA (20 μg/ml) for last 4 hours. Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). NK cells = $CD3^{(-)}$ gate (experiments performed in duplicate for each donor; gated against un-stimulated controls; mean with SEM; n = 8).

3.8. Conclusion

Upon *in vitro* activation, skin-resident lymphocytes readily produced cytokines with distinct cytokine-producing profiles. NKG2D$^+$ cells could be activated under different circumstances *via* NKG2D, which lead to their rapid degranulation, cytokine production and growth factor/chemokine production. Most strikingly, Vδ1$^+$ T cells were activated by NKG2D alone, independent of the TCR, demonstrating innate-like function of human skin-resident T cells. To our knowledge this is the first demonstration in humans of an innate-like, TCR-independent response; shown specifically to be a property of a tissue γδ T cell subset. This was in contrast to NKG2D$^+$ Vδ2$^+$ and CD8$^+$ T_{RM} cells from the same site, which failed to be activated *via* NKG2D alone. Vδ1$^+$ T cells were also rapidly activated by exogenous cytokines, although this capacity was to some extent shared by other "unconventional" lymphocytes and CD8$^+$ T_{RM} cells. Consistent with their T_H1-like/cytotoxic phenotype, skin-resident γδ T cells were also able to mount profound cytolytic responses against transformed cell lines *in vitro*, which was in part dependent on their innate-like activation *via* NKG2D.

Collectively these data identify Vδ1$^+$ T cells as a human ILL population resident within healthy skin, analogous to murine DETC. They had the capacity to directly respond to ligands for the NKG2D receptor and cytokines that are associated with states of tissue stress. The propensity of these cells to respond *en masse* to innate stimuli may compensate for there being lower numbers of them than there are αβ T cells, which most commonly respond as rare, individual clones. Vδ1$^+$ T cells may therefore act as key players in the early immune response to tissue dysregulation within the skin, such as in carcinogenesis.

Chapter Four: Characterising the innate-like response of Vδ1$^+$ T cells to NKG2D ligands

Human skin-resident Vδ1$^+$ T cells had the innate-like capacity to be activated directly *via* NKG2D. This is an unsual property for a T cell to display, since activation *via* the TCR is ordinarily regarded as a requisite "quality control" checkpoint for T cell activation. Interestingly, lymphocytes found resident in murine skin are predominantly T_{RM} cells or ILL, which both show evidence of previous activation, either by antigen priming or their developmental programme, respectively. Indeed, it was reported by our laboratory that murine ILL have an altered mode of TCR responsiveness (Turchinovich and Hayday, 2011; Wencker et al., 2013). Therefore, the molecular basis of the innate-like response of human skin-resident Vδ1$^+$ T cells was investigated.

4.1. IL-15 did not confer innate-like function on Vδ1$^+$ T cells *in vitro*

IL-15 is an important growth factor for certain tissue-resident lymphocytes (De Creus et al., 2002; Edelbaum et al., 1995; Ma et al., 2006; Sprent, 2008); however, it has also been implicated in the development of innate-like CD8$^+$ αβ T cells, both *in vitro* and in disease states in the gut (Hüe et al., 2004; Meresse et al., 2004). In addition, IL-15 can reportedly up-regulate NKG2D expression, and may also synergize with the NKG2D signalling machinery to prime NK cell activation (Tang et al., 2013). The question therefore arose as to whether the innate-like responses of skin-resident Vδ1$^+$ T cells reflected their culture in IL-15.

Explant-isolation of skin-resident lymphocytes in the presence of IL-2 and IL-15 lead to an increase in γδ T cell frequency when compared with *ex vivo* tissue digestion and explant culture without additional cytokines (Figures 2.2 and 2.4). Comparing explant protocols supplemented with either IL-2 or IL-2 and IL-15, there was a significant increase in the frequency of Vδ1$^+$ T cells in the presence of IL-15 (mean 5.0-fold increase ± 2.9 [SD]; n = 7; Figure 4.1A). However, IL-15 did not significantly alter the cell surface expression of NKG2D on Vδ1$^+$ T cells or other lymphocyte subsets, compared with those isolated with IL-2 alone (Figures 4.1B and

C), except for the NK cells (increase from 62.6 % to 78.9 %; p = 0.01) (Figure 4.1C). As this population in fact represented $CD3^-$ lymphocytes, this increase may alternatively represent enrichment of $NKG2D^+$ NK cells within what represents the skin-resident ILC population. Importantly, $V\delta1^+$ T cells that were isolated with only IL-2 supplementation displayed comparable innate-like responses to MICA as those $V\delta1^+$ T cells isolated in the presence of IL-2 and IL-15 (Figure 4.2). Therefore, although IL-15 favoured the isolation of $V\delta1^+$ T cells from skin explants, it did not confer their innate-like function.

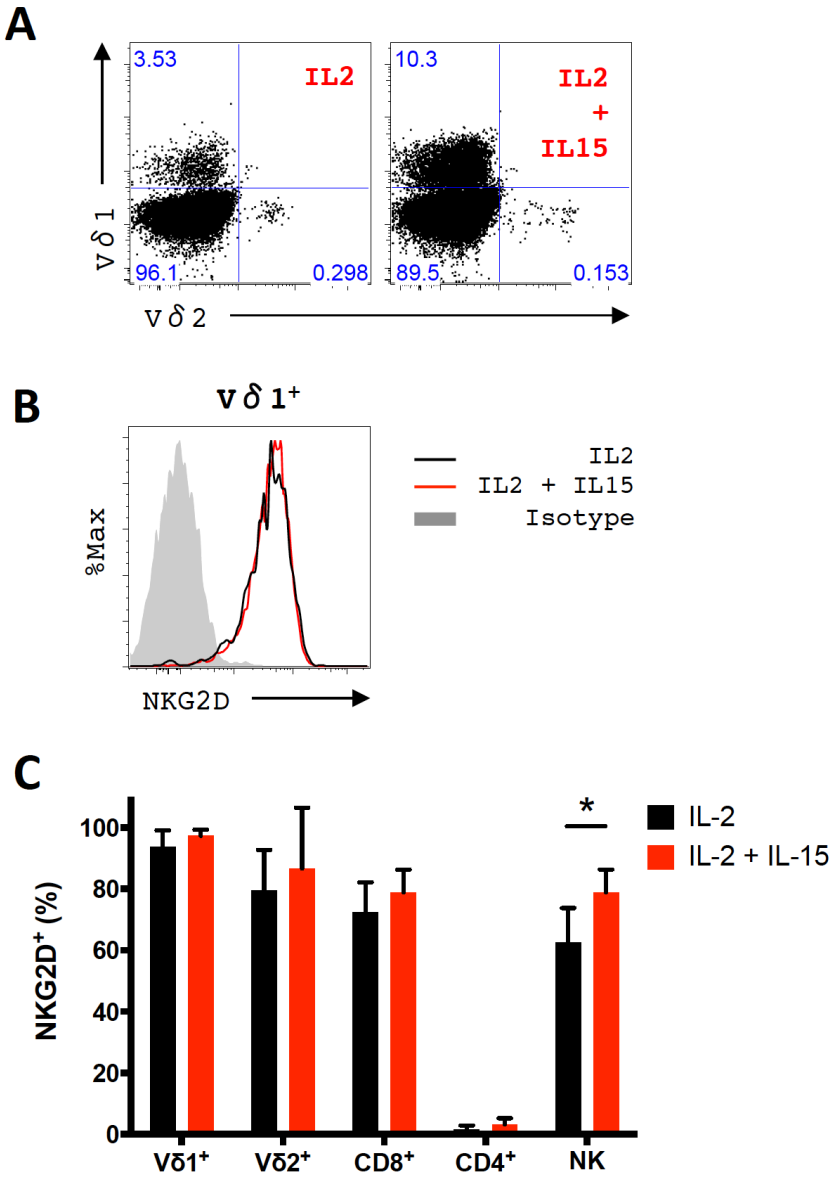

Figure 4.1. Skin explant culture with IL-15 lead to an increase in Vδ1$^+$ T cells, but did not affect NKG2D expression

(A) Representative flow cytometry analysis of Vδ1$^+$ and Vδ2$^+$ T cells subsets following explant isolation with either IL-2 (100 IU/ml) with/without IL-15 (10 ng/ml) (gated on CD3$^+$ events; representative donor of 7). (B) Representative flow cytometry analysis of Vδ1$^+$ T cell surface expression of NKG2D following explant isolation with either IL-2 (100 IU/ml) with/without IL-15 (10 ng/ml) (representative donor of 6). (C) Summary data for cell surface NKG2D expression by skin-resident lymphocytes following explant isolation with either IL-2 (100 IU/ml) with/without IL-15 (10 ng/ml). NK cells = CD3$^{(-)}$ gate (gated against isotype controls; mean with SD; n = 6).

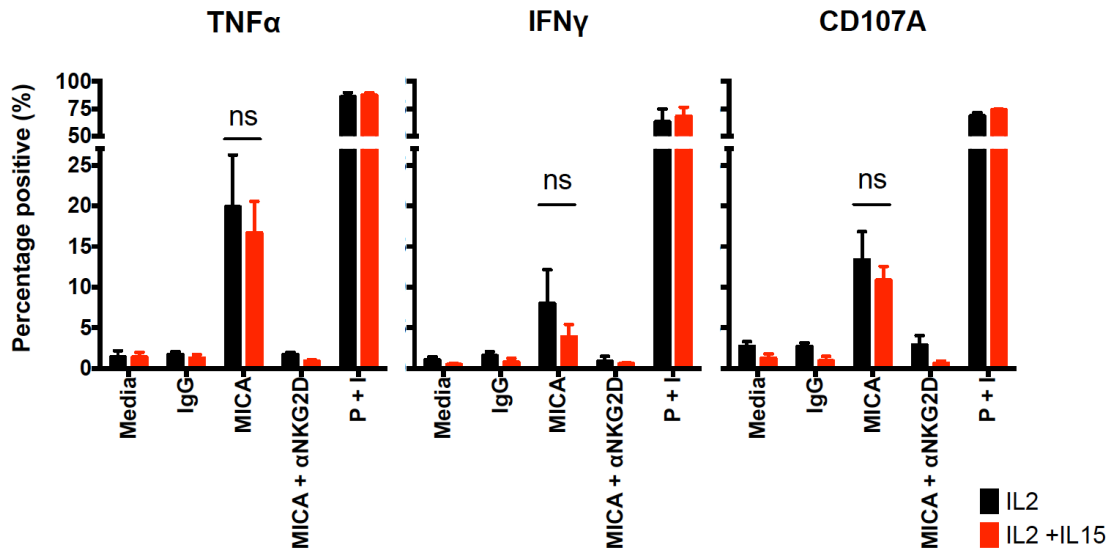

Figure 4.2. Skin explant culture without IL-15 did not affect Vδ1$^+$ T cell innate-like function

Summary data showing intracellular TNFα and IFNγ staining, and cell surface CD107a, following *in vitro* activation with plate-bound recombinant MICA (10 µg/ml), or human IgG control (10 µg/ml), of skin-resident Vδ1$^+$ T cells isolated by explant culture with IL-2 (100 IU/ml) alone, or with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). MICA stimulated cells pre-treated with anti-human NKG2D antibody (10 µg/ml) or isotype-matched control (10 µg/ml). P + I = PMA (10 ng/ml) and ionomycin (1 µg/ml). Assay performed over 6-hours with BFA (20 µg/ml) and IL-2 (100 IU/ml), and in duplicate for each donor (gated against un-stimulated controls; mean with SEM; n = 3).

4.2. NKG2D-mediated activation of Vδ1$^+$ T cells was PI3K-dependent

In mice, NKG2D can signal *via* the DAP12–Syk/ZAP70 pathway to trigger both cytotoxicity and cytokine production (Diefenbach et al., 2002; Gilfillan et al., 2002; Wu et al., 2000), and/or trigger cytotoxic responses by its association with the transmembrane adaptor protein DAP10 that upon ligand engagement signals *via* PI3K or the Grb2-Vav1 complex (Diefenbach et al., 2002; Gilfillan et al., 2002; Ibusuki et al., 2013; Wu et al., 2000). This offers an explanation for how NKG2D engagement might be sufficient to activate mouse T cells by simultaneously providing a signal equivalent to TCR activation [syk/ZAP70] and a signal equivalent to co-stimulation [PI3K].

However, the human NKG2D isoform can only associate with DAP10. Thus, although NKG2D signals *via* the PI3K pathway to trigger cytotoxicity and cytokine production by human NK cells (André et al., 2004; Billadeau et al., 2003; Upshaw et al., 2006), the lack of human NKG2D association with DAP12-Syk/ZAP70 has been hypothesized to underlie the failure of NKG2D engagement to promote human T cell activation.

The innate-like activation of human skin-resident Vδ1$^+$ T cells *via* NKG2D was found to be dependent upon PI3K activity. Treatment with the specific PI3K inhibitor LY294002 significantly impaired both MICA-induced TNFα and IFNγ production, and cytotoxic degranulation (Figure 4.3). Dose-dependent inhibition was seen, with maximal effect at a dose of 50 μM, with inhibition to a level comparable with background activity seen in the IgG control (mean inhibition 66.3 – 72.2 % across multiple donors) (Figure 4.3A – C).

The PI3K pathway also plays a key role in the downstream signalling of multiple receptor-ligand interactions in mammalian cells, including the IL-2 receptor (Ahmed et al., 1997; Moon and Nelson, 2001; Reif et al., 1997; Williamson et al., 1993). In our system, MICA-mediated activation of Vδ1$^+$ T cells was primarily demonstrated in the presence of IL-2 (100 IU/ml). When these assays were performed with skin-resident lymphocytes that had been starved of cytokine for 24 hours, and in the absence of additional IL-2 at the time of activation, the innate-like responsiveness of

Vδ1$^+$ T cells was unchanged (Figure 4.4), indicating that MICA-mediated activation is independent of exogenous IL-2 that may also signal *via* PI3K.

Therefore, blockade of PI3K activity alone, a pathway more commonly associated with T cell co-stimulation, was sufficient to inhibit the innate-like responsiveness of human Vδ1$^+$ T cells, abrogating both the production of pro-inflammatory cytokines and the mobilization of cytotoxic granules.

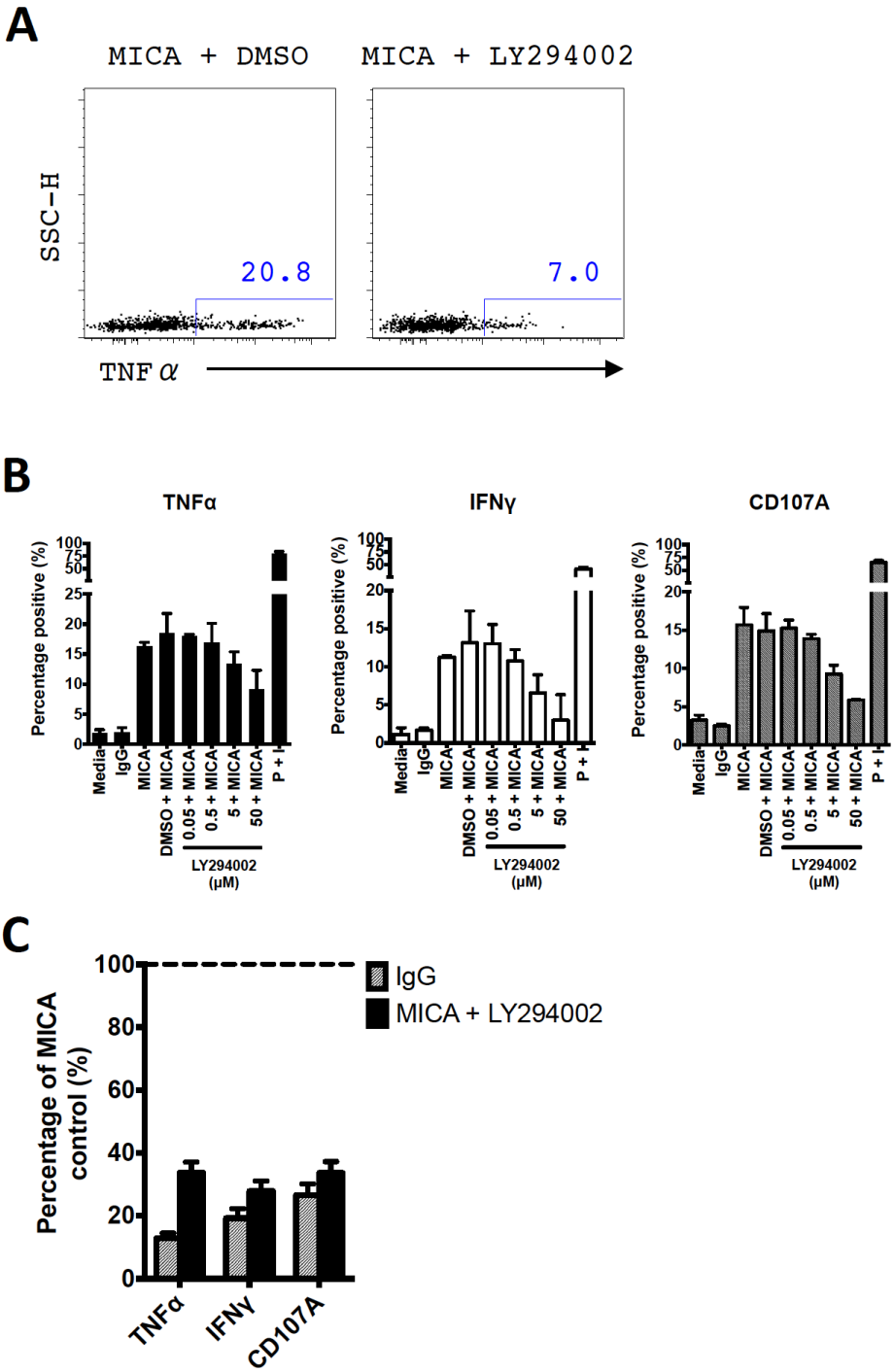

Figure 4.3. NKG2D-mediated activation of skin-resident Vδ1$^+$ T cells was dependent on phosphoinositide 3-kinase (PI3K)

(A) Representative intracellular cytokine staining for TNFα following *in vitro* activation of skin-resident Vδ1$^+$ T cells with plate-bound recombinant MICA (10 μg/ml) in the presence of LY294002 (50 μM) or DMSO control. Gated against un-stimulated controls. (B) Representative data showing intracellular TNFα and IFNγ

staining, and cell surface CD107a, of skin-resident Vδ1$^+$ T cells following *in vitro* activation with plate-bound recombinant MICA (10 μg/ml) or IgG control (10 μg/ml) in the presence of LY294002 or DMSO control (P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml); gated against un-stimulated controls; mean with SD; representative donor of 7). (C) Summary data showing inhibition of Vδ1$^+$ T cell activation following treatment with LY294002 (50 μM), expressed as a percentage of TNFα$^+$, IFNγ$^+$ or CD107a$^+$ cells observed in the DMSO-treated MICA activated control (10 μg/ml), and in comparison with the IgG control (10 μg/ml) (experiments performed in duplicate for each donor; mean with SEM; n = 11 – 13). All cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). All assays performed over 6-hours with BFA (20 μg/ml) and IL-2 (100 IU/ml), following 1 hour pre-treatment with LY294002 or DMSO.

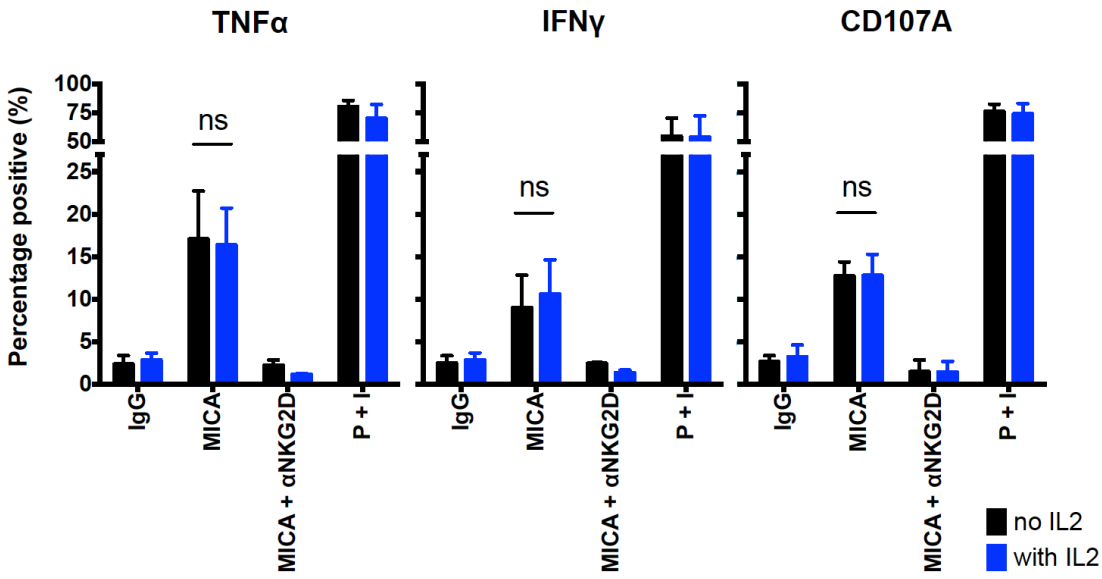

Figure 4.4. NKG2D-mediated activation of skin-resident Vδ1$^+$ T cells was not dependent on the presence of exogenous IL-2 during MICA exposure

Summary data showing intracellular TNFα and IFNγ staining, and cell surface CD107a, of skin-resident Vδ1$^+$ T cells following *in vitro* activation with plate-bound recombinant MICA (10 μg/ml), or human IgG control (10 μg/ml), with or without IL-2 (100 IU/ml). All cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml) and rested in cytokine-free media for 24 hours prior to activation. P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml). MICA stimulated cells pre-treated with anti-human NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). Assay performed over 6-hours with BFA (20 μg/ml) and in duplicate for each donor (gated against un-stimulated controls; mean with SEM; n = 2 – 4).

4.3. NKG2D-mediated activation of Vδ1$^+$ T cells was Lck-independent but required calcineurin function

Strikingly, the responsiveness of skin-resident Vδ1$^+$ T cells to ligands for the NKG2D receptor seemed dependent on NKG2D alone, and independent of the TCR. Such innate-like activation could be blocked with a monoclonal antibody specific for the NKG2D receptor, and was dependent on PI3K activity, which is not typically associated with the TCR signalling complex.

To investigate this further we studied the effect of inhibitors that targeted molecules classically associated with the TCR signalling pathway. Lck is a member of the Src-family of tyrosine kinases, and is a key proximal component of TCR signalling, being responsible for the recruitment of the tyrosine kinase ZAP70 to the signalling complex (van Oers et al., 1996). Inhibition of Lck with the specific LckII inhibitor had no obvious effect on MICA-mediated activation of Vδ1$^+$ T cells (Figure 4.5). Similarly, pre-treatment with the Lck inhibitor did not effect NKG2D-mediated activation of skin-resident NK cells. Thus, the innate-like function of skin-resident Vδ1$^+$ T cells seemed Lck-independent, supporting the notion that this was a TCR-independent process.

As previously discussed, rapid and transient increases in cytosolic calcium concentration are a classical downstream response of T cells following TCR engagement (Weiss et al., 1984). Calcineurin is a calcium-dependent serine threonine phosphatase. Following activation by increases in cytosolic calcium triggers certain nuclear transcription factors, such as the NFAT family, which are central to T cell activation (Rusnak and Mertz, 2000). Interestingly, the innate-like activation of human skin-resident Vδ1$^+$ T cells *via* NKG2D also seemed dependent upon calcineurin activity, as pre-treatment with the specific calcineurin inhibitor ciclosporin A (CsA) inhibited MICA-mediated activation (Figure 4.6). This lead to significant blockade of MICA-induced TNFα and IFNγ production, with maximal effect seen at 80 – 160 nM (79.2 % and 70.6 % inhibition, respectively, at 80 nM) (Figures 4.6B and C). However, CsA was less effective at blocking cytotoxic degranulation at this dose range (55.6 % inhibition at 80nM) (Figures 4.6B and C).

Activation of human NK cells *via* NKG2D requires both PI3K and the Grb2-Vav1 complex, which can reportedly result in dynamic changes in intracellular calcium (Upshaw et al., 2006), comparable to those seen in T cells following TCR engagement. Accordingly, calcineurin was also required for the NKG2D-mediated activation of skin-resident NK cells. Similar dose-dependent inhibition was seen following CsA pre-treatment (Figure 4.7). As with $V\delta1^+$ T cells, CsA was more effective at blocking NK cell cytokine production (maximal inhibition seen at 80 – 160 nM), with incomplete blockade of cytotoxic degranulation seen at this dose range (Figure 4.7).

Our data indicates that recognition of MICA by $V\delta1^+$ T cells leads to calcineurin-dependent events, which may include the activation of transcription factors such as NFAT. NFAT is considered an important regulator of IL-2-production (Peng et al., 2001), and is capable of binding multiple regulatory elements at the *IL-2* gene transcription start site (Rooney et al., 1995). Consistent with this, skin-resident $V\delta1^+$ T cells were able to produce IL-2 upon MICA-mediated activation (Figures 4.8A and B), albeit a small but discrete subset of cells (5.2 % ± 1.3). Production of this important T cell growth factor was limited to $V\delta1^+$ T cells, and was not seen in the NK cell population, which also has the capacity to respond to MICA. Interestingly, although $V\delta1^+$ T cells also had the capacity to produce IL-2 following TCR cross-linking, this was somewhat less (10.5 % ± 0.6) than for other T cell subsets (47.7 % ± 5.2 for $V\delta2^+$ T cells; 23.6 % ± 4.2 for $CD8^+$ T_{RM}; and 20.7 % ± 4.6 for $CD4^+$ T cells) (Figures 4.8A and B).

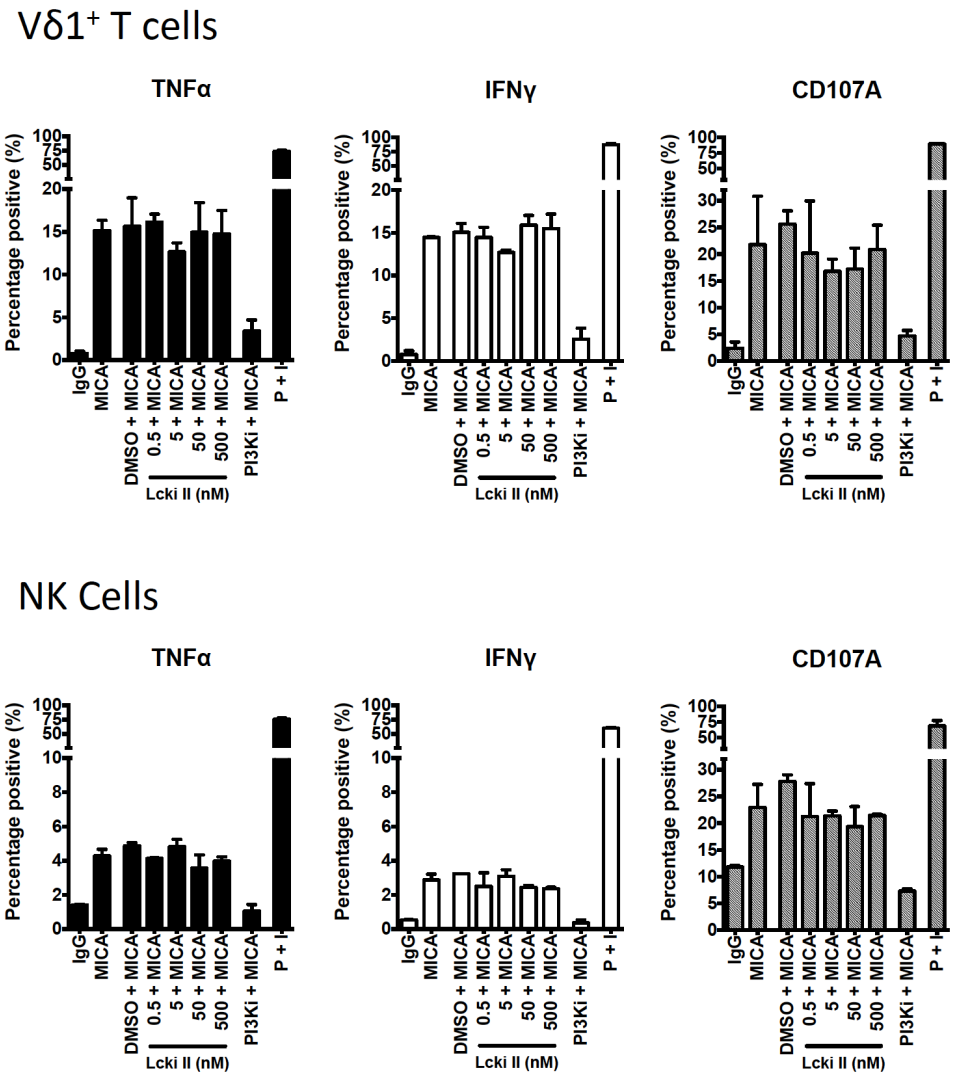

Figure 4.5. NKG2D-mediated activation of skin-resident Vδ1$^+$ T cells and NK cells was Lck independent

Representative data showing intracellular TNFα and IFNγ staining, and cell surface CD107a, of skin-resident Vδ1$^+$ T cells and NK cells following *in vitro* activation with plate-bound recombinant MICA (10 μg/ml) or IgG control (10 μg/ml) in the presence of Lck inhibitor (Lck II inhibitor; Calbiochem 428206) or DMSO control (PI3Ki = LY294002 (50 μM); P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml); mean with SD; representative donor of 4). All cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). All assays performed over 6-hours with BFA (20 μg/ml) and IL-2 (100 IU/ml), following 1 hour pre-treatment with Lck inhibitor, LY294002 or DMSO. Gated against un-stimulated controls.

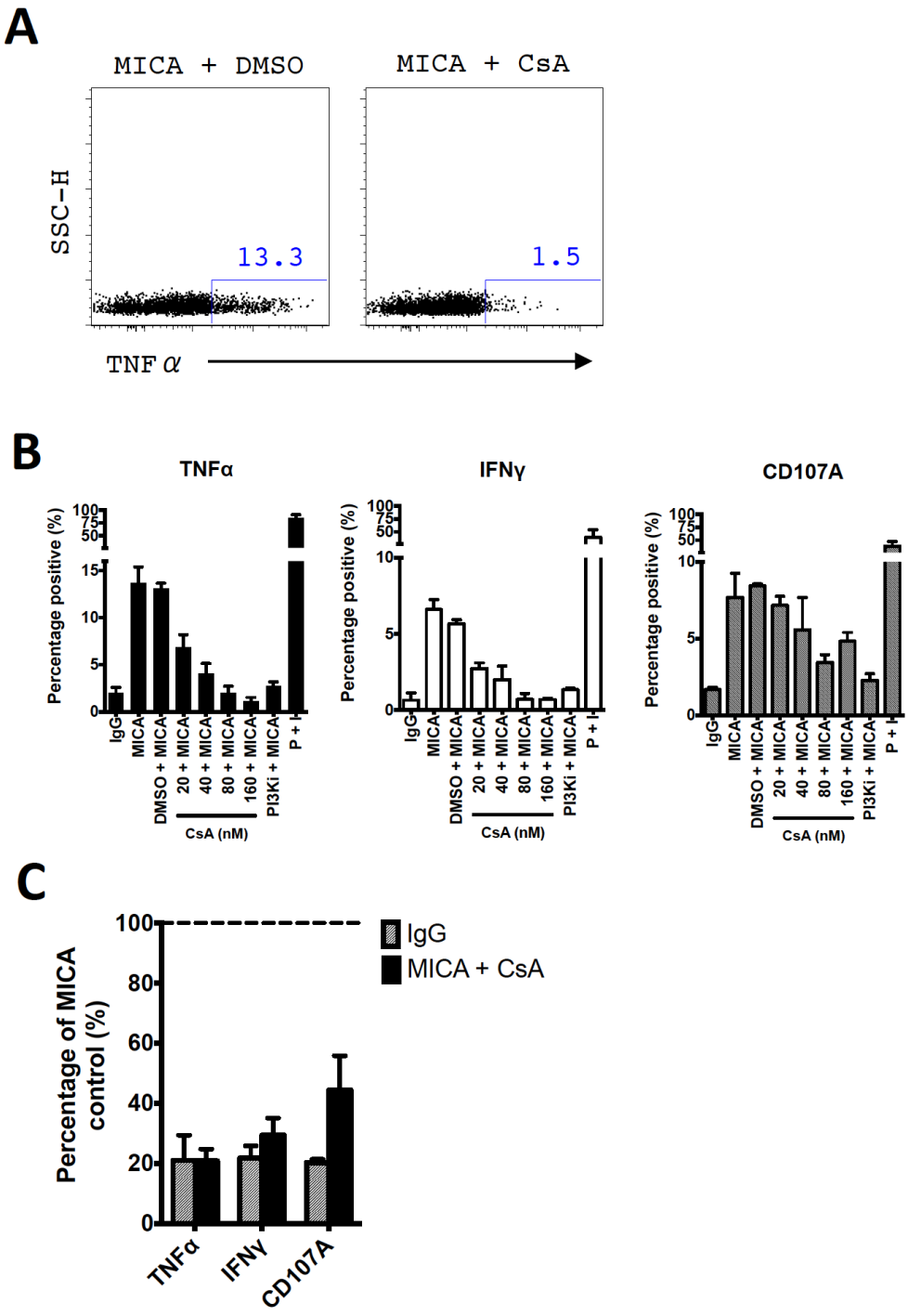

Figure 4.6. NKG2D-mediated activation of skin-resident Vδ1$^+$ T cells was calcineurin-dependent

(A) Representative intracellular cytokine staining for TNFα following *in vitro* activation of skin-resident Vδ1$^+$ T cells with plate-bound recombinant MICA (10 μg/ml) in the presence of Ciclosporin A (CsA) (80 nM) or DMSO control. Gated against un-stimulated controls. (B) Representative data showing intracellular TNFα

and IFNγ staining, and cell surface CD107a, of skin-resident Vδ1$^+$ T cells following *in vitro* activation with plate-bound recombinant MICA (10 μg/ml) or IgG control (10 μg/ml) in the presence of CsA or DMSO control (PI3Ki = LY294002 (50 μM); P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml); gated against un-stimulated controls; mean with SD; representative donor of 3). (C) Summary data showing inhibition of Vδ1$^+$ T cell activation following treatment with CsA (80 nM), expressed as a percentage of TNFα$^+$, IFNγ$^+$ or CD107a$^+$ cells observed in the DMSO-treated MICA activated control (10 μg/ml), and in comparison with the IgG control (experiments performed in duplicate for each donor; mean with SEM; n = 3 – 5). All cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). All assays performed over 6-hours with BFA (20 μg/ml) and IL-2 (100 IU/ml), following 1 hour pre-treatment with CsA, LY294002 or DMSO.

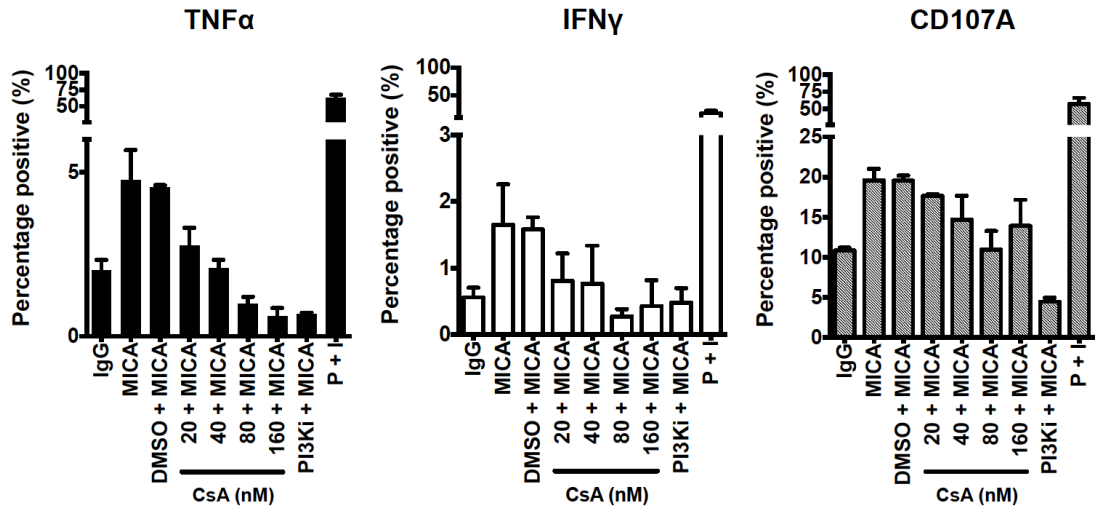

Figure 4.7. NKG2D-mediated activation of skin-resident NK cells was calcineurin-dependent

Representative data showing intracellular TNFα and IFNγ staining, and cell surface CD107a, of skin-resident NK cells following *in vitro* activation with plate-bound recombinant MICA (10 μg/ml) or IgG control (10 μg/ml) in the presence of CsA or DMSO control (PI3Ki = LY294002 (50 μM); P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml); mean with SD; representative donor of 3). All cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). All assays performed over 6-hours with BFA (20 μg/ml) and IL-2 (100 IU/ml), following 1 hour pre-treatment with CsA, LY294002 or DMSO. Gated against un-stimulated controls.

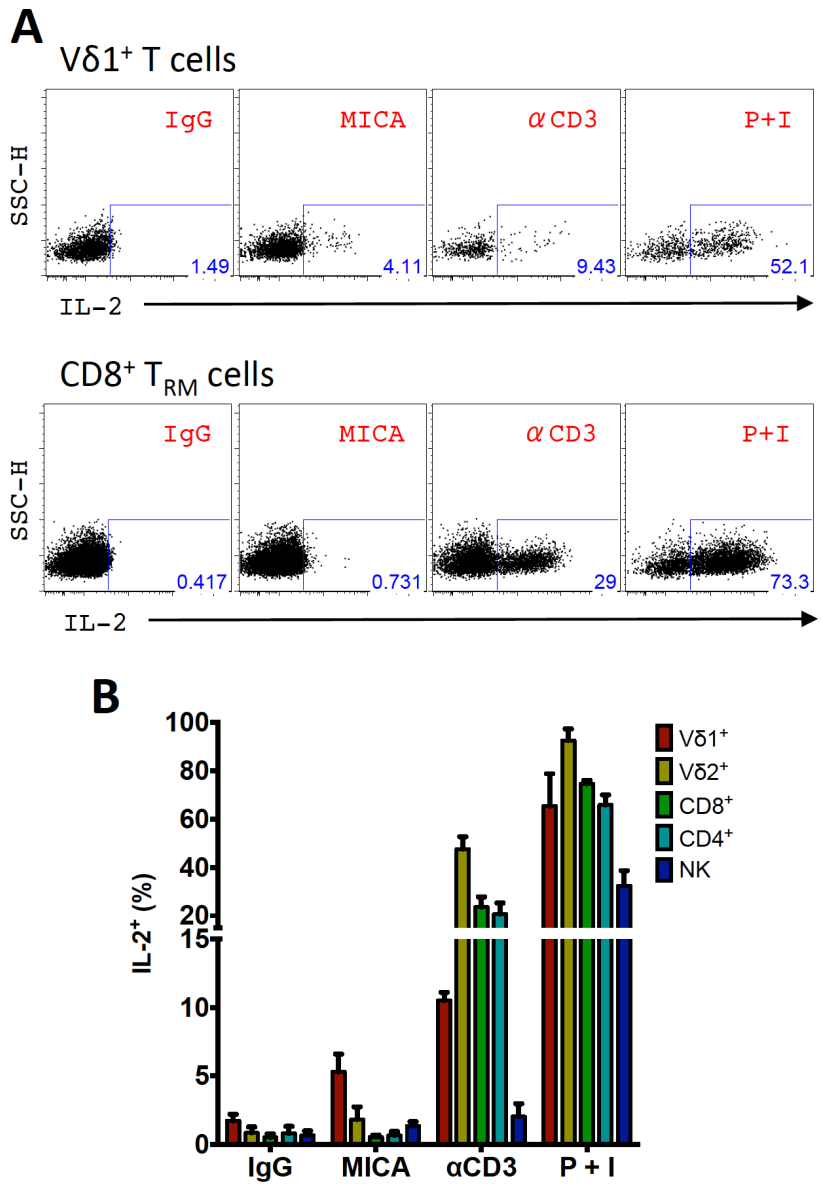

Figure 4.8. Skin-resident Vδ1$^+$ T cells have the capacity to produce IL-2 following both innate-like and TCR-mediated activation

(A) Representative flow cytometry analysis of Vδ1$^+$ and CD8$^+$ T_{RM} cells, and (B) summary data for skin-resident lymphocytes, showing intracellular IL-2 staining following *in vitro* activation with plate-bound human IgG control (10 μg/ml), recombinant MICA (10 μg/ml), anti-human CD3 antibody (5 μg/ml) or soluble PMA (10 ng/ml) and ionomycin (1 μg/ml) (P + I). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml), and then rested for 24 hours in media with no added cytokines. 6-hour activation with BFA (20 μg/ml) (experiments performed in duplicate for each donor; gated against un-stimulated controls; mean with SEM; n = 3).

4.4. Conclusion

Human skin-resident Vδ1$^+$ T cells had features of ILL, being capable of innate-like responses *via* NKG2D alone, analogous to DETC. Although IL-15 promoted the isolation of Vδ1$^+$ T cells from skin explants, their innate-like function was not a result of this condition. Strikingly, such functional capacity seemed independent of the TCR, as the Vδ1$^+$ T cells had not knowingly had their TCR manipulated during either their culture or isolation, and pre-treatment with an Lck inhibitor had little effect on their innate-like responsiveness. In addition, NKG2D-mediated activation could be blocked by anti-NKG2D antibody alone. This is a fundamental observation, as the TCR is conventionally considered the key activatory "checkpoint" for T cells, which, in the periphery, usually determines their reactivity to states of dysregulation.

In addition, MICA-induced activation of Vδ1$^+$ T cells was dependent on the PI3K pathway. It was striking that PI3K activity was sufficient for their full activation, including both cytokine production and cytotoxic degranulation, as this proximal signalling pathway is usually associated with T cell co-stimulatory signals. The innate-like responsiveness of Vδ1$^+$ T cells was also dependent upon calcineurin, and lead to IL-2 production, which may indicate that NFAT is required for their effector function. These are key components of αβ T cell activation that are classically seen downstream of TCR engagement. It is therefore striking that the innate-like activation of Vδ1$^+$ T cells *via* NKG2D also lead to calcineurin-dependent events, highlighting this as a novel mode of tissue-resident T cell activation.

Chapter Five: Identification of an additional novel skin-resident γδ T cell subset

In delineating the skin-resident lymphocyte population, we had focused on the major subsets of γδ T cells reported in human blood and tissues, which express either the Vδ1 or Vδ2 TCR chain. However, a more detailed, back-up analysis of the skin-resident γδ T cell compartment revealed an additional population of γδ T cells, which expressed neither Vδ1 nor Vδ2 TCR, representing a hitherto undescribed T cell population in human skin. We therefore sought to define key characteristics of these cells, in line with our previous work that had identified $V\delta1^+$ T cells as human skin-resident ILL.

5.1. A novel population of skin-resident $V\delta1^-/V\delta2^-$ γδ T cells that predominantly express the Vδ3 TCR

An alternative gating strategy was employed to analyse the skin-resident γδ T cell compartment, whereby cells were co-stained with an antibody specific for the γδ TCR (pan-TCRγδ), in addition to antibodies specific for either Vδ1 or Vδ2 TCR. This revealed a novel population of $TCR\gamma\delta^+$ $V\delta1^-/V\delta2^-$ double-negative (DN) cells, that were reproducibly found across a large number of donors (Figures 5.1A and B). Although less frequent than the predominant $V\delta1^+$ population (mean 76.4 % ± 4.9 [SEM] of γδ T cells), they made up a significant component of the skin-resident γδ T cell compartment (20.0 % ± 4.3) and were more numerous than the rare $V\delta2^+$ subset (3.7 % ± 0.8) (Figure 5.1B).

Strikingly these cells predominantly expressed the Vδ3 TCR chain (70.2 % and 59.6 % $V\delta3^+$ in the two donors analysed) (Figure 5.1B); however, this also revealed an additional small population of pan-$TCR\gamma\delta^+$ $V\delta1^-/V\delta2^-/V\delta3^-$ T cells. Vδ3 TCR expression has been reported on genomic analysis of human gut (Holtmeier et al., 1995), however such a population has not been previously reported in human skin, or readily isolated and characterised in any detail. Thus, the identification of "non-Vδ1/Vδ2" γδ T cells in the skin, that largely expressed the Vδ3 TCR chain, is both unanticipated and interesting.

5.2. Skin-resident Vδ1^{-}/Vδ2^{-} DN γδ T cells resembled Vδ1^{+} T cells

Skin-resident Vδ1^{-}/Vδ2^{-} DN γδ T cells expressed high levels of the NKG2D receptor, equivalent to those expressed by Vδ1^{+} T cells (5.2A). In addition, Vδ1^{-}/Vδ2^{-} DN γδ T cells lacked expression of the classical co-stimulatory molecule CD28, consistent with our previous observation that the limited CD28 expression seen in skin-derived γδ T cells was expressed by Vδ2^{+} T cells (5.2B). Therefore, both γδ T cell subsets predominantly found within the skin-resident lymphocyte compartment showed comparable expression profiles of these key regulatory molecules, and were distinct from Vδ2^{+} T cells, which are more commonly found circulating in the peripheral blood.

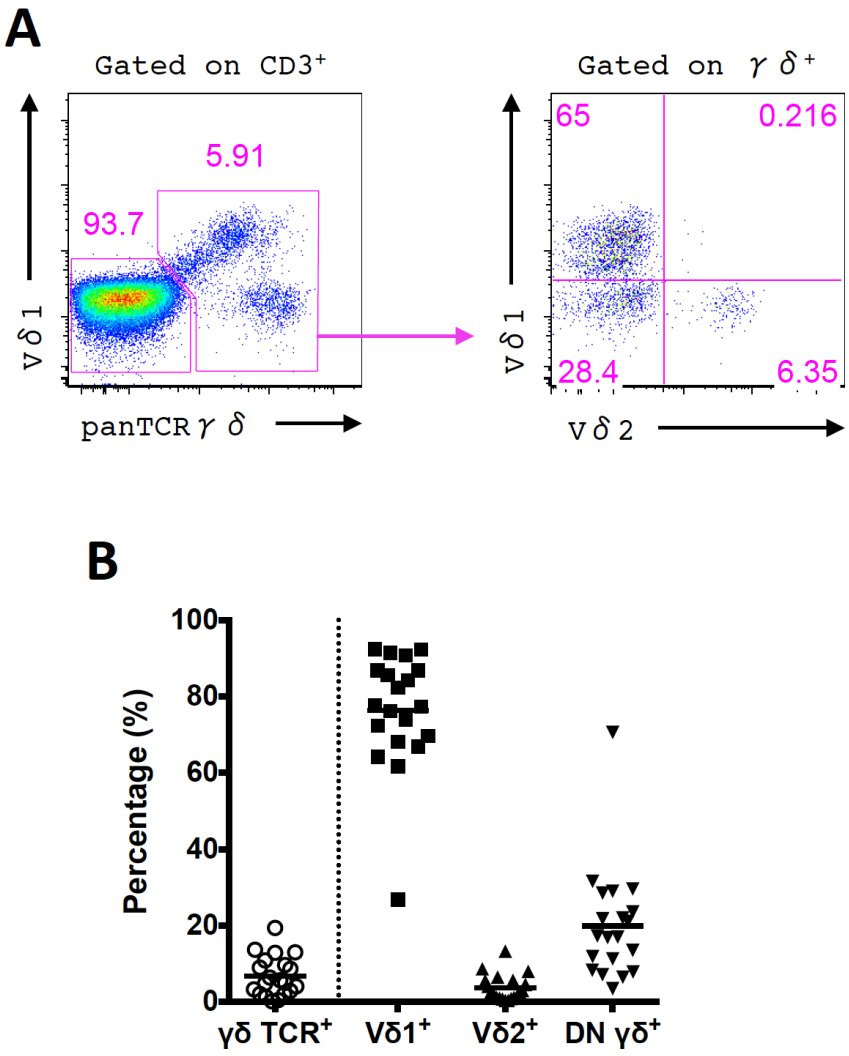

Figure 5.1. Alternative gating of skin-resident γδ T cells identified a novel Vδ1$^-$ /Vδ2$^-$ double-negative (DN) γδ T cell population

(A) Representative flow cytometry analysis demonstrating gating strategy that revealed a novel Vδ1$^-$/Vδ2$^-$ DN γδ T cell population. (B) Summary data showing the frequency of skin-resident γδ T cells (expressed as a percentage of CD3$^+$ events), and the composition of the skin-resident γδ T cell compartment as delineated by Vδ1 and Vδ2 TCR chain expression (expressed as a percentage of pan-TCRγδ$^+$ events; mean indicated; n = 20). Cells isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml).

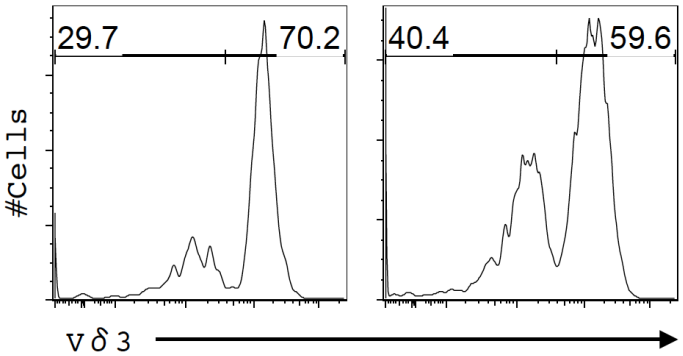

Figure 5.2. Skin-resident Vδ1^{-}/Vδ2^{-} DN γδ T cells predominantly expressed the Vδ3 TCR chain

Flow cytometry analysis showing Vδ3 TCR chain expression by pan-TCRγδ^{+} Vδ1^{-} Vδ2^{-} T cell subset in two independent donors. Cells isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml).

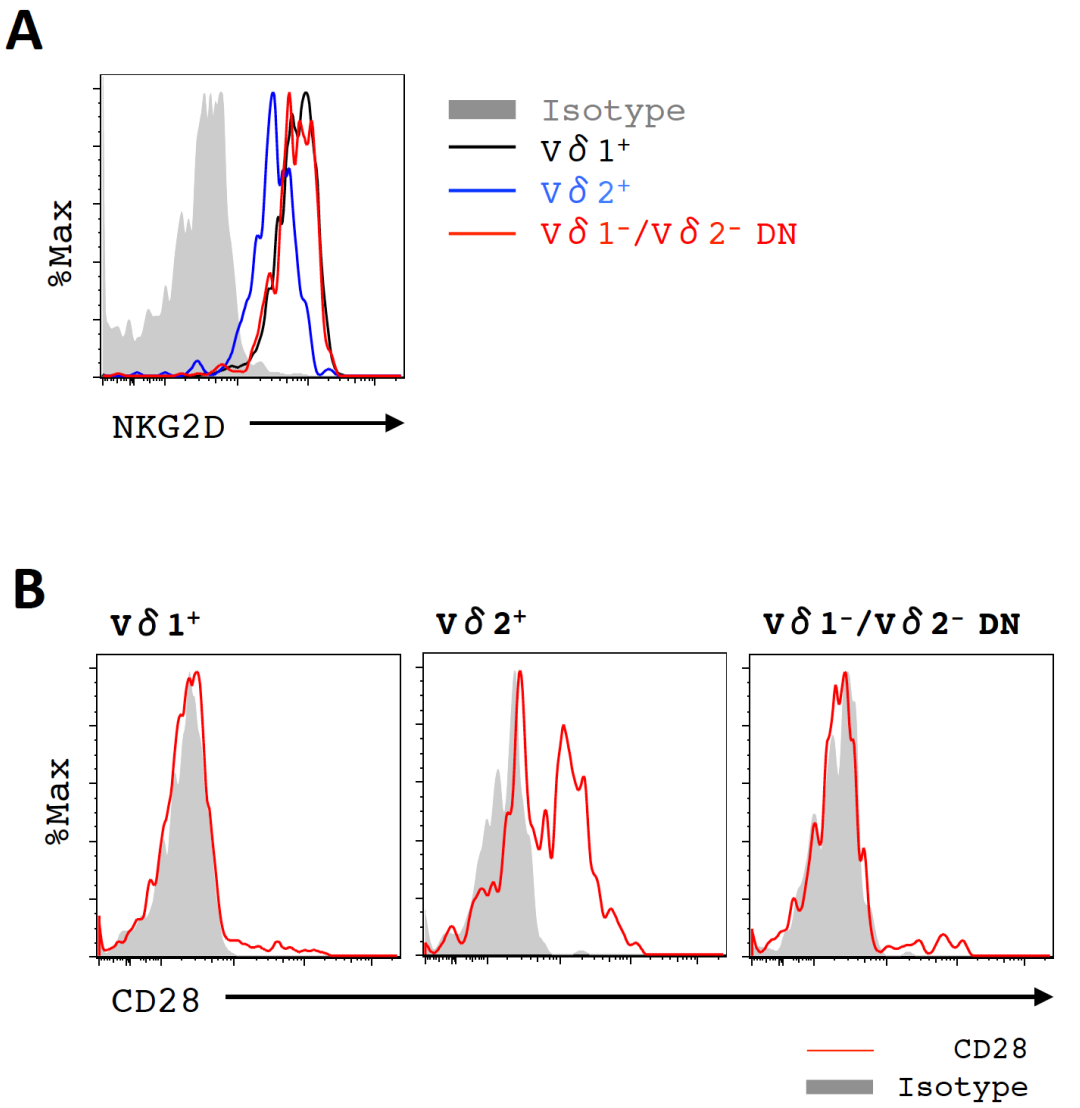

Figure 5.3. Skin-resident Vδ1$^-$/Vδ2$^-$ DN γδ T cells resembled skin-resident Vδ1$^+$ T cells, expressing high levels of NKG2D and being CD28$^-$

Representative flow cytometry analysis of the relative expression of (A) NKG2D and (B) CD28 by skin-resident Vδ1$^-$/Vδ2$^-$ DN γδ T cells in comparison with Vδ1$^+$ and Vδ2$^+$ T cells, isolated following explant culture with IL2 (100 IU/ml) and IL15 (10 ng/ml) (Representative of 9 or 5 donors, respectively).

5.3. Skin-resident Vδ1$^-$/Vδ2$^-$ DN γδ T cells showed innate-like responsiveness to recombinant MICA and exogenous cytokines

We have shown previously that skin-resident Vδ1$^+$ T cells have the innate-like capacity to be activated *via* NKG2D alone. Strikingly, Vδ1$^-$/Vδ2$^-$ DN γδ T cells also responded to plate-bound MICA, which was blocked following pre-treatment with anti-NKG2D antibody, confirming that this activation was also NKG2D-dependent (Figure 5.3). MICA-mediated activation led to cytotoxic degranulation (increased CD107a expression) (Figure 5.3), and both TNFα and IFNγ production, which were also seen following activation with PMA and ionomycin (Figure 5.4). This suggested that Vδ1$^-$/Vδ2$^-$ DN γδ T cells shared the T_H1-like bias of skin-resident Vδ1$^+$ T cells. Vδ1$^+$ T cells seemed more responsive to MICA than the Vδ1$^-$/Vδ2$^-$ DN population, with more cells staining for CD107a, TNFα or IFNγ ($p = 0.02 – 0.0002$); however, this may reflect heterogeneity in the latter subset, which did not universally express the Vδ3 TCR chain, and may thereby contain γδ T cell subsets with different functional capacities.

An additional innate property of skin-resident γδ T cells, CD8$^+$ T_{RM} cells and NK cells was their response to a combination of IL-18 and IL-12, which are IL-1 family and STAT-activating cytokines, respectively. Consistent with this, skin-resident Vδ1$^-$/Vδ2$^-$ DN γδ T cells also responded to IL-18 and IL-12, making a robust IFNγ response (35.6 % ± 14.4 IFNγ$^+$) (n = 3; Figure 5.5). Their innate response to IL-18 and IL-12 was of comparable magnitude to the innate activation seen in the Vδ1$^+$ T cell subset (Figure 5.5B). Interestingly, all skin-resident γδ T cell subsets also displayed some response to IL-12 alone to produce IFNγ; however, this was a more modest effect than when activated in combination with IL-18 (mean 8.3 – 14.0 % IFNγ$^+$).

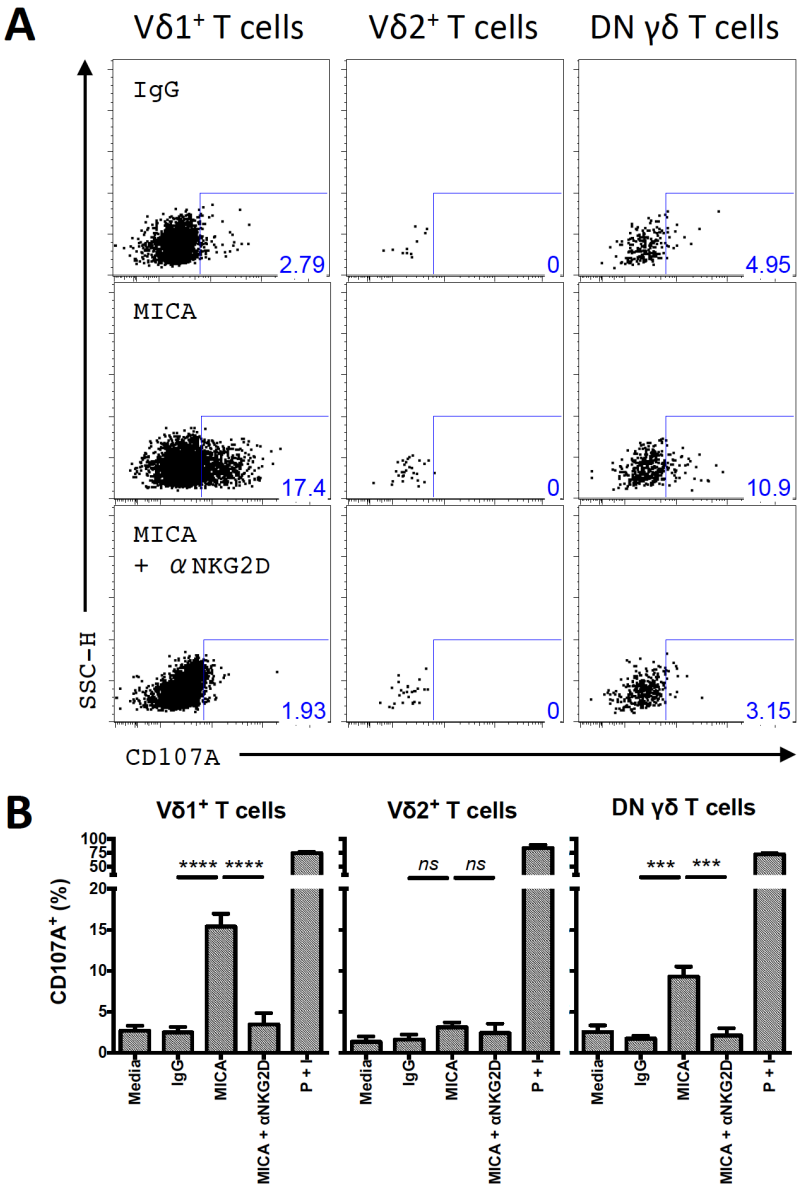

Figure 5.4. Skin-resident Vδ1⁻/Vδ2⁻ DN γδ T cells also showed innate-like responsiveness to recombinant MICA

(A) Representative cell surface staining and (B) summary data for CD107a expression following *in vitro* activation of skin-resident γδ T cells with plate-bound recombinant MICA (10 μg/ml) or human IgG control (10 μg/ml). Cells isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). MICA stimulated cells pre-treated with either anti-human NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml). 6-hour activation with BFA (20 μg/ml) and IL-2 (100 IU/ml) (experiments performed in duplicate for each donor; gated against un-stimulated controls; mean with SEM; n = 5 – 8).

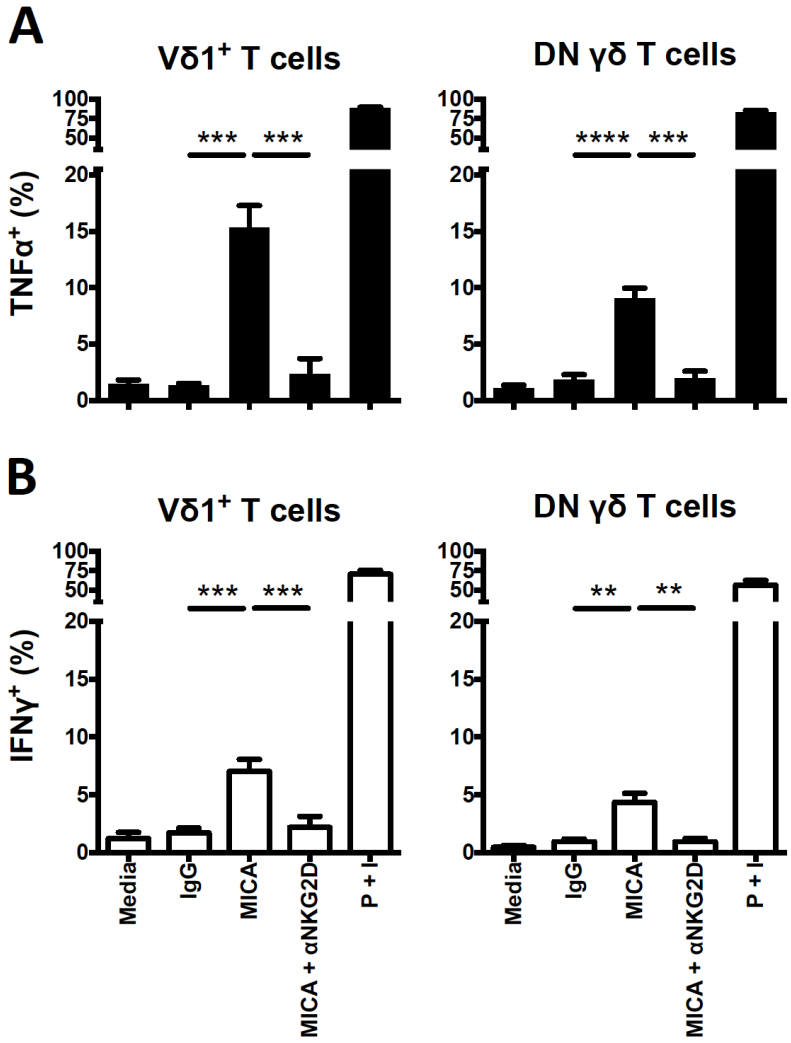

Figure 5.5. Skin-resident Vδ1⁻/Vδ2⁻ DN γδ T cell innate-like responsiveness to MICA was comparable to that of Vδ1⁺ T cells

Summary data for intracellular TNFα and IFNγ, following *in vitro* activation of skin-resident Vδ1⁺ and Vδ1⁻/Vδ2⁻ γδ T cells with plate-bound recombinant MICA (10 μg/ml) or human IgG control (10 μg/ml). Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml). MICA stimulated cells pre-treated with anti-human NKG2D antibody (10 μg/ml) or isotype-matched control (10 μg/ml). P + I = PMA (10 ng/ml) and ionomycin (1 μg/ml). 6-hour activation with BFA (20 μg/ml) and IL-2 (100 IU/ml) (experiments performed in duplicate for each donor; mean with SEM; n = 5 – 8).

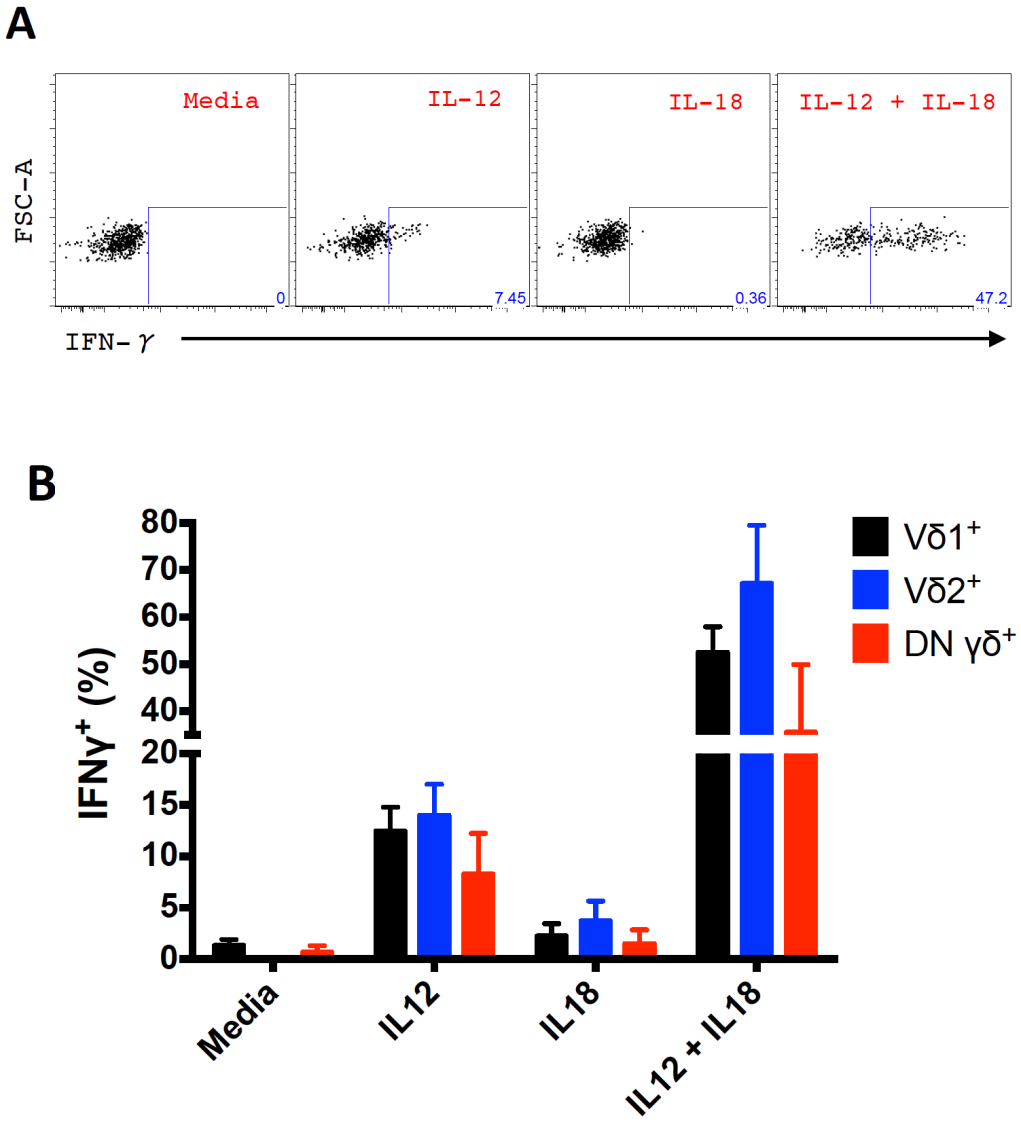

Figure 5.6. Skin-resident Vδ1⁻/Vδ2⁻ DN γδ T cells showed innate responsiveness to exogenous cytokine, comparable to other skin-resident γδ T cell populations

(A) Representative flow cytometry analysis of intracellular IFNγ staining following *in vitro* activation of skin-resident Vδ1⁻/Vδ2⁻ T cells with IL-12 (100 ng/ml) and/or IL-18 (100 ng/ml) (representative of 3 donors). (B) Summary data showing intracellular IFNγ staining following *in vitro* activation of skin-resident γδ T cell subsets with IL-12 (100 ng/ml) and IL-18 (100 ng/ml). 24-hour activation with BFA (20 μg/ml) for last 4 hours. Cells were isolated by explant culture with IL-2 (100 IU/ml) and IL-15 (10 ng/ml) (experiments performed in duplicate for each donor; gated against un-stimulated control; mean with SEM; n = 3).

5.4. Conclusion

Further in depth analysis of the skin-resident γδ T cell compartment revealed a third population of γδ T cells, which was characterised by their lack of both Vδ1 and Vδ2 TCR chain expression. This is an undescribed lymphocyte population in human skin, and these cells were found to predominantly, but not exclusively, express the Vδ3 TCR chain. This novel population largely resembled skin-resident $V\delta1^+$ T cells, expressing high levels of NKG2D; and showing comparable innate-like responsiveness to MICA, *via* the NKG2D receptor, and to particular exogenous cytokines.

It is therefore striking that the predominant populations of skin-resident γδ T cells share the distinct and unusual capacity to respond directly to MHC-I-like "stress" ligands, independent of the TCR. This was not seen in other $NKG2D^+$ T cell subsets found within the skin, including $V\delta2^+$ and $CD8^+$ αβ T_{RM} cells. This unconventional mode of T cell activation places skin-resident γδ T cells in a critical position, primed to directly sense tissue dysregulation, and to mediate potent early pro-inflammatory and cytolytic responses.

Chapter Six: Discussion

In this thesis, we have sought to develop a better understanding of human skin as an immunological organ. In particular, we investigated whether human skin displayed a signatory characteristic of body surface immunity, first demonstrated in mouse skin; namely the presence of a distinct T cell compartment that could respond rapidly and in large numbers to innate markers of tissue perturbation, rather than requiring clonal, antigen-specific activation. Such a lymphoid stress-surveillance response (LSSR) would have implications for the immunology of human skin infections, inflammation, chemical sensitisation, and malignancy. Although an LSSR was demonstrated for TCR$\gamma\delta^+$ dendritic epidermal T cells (DETC) of mouse skin, no such overt morphological compartment is present in human skin, leaving this key question unanswered.

To address it, we have isolated and characterised the T cell compartments from the skin of over one hundred donors. This has revealed that human skin contains a large compartment of γδ T cells, which are highly distinct from blood γδ T cells. Strikingly, these cells uniquely displayed strong, innate-like responsiveness to ligands for the NKG2D receptor, independent of the TCR. We believe that this is the first description of human, tissue-resident innate-like T lymphocytes.

6.1. Human skin contains a distinct population of γδ T cells with innate-like functional potential

Our study has shown that healthy human skin contains a large and complex T cell compartment, which displayed certain invariable characteristics across all donors. In particular there was a substantial population of γδ T cells that were reproducibly present within the skin. Although these cells were less frequent than αβ T cells, it is estimated that human skin contains up to 20 billion lymphocytes (Clark et al., 2006b), and therefore γδ T cells constitute a significant immune compartment. This is a striking finding as since the first reports that murine tissues contained large numbers of lymphocytes that express the γδ TCR there have been efforts to identify

equivalent populations within human tissues, such as the skin. However, up until now, previous studies have been significantly limited, as conventional tissue disaggregation protocols can greatly distort and/or destroy the associated T cell compartment(s) (Steinert et al., 2015); and the combination of sub-optimal reagents and non-uniform lymphocyte morphology has made γδ T cell quantitation difficult by immunohistochemistry (Bos et al., 1990; Foster et al., 1990; Groh et al., 1989; Spencer and Isaacson, 1989).

To overcome these challenges, we made use of a skin explant protocol that was first developed by Clark *et al*. for the study of skin-resident αβ T cells (Clark et al., 2006a). We found that this method, particularly with addition of the cytokines IL-2 and IL-15, significantly increased the number of viable skin-resident lymphocytes isolated, including γδ T cells. Although these conditions modestly favoured γδ T cell isolation when compared with tissue digestion methods, these cytokines represent growth and survival factors that can be produced by lymphocytes and/or stromal cells under physiological conditions, and are essential for the development of certain "unconventional" lymphocytes in mice and humans (De Creus et al., 2002; Edelbaum et al., 1995; Ma et al., 2006; Sprent, 2008). In addition, these cytokines did not seem to significantly alter cell surface or functional parameters that were studied. Therefore, this offered a powerful system with which we could isolate and characterise this largely under-studied lymphocyte subset.

The skin-resident γδ T cell compartment was quite distinct from the blood, which is dominated by Vγ9Vδ2 T cells. Conversely, skin-resident γδ T cells predominantly expressed the Vδ1 TCR chain. We also identified a small but consistent population of $V\delta1^{-}/V\delta2^{-}$ DN γδ T cells, which largely expressed the Vδ3 TCR chain, but also contained a further population of γδ T cells that expressed neither of the Vδ1-3 TCR chains. $V\delta1^{+}$ T cells have been previously reported as the major human γδ T cell subset in the skin (Ebert et al., 2006; Holtmeier et al., 2001; Toulon et al., 2009), and the gut (Holtmeier et al., 1995). Although Vδ3 TCR expression has been reported following genomic analysis of gut tissue (Holtmeier et al., 1995), with rare Vδ3 and Vδ5 TCR expression seen in blood-derived γδ T cell lines (Deniger et al., 2014;

Fisher et al., 2014), the Vδ1^{-}/Vδ2^{-} DN γδ T cell subset has not been previously described in the skin.

Lymphocyte activation conventionally requires antigen recognition *via* antigen receptors, such as the TCR (Janeway, 1989). This serves as a requisite "quality control" checkpoint that selects for cells reactive for foreign peptides, and prevents the expansion of those with inappropriate specificities. Conventional TCR-mediated activation is also tightly regulated, and requires additional co-stimulatory ligands and cytokines, which prevent these cells from participating in early innate immune responses (Janeway, 1989). It was work in our laboratory that first lead to the concept of lymphoid stress surveillance; whereby epidermal γδ T cells (DETC) in mice have the innate-like capacity to directly sense early markers of tissue dysregulation, such as the acute up-regulation of NKG2D ligands *in vivo* (Hayday, 2009; Strid et al., 2011; 2008).

We therefore characterised the skin-resident lymphocyte compartment for molecules that may regulate their activation, and found that skin-resident γδ T cells, CD8^{+} T_{RM} and NK cells uniformly expressed the activatory NKG2D receptor. Although all skin-resident lymphocytes readily produced cytokines following activation *in vitro* with various stimuli, NKG2D^{+} T cells responded under different circumstances to ligands for the NKG2D receptor. Strikingly, only the predominant Vδ1^{+} and Vδ1^{-} /Vδ2^{-} DN γδ T cell subsets were activated *via* the NKG2D receptor alone, independent of the TCR (Figure 6.1). This responsiveness was not limited to MICA, and was likewise activated by ULBPs. Under these conditions, Vδ1^{+} and Vδ1^{-}/Vδ2^{-} DN γδ T cells showed a robust effector responses, producing TNFα, IFNγ, cytolytic mediators and additional growth factors/chemokines. Although skin-resident NK cells were also activated *via* NKG2D, inducing primarily and cyctolytic/CCL4 response, this demonstrated a highly unusual and specific innate-like capacity of tissue-resident γδ T cells within the T cell compartment. Such innate-like responses were not displayed by NKG2D^{+} Vδ2^{+} or CD8^{+} T_{RM} cell subsets, which required concurrent TCR antigen receptor signalling for their activation, as has been reported elsewhere (Figure 6.1) (Groh et al., 2001; Nedellec et al., 2010; Roberts et al., 2001).

This indicates that the predominant skin-resident γδ T cell subsets have the capacity to directly respond in conditions of tissue stress, and can therefore participate in the early afferent immune response. Moreover, because innate myeloid cells do not express NKG2D, one may conclude that the TCRγδ^{+} ILL of human skin provide a rapid afferent response to forms of perturbation that may go unnoticed by the myeloid compartment. Hence, the LSSR qualitatively expands the immunobiology of human skin.

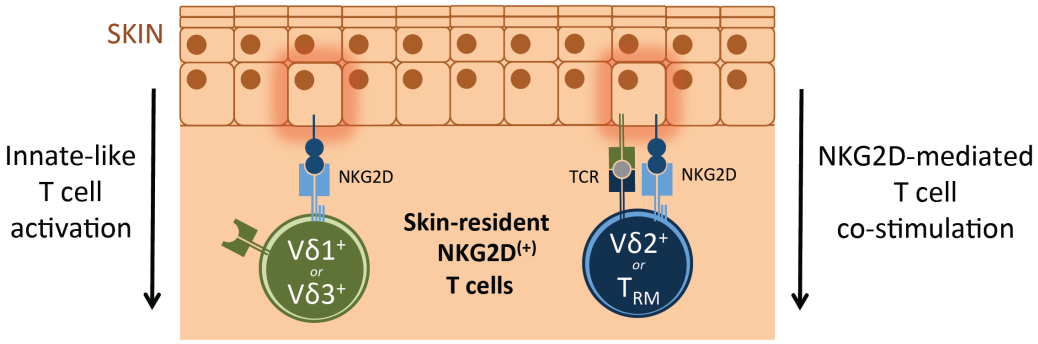

Figure 6.1. NKG2D can activate human skin-resident innate-like γδ T cells, and co-stimulate the activation of skin-resident CD8^{+} αβ T_{RM} and Vδ2^{+} T cells
The predominant skin-resident γδ T cell populations have the innate-like capacity to respond to MHC-I-like molecules *via* NKG2D alone, compatible with a human LSSR. Such innate stimuli may also serve as key co-stimulatory signals to CD8^{+} T_{RM} cells, and rare skin-resident Vδ2^{+} T cells, under dysregulated conditions where these cells also receive a TCR-mediated signal.

NKG2D has previously been implicated in human Vδ1^{+} and Vδ1^{-}/Vδ2^{-} γδ T cell recognition of MICA expressing tumour cells; however, this was primarily shown using γδ T cell lines. These cells, in their generation, have either undergone heavy manipulation through their TCR (Bauer et al., 1999; Deniger et al., 2014; Groh et al., 1998; 1999); or were isolated as activated, expanding clones from patients with viral

infections (Knight et al., 2010; 2012a). Although these cells reportedly showed evidence of innate-like function (Bauer et al., 1999; Deniger et al., 2014; Knight et al., 2010; 2012a), the conditions under which these cells were derived could have significantly altered their subsequent response mode(s). It has also been reported that $V\delta2^+$ and $CD8^+$ αβ T cell lines can acquire innate-like function following their pre-activation, either *via* the TCR or following prolonged culture with cytokine(s) (Lanca et al., 2010; Maccalli et al., 2007; Meresse et al., 2004; Rincon-Orozco et al., 2005; Shafi et al., 2011; Verneris et al., 2004). It was therefore striking that under the conditions by which we could isolate skin-resident lymphocytes, with no conscious manipulation of the TCR, only certain γδ T cell subsets had the innate-like capacity to directly respond to MHC-I-like molecules *via* NKG2D. We therefore feel that this represents the first true demonstration of human tissue-resident ILL, which is a fundamental observation.

Skin-resident $V\delta1^+$ and $V\delta1^-/V\delta2^-$ γδ T cells also displayed robust innate responses to the cytokines IL-18 and IL-12, to produce IFNγ. The response to IL-1 family and STAT activating cytokines is considered key feature of murine ILL biology, and strikingly DETC respond to the same discrete cytokine combination as human $TCR\gamma\delta^+$ ILL to produce IFNγ (Sugaya et al., 1999). However, this response was not unique and was also displayed by $V\delta2^+$, $CD8^+$ T_{RM} and NK cell subsets, which has been reported elsewhere for these populations in the blood (Berg et al., 2002; Carter and Murphy, 1999; Fehniger et al., 1999; Nussbaumer et al., 2011). IL-18 and IL-12 are key mediators of tissue stress, and are secreted by both activated myeloid cells (Gracie et al., 2003; Stoll et al., 1998), and skin stromal cells, such as keratinocytes (Aragane et al., 1994; Müller et al., 1994; Naik et al., 1999). Therefore, such cytokine-induced activation may permit skin-resident $TCR\gamma\delta^+$ ILL, in addition to certain other lymphocyte subsets, to rapidly sense dysregulation within the tissue, such as during infection, thus complementing other modes of lymphoid stress surveillance. Establishing if there is any synergy between these different modes of ILL activation is clearly of interest.

LSSR represents a key component of host immunoprotection, and mice that lack DETC show dysregulated skin homeostasis, with evidence of spontaneous skin

inflammation, impaired wound healing and increased susceptibility to skin carcinogenesis (Girardi et al., 2001; 2002; Jameson et al., 2002; Strid et al., 2008). LSSR can also effect systemic immune responses, such as the atopic response to cutaneous antigen exposure (Strid et al., 2011). It is therefore reasonable to assume that human skin-resident γδ T cells with innate-like function may likewise contribute to host immune responses and immunopathologies, as are often associated with atopy. The capacity of these cells to respond *en masse* to innate stimuli, as opposed to responding clonally to antigen, may compensate for there being less γδ T cells than αβ T_{RM} cells in the tissue. Not only does our data revise the current view of human skin immunobiology, but it has implications for our understanding of human lymphocyte biology, indicating that the activation of certain human T cell subsets in the tissue may not be constrained by conventional antigen recognition *via* the TCR.

6.2. Factors that regulate the innate-like responsiveness of skin-resident γδ T cells

The skin-resident lymphocyte compartment in mice is predominantly made up of T_{RM} cells and γδ T cells, which interestingly both show evidence of previous activation, by either antigen priming or their developmental programme, respectively. We found that skin-resident αβ T cells had features of antigen-experienced T_{RM}, based upon their expression of receptors associated with skin homing/residence and markers that indicate prior activation and acquisition of "memory status". This was consistent with data reported elsewhere (Clark et al., 2012; 2006a; Gaide et al., 2015). Interestingly, human skin-resident γδ T cells also displayed many of these features. They expressed skin-homing receptors, but also predominantly displayed a non-terminally differentiated "memory" phenotype (Dieli et al., 2003; Sallusto et al., 1999). They also expressed the markers CD69 and PD-1, which are conventionally considered markers of lymphocyte activation and exhaustion, respectively; however, they are also widely reported as features of skin-resident T_{RM}, in both humans and mice (Clark et al., 2006a; Mackay et al., 2015; Schenkel et al., 2014; Utzschneider et al., 2013).

Although NKG2D expression was displayed by all γδ T cell subsets, interestingly expression was higher in the predominant skin-resident Vδ1$^+$ T cell and Vδ1$^-$/Vδ2$^-$ γδ T cell populations, which lacked expression of CD28 and were capable of innate-like responses *via* NKG2D alone. Vδ2$^+$ T cells, which did not show innate-like function, expressed lower levels of NKG2D and some cells expressed CD28. Although some CD8$^+$ T_{RM} cells also expressed CD28, loss of CD28 expression is association with the development of memory CD8$^+$ αβ T cells (Azuma et al., 1993), and interestingly NKG2D expression was higher on CD28$^-$ cells. For skin-resident T cells this may reflecting a transition in the co-stimulatory requirement(s) of these cells towards tissue-associated factors, such as NKG2D ligands. Within γδ T cell subsets, possibly lower expression of NKG2D is a feature of T cells in which it functions primarily as a co-stimulator for the antigen receptor.

Skin-resident γδ T cells displayed a distinct TCR Vδ chain usage profile, predominantly expressing Vδ1 and Vδ3 TCR chains. Mouse skin contains several distinct populations of γδ T cells that localise to different anatomical compartments, with the vast majority of DETC expressing the monoclonal Vγ5Vδ1$^+$ TCR (Asarnow et al., 1988; Carding and Egan, 2002). DETC development in the thymus is dependent on the butyrophillin-like molecule Skint-1 that requires TCR-mediated activation (Barbee et al., 2011; Boyden et al., 2008; Lewis et al., 2006), and during this process they acquire expression of skin-homing markers (Jiang et al., 2010). In the skin, DETC show evidence of atypical TCR responsiveness; constitutively engaging with tissue components with evidence of tonic signalling (Chodaczek et al., 2012); and displaying attenuated responses to TCR agonists *in vitro* – a feature shared with other murine ILL subsets (Turchinovich and Hayday, 2011; Wencker et al., 2013). This unconventional mode of ILL development and peripheral "activated-yet-resting" state may remove the TCR as a primary checkpoint, and permit DETC to respond directly to innate stimuli, such as NKG2D ligands or combinations of IL-1 family and STAT-activating cytokines (Strid et al., 2011; Wencker et al., 2013).

Although the reactivity of human Vγ9Vδ2$^+$ T cells to phosphoantigen has been characterised in some detail, with recent focus on the butyrophilin molecule

BTN3A1 (Reviewed in (Vantourout and Hayday, 2013)), the reactivity of "non-Vδ2" T cells is not well understood (Hayday and Vantourout, 2013). Vδ1$^+$ T cell clones have been reported to both recognise CD1-associated lipids and MICA directly *via* their TCR (Luoma et al., 2013; Uldrich et al., 2013; Wu et al., 2002; Xu et al., 2011; Zhao et al., 2006); however, such responses are not ubiquitous, having only been demonstrated in rare subsets, and with some overlap in apparent TCR specificity reported (Hayday and Vantourout, 2013). It may be that the selective focusing of human γδ T cells with certain Vδ chains to the tissue, which have a "pre-activated" T_{RM}-like phenotype, in fact represents an alternative developmental programme; whereby γδ T cell subsets reactive to shared elements populate the skin, where they have specialised innate-like effector functions, analogous to DETC. Clearly, the identification of ligands for the skin-associated Vδ1 and Vδ3 TCRs remains a high priority.

As discussed, normal murine ILL development requires components of TCR signalling, subsequent to which antigen receptor responses are attenuated, which can result in qualitative differences in intracellular calcium flux and reduced IL-2 production (Turchinovich and Hayday, 2011; Wencker et al., 2013). Human skin-resident γδ T cells displayed acute, transient fluxes in intracellular calcium following TCR engagement, comparable to CD8$^+$ T cells. Nonetheless, the amplitude of calcium flux was quantitatively less for both subsets than for CD4$^+$ T cells. In addition, although γδ TCR cross-linking induced effector responses, this provoked conspicuously less IL-2 production by innate-like γδ T cells than by other T cell subsets. IL-2 synthesis is predominantly induced by the TCR signalling, regulated downstream by calcineurin and NFAT (Peng et al., 2001; Rooney et al., 1995; Rusnak and Mertz, 2000; Weiss et al., 1984). While these data did not irrefutably demonstrate attenuated TCR signalling in human skin-resident ILL, they suggested that human and murine ILL share some features of TCR responsiveness that merit better understanding. Again this can follow from the identification of ligands for the skin-associated Vδ1 and Vδ3 TCRs.

NKG2D-mediated activation of human skin-resident γδ T cells was dependent on PI3K signalling, which was required for both cytokine production and cytotoxic

degranulation. The human NKG2D isoform can only associate with the adaptor protein DAP10, which is known to signal *via* PI3K (Billadeau et al., 2003; Upshaw et al., 2006; Wu et al., 2000). The PI3K pathway is usually associated with co-stimulatory molecules, such CD28 and the IL-2 receptor (Ahmed et al., 1997; Appleman et al., 2002; Moon and Nelson, 2001; Reif et al., 1997; Williamson et al., 1993). It was therefore striking that NKG2D could seemingly induce full T cell activation *via* NKG2D/DAP10-PI3K signalling alone, in the absence of overt TCR signalling. Murine NKG2D can signal *via* both the DAP10-PI3K or DAP12-Syk/ZAP10 pathways, which was thought to permit the innate-like activation of murine γδ T cells, with Syk/ZAP10 signalling activating components downstream of the TCR (Ibusuki et al., 2013).

Interestingly, MICA-mediated activation was also calcineurin dependent and led directly to the production of IL-2. Although IL-2 is predominantly activated following TCR-engagement (Peng et al., 2001; Rooney et al., 1995; Rusnak and Mertz, 2000; Weiss et al., 1984) (*see above*), it has been reported that human NKG2D signalling can alone induce changes in cytosolic calcium, via the Vav1-Grb2 complex, which could activate calcineurin/NFAT (Upshaw et al., 2006). This is of clinical interest, as calcineurin antagonists are widely used to treat inflammatory dermatoses, and would therefore also inhibit ILL activation. In addition, MICA-mediated Vδ1$^+$ T cell activation was independent of the tyrosine kinase Lck, which is a key component of the proximal TCR-signalling complex (van Oers et al., 1996), which further argues that MICA-mediated activation did not require concurrent TCR signalling.

Our data does not however preclude the notion that skin-resident ILL may still have undergone prior "atypical" activation *via* their TCR, either during development and/or within the tissues, which may license their innate-like responsiveness. Such a developmental "checkpoint" may explain why, despite Vδ1$^+$ and Vδ1$^-$/Vδ2$^-$ DN γδ T cells having a robust innate-like response to either NKG2D ligands or discrete cytokine combinations, not all cells seemed to respond and there is some inter-individual variation. Such a developmental programme may also account for the effector response of some γδ T cell cells being skewed towards either cytokine production or cytotoxic degranulation upon innate-like activation, which may reflect

different states of developmental/peripheral maturation, analogous to NK cells or murine ILL (Caligiuri, 2008; Chan et al., 2007; Cooper et al., 2001; Wencker et al., 2014). However, their peripheral activation *via* NKG2D *in vitro* seems independent of concurrent TCR antigen receptor signalling, with NKG2D capable of directly activating key signalling pathways that lead to ILL cytokine production and cytotoxicity.

6.3. Implications of human innate-like γδ T cells for skin immunobiology

Skin lymphocyte immunobiology has been largely shaped by a model of adaptive immunity. However, the identification of lymphocyte populations that display innate responses within the tissues, such as ILL and ILCs, has challenged this view. Our first description in the human skin of innate-like γδ T cells, analogous to those found in murine tissues, implicates this previously unappreciated aspect of lymphocyte immunobiology in host immune responses and inflammatory disease.

Skin-resident γδ T cells had a predominant T_H1-like bias, and upon their innate-like activation *via* NKG2D produced TNFα, IFNγ, cytolytic mediators and additional growth factors/chemokines. This phenotype was strikingly similar to that seen in DETC, which are biased toward cytolysis, and the production of IFNγ, IL-13, and a discrete subset of comparable chemokines (Boismenu et al., 1996b; Shires et al., 2001; Strid et al., 2011; Turchinovich and Hayday, 2011). This effector profile and innate-like response mode is consistent with a potential to recognise and attack malignant cells, which commonly upregulate NKG2D ligands, and to recruit other components of the immune system (Gasser et al., 2005; Groh et al., 1999). Consistent with this, mice specifically lacking DETC show an increased susceptibility to skin carcinogens that equates to that of mice lacking all γδ T cells, and that is more profound than that of mice lacking αβ T cells (Girardi et al., 2001; Strid et al., 2008).

Human skin-resident γδ T cells also displayed profound cytolytic responses towards cancer cell lines *in vitro*, which was in part mediated by their capacity for lymphoid

stress surveillance *via* NKG2D. Human γδ T cells have been reported within the immune cell infiltrate of many solid tumours, including malignant melanoma (Cordova et al., 2012; Maccalli et al., 2007; Paschen et al., 2009); however, they often only comprise a relatively minor subset, and their true significance is not well understood (Cordova et al., 2012; Gentles et al., 2015). Despite this, in a recent meta-analysis that determined the intratumoural leukocyte compartment of 25 different human cancers across approximately 6 000 samples, γδ T cells strikingly emerged as the most significant favourable cancer-wide prognostic population (Gentles et al., 2015). In the same study, transcriptomic analyses of approximately 18 000 samples from 39 malignancies identified *KLRK1*, which encodes human NKG2D, as one of the ten genes that were most significantly associated with favourable survival (Gentles et al., 2015).

Therefore, the demonstration of lymphoid stress surveillance by human NKG2D^{+} γδ T cells in the skin may help clarify the perplexing issue that surrounds the immunogenicity of solid tumors, and in part explain the prognostic benefit of infiltrating γδ T cells in the host response to cancer(s). In addition, the capacity to mount effective immune responses against carcinogenesis *via* LSSR may constitute a selective pressure that drives cancers, such as melanoma, to develop reported immunoevasion strategies that target the NKG2D axis (Heinemann et al., 2012; Oppenheim et al., 2005; Paschen et al., 2009; Raffaghello et al., 2004). Although blocking NKG2D impaired recognition of cancer cells by skin-resident γδ T cells, this did not completely abrogate their response. This seemingly implies other modes by which the cells can mediate LSSR, including *via* other innate receptor-ligand interactions. A full cell surface proteomic assessment of skin Vδ1 cells is called for, so as to reveal other receptor-ligand axes that mediate the cells' innate-like responsiveness. At the same time, the cells may have undergone prior activation within the tissue or during their isolation. Clearly the nature of such human LSSR responses merits further study, in particular factors that may regulate effective γδ T cell immune surveillance in the skin, and potentially other tissues, during early carcinogenesis and/or established malignant disease.

Skin-resident γδ T cells, in some individuals, were a source of the type-2 cytokine IL-13, which could also be provoked following activation *via* NKG2D. Similarly, $CD8^+$ T_{RM} cells also produced IL-13 in certain individuals, when co-stimulated by NKG2D engagement. In mice, NKG2D-dependent cross talk between dysregulated epithelial cells and DETC was required for normal local and systemic atopic responses to cutaneous antigen (Strid et al., 2011). DETC IL-13-production was a key feature of this, and LSSR enhanced systemic IgE responses (Strid et al., 2011). This not only implicates LSSR in the afferent induction of T_H2 responses, but also links atopy with early immune responses to tissue damage and carcinogenesis. It was therefore striking that IL-13 production was a feature of the human skin-resident lymphocyte response to NKG2D. Although this was not a consistent observation, which may indicate undisclosed atopic disease amongst donors, this still indicates that type-2 immunity may be an important component of human LSSR. Establishing the contribution of such a response to lymphoid stress surveillance, and also atopic skin disease, is clearly of interest.

Human skin-resident γδ T cell cells were not a source of IL-17. This could be the result of bias introduced during the isolation of these cells; however, under these conditions a range of effector functions were seen in the αβ T cell compartment, including T_H17 cells. The lack of human γδ T cell-derived IL-17 was is in contrast to dermal γδ T cells in mice, which produce IL-17 in response to IL-1β and IL-23, resulting in a psoriasiform dermatitis (Cai et al., 2011; Mabuchi et al., 2011; Martin et al., 2009; Sumaria et al., 2011; Sutton et al., 2009). $V\gamma9V\delta2^+$ T cells in the blood and skin of patients with psoriasis have been reported as a potential source of IL-17 (Cai et al., 2011; Laggner et al., 2011), although $V\delta2^+$ T cells were rare in healthy skin. The absence of IL-17 production by human ILL was consitent with a potential immunoprotective role, especially as in certain murine cancer models, IL-17-producing γδ T cells have been reported to have a paradoxical role, and may in fact promote tumorigenesis (Carmi et al., 2011; Ma et al., 2014; Rei et al., 2015; Wakita et al., 2010).

Although skin-resident γδ T cells displayed an effector response compatible with an effective cancer immune surveillance, many other states of tissue "stress" may lead to the up-regulation of NKG2D ligands in the skin, which could therefore activate

the innate immune compartment. These may include common environmental exposures, such as UV irradiation (Nice et al., 2009; Vantourout et al., 2014) or chemical sensitisers (Nielsen et al., 2015); or the response to certain infections, such as viruses (Draghi et al., 2007; Knight et al., 2012a). NKG2D ligands have also been implicated in the pathogenesis of common inflammatory skin diseases, such as psoriasis (Knight et al., 2012b; Pollock et al., 2013; 2011; Song et al., 2013), and alopecia areata (Ito et al., 2007; Petukhova et al., 2010; Xing et al., 2014). Interestingly IL-15 expression is also reportedly increased at times of skin dysregulation, such as following UV irradiation (Mohamadzadeh et al., 1996); or during pathological changes, such as in alopecia areata (Ruckert et al., 2000; Xing et al., 2014). IL-15 acted as a γδ T cell growth factor, favouring their isolation from skin explants. It is therefore striking that at times of tissue "stress", when NKG2D ligands may also be expressed; stromal cells produce local factors that may also support the recruitment of skin-resident ILL that are capable of a coordinated response to such innate stimuli.

Therefore, having established that human skin contains a significant, reproducible population of innate-like γδ T cells, that have the capacity to participate in early immune responses to tissue "stress", it would clearly be of considerable interest to establish the contribution of these cells to tissue homeostasis and inflammatory skin disease. In addition, such human ILL biology may not be limited to the skin, and may represent a common feature of other human barrier tissues that are populated with significant numbers of γδ T cells, such as the gut – addressing this is a high priority.

6.4. Conclusions

In this thesis, we sought to develop a better understanding of human skin as an immunological organ. In particular, we investigated if human skin was populated with a distinct T cell compartment that had the capacity to make rapid, innate-like responses to markers of tissue "stress", analogous to the LSSR in mouse skin. This has led us to make what we believe is the first description of human innate-like, tissue-resident T cells. This offers a new perspective on tissue immune surveillance, and has implications for future studies in human skin immunobiology.

Through the analysis of over one hundred healthy donors, we have established that the skin contains a distinct population of γδ T cells, which predominantly expressed the Vδ1 and Vδ3 TCR chains. These cells displayed a unique capacity to become robustly activated by ligands for the NKG2D receptor, independent of the TCR, which is a feature of ILL. This capacity permits skin-resident γδ T cells to directly sense tissue dysregulation and participate in the early afferent immune response. As these cells respond *via* NKG2D, they may become activated under conditions not sensed by other innate myeloid cells in the skin, thereby qualitatively expanding the immunobiology of human skin. The TCRγδ$^+$ ILL also responded robustly to discrete cytokines. Although γδ T cells are less frequent than αβ T_{RM} cells in the tissue, their capacity to respond *en masse* to innate stimuli, as opposed to responding clonally to antigen, may compensate for this.

The innate-like responsiveness that skin-resident γδ T cell displayed is a highly unusual mode of lymphocyte activation, as the TCR antigen receptor conventionally provides a "quality control" checkpoint that regulates the peripheral activation of T cells. Murine ILL undergo TCR-mediated activation during their development, which is thought to remove this primary checkpoint and permit their subsequent innate-like activation. Interestingly, human skin-resident γδ T cells shared some features with murine ILL suggestive of an "activated-yet-resting" state; however, further characterisation of the skin-associated Vδ1$^+$ and Vδ3$^+$ TCRs is merited.

It was striking that the robust effector response elicited *via* NKG2D in human skin-resident γδ T cells was analogous to that seen in DETC, which are the prototypic effectors of LSSR in murine skin. LSSR represents key components of host immunoprotection in the mouse, which can also affect systemic immune responses, such as atopy. Our findings that human skin-resident γδ T cells can participate in immune responses compatible with LSSR, strongly argues for its existence. This revises our current view of skin immunobiology, and needs to be considered in the future study of malignancy, infection, inflammation and atopy in human skin.

Chapter Seven: Materials and Methods

7.1. Normal human skin

Human skin samples were obtained as discarded material after cutaneous or reconstructive surgery (St Thomas' Hospital, London) under ethical approval of the Guy's and St Thomas' NHS Foundation Trust Research Ethics Committee (06/Q0704/18), adhering to the principles of the Declaration of Helsinki. Participants were adult patients that were undergoing elective mastopexy, mastectomy, breast reconstructive surgery or abdominoplasty. Truncal (breast or abdominal) skin was used. Data from 101 donors is presented in this thesis. Ninety-eight (97 %) donors were female with a median age of 49 years (range 20 – 77 years). Eighty-one (80.2 %) donors were White, sixteen (15.8 %) Black and four (4 %) Asian.

Relevant comorbidities were noted (from patient history and electronic patient records). Given the clinical setting in which participants were recruited, fifty-nine (58.4 %) donors had a previous or concurrent diagnosis of breast cancer. Participants were excluded if they had known active skin disease, were on systemic immunosuppressive medications, or had received cytotoxic chemotherapy within six months of surgery.

7.2. *Ex vivo* isolation of lymphocytes from human skin by tissue disaggregation

Samples of adult human skin were obtained within 3 – 6 hours of surgery. Subcutaneous fat was removed and the remaining skin tissue (dermis with epidermis) was minced into fragments measuring approximately 1 mm x 1 mm. Skin fragments were added to 5 ml of RPMI 1640 with collagenase D (1 mg/ml; Roche) and DNase (0.1mg/ml; Roche), and incubated at 37°C on a shaker for 30 minutes. Enzymatically digested tissue was subsequently mechanically disaggregated using a gentleMACS® dissociator (MiltenyI Biotec). For this the tissue digest was made up to 10 ml with RPMI 1640 and transferred to a gentleMACS® tube. This was processed for 60 seconds using programme

m_spleen_04. Tissue fragments and supernatant were passed through a 70-µm filter (BD Biosciences) into a 50ml centrifuge tube (Corning). Digestion was halted by addition of two 10 ml aliquots of cold PBS/10 mM EDTA, which were used to wash the gentleMACS® tube and also passed through the 70-µm filter. Cells were isolated by centrifugation (1600 rpm for 15 minutes at 4 ^{o}C). The cell pellet was re-suspended in 1 ml of Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma) and incubated at room temperature for 5 minutes. Lysis was stopped by the addition of 1 ml of PBS. Samples were pooled at this stage. Cells were isolated by centrifugation (1600 rpm for 10 minutes). The cell pellet was re-suspended in RPMI 1640 for subsequent analysis by flow cytometry. When cell counts were required, leukocytes were counted by; (1) trypan blue stain (0.4 %) (Life Technologies) using a haemocytometer, or (2) CASY® Model TT cell counter and analyser (Roche).

7.3. Isolation of lymphocytes from human skin by three-dimensional explant culture

A three-dimensional skin explant protocol was established, as described elsewhere (Clark et al., 2006a). The 9 mm x 9 mm x 1.5 mm Cellfoam Matrices (Cytomatrix Pty Ltd, Victoria, Australia) were autoclaved, then incubated in a solution of 100 mg/ml rat tail collagen I (BD Biosciences) in PBS for 30 minutes at room temperature, followed by one rinse in PBS. Samples of adult human skin were obtained within 3 – 6 hours of surgery. Subcutaneous fat was removed and the remaining skin tissue was minced into fragments measuring approximately 1 mm x 1 mm. Approximately five skin fragments were placed and pressed down onto the surface of each matrix. Each matrix was placed into a separate well of a 24-well plate (Corning) containing 2 ml of 'Skin-T' media [Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies) with 10% heat-inactivated foetal bovine serum (Life Technologies), L-glutamine (292 µg/ml; Life Technologies), penicillin (100 units/ml; Life Technologies), streptomycin (100 µg/ml; Life Technologies), and 2-mercaptoethanol (3.5 µl/L; Life Technologies)]. For the first 7 days of culture Amphotericin B (2.5 µg/ml; Life Technologies) was added to the media. Media was refreshed three times per week. For feeding, the upper 1 ml of media was aspirated from each well and replaced with fresh media. For cultures treated with IL-2 and/or

IL-15, cytokines were added from the initiation of culture and on each media change until the isolation of lymphocytes at 21 days. Human recombinant IL-2 was added at 100 IU/ml (Proleukin; Novartis Pharmaceuticals UK Ltd). Human recombinant IL-15 was added at 10 ng/ml (Biolegend). Up to 96 wells (four 24-well plates) were set up in culture for each donor.

To isolate the lymphocytes, the matrices were transferred to a 50ml centrifuge tube (Corning) containing 10 ml Hanks Balanced Salt Solution (HBSS; Life Technologies) with 0.01 mM HEPES (up to 12 matrices/tube). The matrices were rinsed with the cell suspension using a 10 ml pipette, and the cell suspension passed through a 70-µm filter (BD Biosciences) into a fresh 50ml centrifuge tube (Corning). The 'washing' of the matrices was repeated two further times. The media from the culture well was also aspirated and passed through a 70-µm filter (BD Biosciences) into fresh 50 ml centrifuge tube (Corning). The wells were washed two further times with 1ml of 0.01 mM HEPES/HBSS and passed through a 70-µm filter (BD Biosciences). Cells were subsequently isolated by centrifugation (1600 rpm for 15 minutes). The pellet was re-suspended in 'Skin-T' media. Often, for each donor there were multiple centrifuge tubes. Therefore at this stage samples were pooled in a 14 ml centrifuge tube (Corning) and isolated by further centrifugation (1600 rpm for 10 minutes). The final cell pellet was re-suspended in 'Skin-T' media for subsequent flow cytometry analysis and/or functional studies. When cell counts were required leukocytes were counted by; (1) trypan blue stain (0.4 %) (Life Technologies) using a haemocytometer, or (2) CASY® Model TT cell counter and analyser (Roche).

7.4. Isolation of peripheral blood mononuclear cells by density centrifugation

Mononuclear cells were isolated from the peripheral blood of healthy donors that were sourced via the National Health Service Blood & Transplant (NHSBT) service as component donation cones (Customer number: P411). Donors had been consented for their blood to be used in biomedical research, in accordance with the principles of the Declaration of Helsinki.

To isolate peripheral blood mononuclear cells (PBMC), donor blood was drained from the component donation cone into a 50ml centrifuge tube (Corning). This was diluted with PBS, and split between additional centrifuge tubes, to a total volume of 240 ml. 30 ml aliquots of diluted blood suspension were carefully layered onto 15 ml of Ficoll-Paque PLUS (GE Healthcare Life Sciences) in 50ml centrifuge tubes (Corning). This was centrifuged at 2000 rpm for 25 minutes at 20 ^{o}C in a swinging-bucket rotor with the brake disengaged. The upper layer was then carefully aspirated to leave the peripheral blood mononuclear cell (PBMC) layer un-disturbed at the interphase. The mononuclear layer was carefully transferred to a new 50ml centrifuge tube (Corning). This was then diluted with 50 ml of PBS and centrifuged at 1600 rpm for 15 minutes at 20 ^{o}C. The supernatant was carefully removed completely, and the wash step repeated two further times. The cell pellet was then re-suspended in an appropriate volume of plain RPMI and counted using a CASY® Model TT cell counter and analyser (Roche).

Mononuclear cells were subsequently diluted at the required cell density in RPMI mixed at a 1:1 ratio with FCS containing 20 % DMSO. The cell suspension was then aliquoted into 2 ml cryovials (Corning), placed into a Mr Frosty freezing container (Thermo Scientific Nalgene) and cryopreserved at -80 ^{o}C.

When required, cryovials containing PBMCs were thawed at 37^{o}C. They were subsequently slowly diluted into 15 ml of RPMI pre-warmed to 37^{o}C. Cells were rested for 30 minutes at 37^{o}C and then centrifuged at 1600 rpm for 15 minutes at 20 ^{o}C. Cells were re-suspended in an appropriate volume of RPMI to ensure an approximate density of 10 x 10^{6}/ml, counted using a CASY® Model TT cell counter and analyser (Roche) and used as required.

7.5. Flow cytometry assays

Flow cytometry analysis of lymphocytes was performed using directly conjugated monoclonal antibodies (Tables 7.1 – 7.3). Flow cytometry staining was performed in a 96-well round bottom plate (Corning). For extracellular staining, cells were first stained with a fixable viability stain – either Aqua LIVE/DEAD cell stain (Life

Technologies) (1 in 1000 dilution for 10 minutes at room temperature), or Fixable Viability Dye eFluor® 780 (eBioscience) (1 in 500 dilution for 10 minutes at room temperature). After washing off the viability stain (addition of 150 µl flow cytometry buffer [PBS with 2 % foetal bovine serum and 2 mM EDTA] and centrifuged at 2000 rpm for 1 minute), cells underwent Fc receptor blockade with a specialised human IgG solution (Human TruStain FcX™; Biolegend) (1 in 20 dilution for 5 – 10 minutes at room temperature). After washing off the Fc-block, cells were incubated with directly conjugated monoclonal antibodies (30 minutes at room temperature) specific for cell surface proteins of interest or isotype-matched controls (Table 7.1 and 7.2). After washing off the conjugated monoclonal antibodies, cells were re-suspended in BD CellFIX (BD Biosciences) and refrigerated at 4 ^{o}C in the dark until analysis. Prior to analysis the fixative was washed off (centrifugation at 2000 rpm for 1 minute) and the cells re-suspended in flow cytometry buffer.

When required, intracellular cytokine staining was done following at least 20 minutes incubation in BD CellFIX (BD Biosciences) (50 µl) at 4 ^{o}C. Cells were washed with the addition of 200 µl flow cytometry buffer per well and centrifuged at 2000 rpm for 1 minute. Cells were then incubated at room temperature for 30 minutes in Permeabilization Wash Buffer (Biolegend). After washing off the permeabilization buffer, cells were incubated with directly conjugated monoclonal antibodies specific for cytokines of interest (Table 7.3) diluted in permeabilization buffer for 25 minutes at room temperature. The conjugated monoclonal antibodies were then washed off (150 µl flow cytometry buffer per well and centrifuged at 2000 rpm for 1 minute) and the cells re-suspended in flow cytometry buffer and refrigerated at 4 ^{o}C in the dark until analysis.

Experiments were acquired on Becton Dickinson FACSCanto II instruments (3-laser; up to 10 parameter data acquisition). Data were analysed using BD CellQuest (BD Biosciences) and Flowjo (Tree Star Inc.) software. Lymphocytes were gated based on their light scatter properties; dead cells were excluded using Aqua LIVE/DEAD stain (Life Technologies) or Fixable Viability Dye eFluor® 780 (eBioscience); and doublets were excluded on side scatter width/side scatter area plots.

7.6. Lymphocyte functional assays

For the functional analysis of skin-resident lymphocytes, cells isolated by 21-day explant culture with IL2 (100 IU/ml) and IL-15 (10 ng/ml) were used unless otherwise stated in the figure legend. Isolated lymphocytes were re-suspended in Skin-T media at a concentration of 1 x 10^6 cells/ml.

For PMA-ionomycin assays, lymphocytes were added in 200 µl of Skin-T media (1 x 10^6 cells/ml) to each well of a 96-well round bottom plate (Corning), with/without PMA (10ng/ml; Sigma) and ionomycin (1 µg/ml; Sigma). Cells were incubated at 37 ^oC. After one-hour brefeldin A (20 µg/ml; Sigma) was added. Cells were incubated for a total of 5 hours after which they were isolated for analysis of cytokine production.

For plate-bound anti-human CD3 assays, 96-well flat bottom plates (Corning) were pre-coated with anti-human CD3 antibody (5 µg/ml; clone OKT3; Biolegend) or isotype-matched control IgG2a antibody (5 µg/ml; clone MOPC-173; Biolegend) diluted in 50 µl of PBS and incubated overnight at 4 ^oC. Prior to activation, excess anti-human CD3 antibody was removed by gentle washing with 200 µl of PBS. Lymphocytes were added in 200 µl of Skin-T media (1 x 10^6 cells/ml) to each well of the activation plate and incubated at 37 ^oC. After one-hour brefeldin A (20 µg/ml; Sigma) was added. Cells were incubated for a total of 6 hours after which they were isolated for analysis of cytokine production.

For plate-bound NKG2D ligand assays, 96-well flat bottom plates (Corning) were pre-coated with recombinant human MICA (10 µg/ml; R&D), recombinant human ULBP2 (10 µg/ml; R&D) or recombinant human ULBP3 (10 µg/ml; R&D) diluted in 50 µl of PBS and incubated overnight at 4 ^oC. Human polyclonal IgG (10 µg/ml; Sigma) was used as a control. Where 'low-dose' anti-human CD3 antibody was used, anti-human CD3 antibody (50 ng/ml; clone OKT3; Biolegend) was diluted in 50 µl of PBS and incubated overnight at 4 ^oC. Prior to activation, excess plate-bound proteins were removed by gentle washing with 200 µl of PBS. Lymphocytes had been isolated from explant culture 24 hours prior to activation, and were rested in plain Skin-T media (without cytokine). Lymphocytes were then added in 200 µl

of Skin-T media (1 x 10^6 cells/ml) to each well of the activation plate and incubated at 37 ^oC, in the presence of brefeldin A (20 μg/ml; Sigma) and IL-2 (100 IU/ml; Novartis Pharmaceuticals UK Ltd). Lymphocytes were pret-treated with anti-NKG2D antibody (10 μg/ml; clone 1D11; Biolegend) or IgG1 isotype-matched control (10 μg/ml; clone MOPC-21; Biolegend) where indicated. PMA (10ng/ml; Sigma) and ionomycin (1 μg/ml; Sigma) were used as a positive control and added at the start of the activation assay. Cells were incubated for a total of 6 hours after which they were isolated for analysis of cytokine production.

Where CD107a, a cell surface marker of cytotoxic degranulation (Alter et al., 2004), was used as a functional read-out, phycoerythrin(PE)-conjugated anti-human CD107a antibody (1 in 40 dilution; BD Biosciences) was added at the start of the activation assay.

For cytokine activation assays, lymphocytes were added in 200 μl of Skin-T media (1 x 10^6 cells/ml) to each well of a 96-well round bottom plate (Corning) with/without IL-12 (100 ng/ml; PeproTech) and/or IL-18 (100 ng/ml; Medical and Biological Laboratories). Cells were incubated for a total of 24 hours, with brefeldin A (20 μg/ml; Sigma) added for the last 4 hours, after which they were isolated for analysis of cytokine production.

7.7. Inhibitor assays

For inhibitor assays, lymphocytes were pre-treated with the indicated inhibitor (pre-diluted to required concentration in Skin-T media), or a DMSO vehicle control, and incubated at 37 ^oC for 1 hour prior to activation. Inhibitors used were LY294002 (#9901; Cell Signaling Technology), Lck II inhibitor (#428206; Calbiochem) and Ciclosporin A (Sigma). Lymphocytes were then activated as outlined above for plate-bound NKG2D ligand assays.

7.8. Intracellular calcium mobilisation assays

For assays of intracellular calcium (Ca^{2+}) mobilization, cells were loaded for 45 min at 37 °C with the membrane-permeable fluorescent Ca^{2+} indicator dye Indo-1 AM (Life Technologies) at a concentration of 1 μM in RPMI medium with no FCS. Thereafter, cells were stained for surface markers (Vδ1 TCR, Vδ2 TCR, CD8α and NKp46; antibodies identified below) and kept on ice. Before stimulation, cell aliquots were allowed to equilibrate to 37 °C for 5 min and then were analyzed by flow cytometry. After acquisition of background intracellular Ca^{2+} concentrations for 30 seconds, cells were stimulated with biotinylated anti-CD3ε (10 μg/ml; UCHT1; BioLegend) and/or biotinylated anti-NKG2D (10 μg/ml; 1D11; BioLegend) and then were cross-linked by the addition of streptavidin (20 μg/ml; Life Technologies). Ionomycin was added (2 μg/ml; Sigma) as a positive control after 540 s. Samples were acquired on a LSRFortessa (BD) and were analysed using FlowJo software (Tree Star Inc.).

7.9. Cytotoxicity assays

For cytotoxicity assays, to increase overall cell number, skin-resident lymphocytes isolated from explant cultures were cultured for a further 14 days in RPMI 1640 with 10% heat-inactivated foetal bovine serum (Life Technologies), L-glutamine (292 μg/ml; Life Technologies), penicillin (100 units/ml; Life Technologies), streptomycin (100 μg/ml; Life Technologies), 2-mercaptoethanol (3.5 μl/L; Life Technologies)], IL2 (100 IU/ml) and IL-15 (10 ng/ml). This permitted fluorescence-activated cell sorting of the T cell subsets. This was done by negative sorting, with skin-derived γδ T cells isolated by excluding $TCR\alpha\beta^+$ cells (PE-conjugated anti-human TCRαβ antibody; Clone IP26; Biolegend), and αβ T cells isolated by excluding $TCR\gamma\delta^+$ cells (PE/Cy7-conjugated anti-human TCRγδ antibody; clone IMMU510; Beckman Coulter). Target cell lines used were sourced from Cancer Research UK Cell Services (Clare Hall, London). Target cells were seeded at 10 000 cells/well of 96-well flat bottom plate (Corning) 24 hours prior to killing assay. Sorted skin-derived lymphocytes (effectors) were added to target cells at increasing effector-to-target (E:T) ratios in 200 μl of Skin-T media with IL-2 (100 IU/ml).

Lymphocytes were pre-treated with anti-NKG2D antibody (10 μg/ml; clone 1D11; Biolegend) or IgG1 isotype-matched control (10 μg/ml; clone MOPC-21; Biolegend) where indicated. Staurosporine (50 μM; Sigma) was used as a positive control for target cell apoptosis. Cells were incubated at 37 ^{o}C for 24 hours, after which the supernatants from each well were collected and stored at -20^{o}C until further analysis. Target cell apoptosis was measured using an ELISA for the epithelial cell-specific caspase-cleaved cytokeratin 18 (M30 CytoDEATH^{TM} ELISA, Peviva).

7.10. Statistical analyses

Phenotypic characterization and mechanistic studies on cells derived from human tissues and blood were performed *in vitro*, using assays without blinding or randomization. Summary data is primarily based on the mean values for each group shown, with either standard deviation [SD] (where individual data points for each donor) or standard error of the mean [SEM] (where data points represent mean value of replicate values for each donor) demonstrated. Statistical differences between two independent groups were determined by Student's *t* test, which assumes a Gaussian distribution of the data, using Prism 6.0 software (GraphPad Software). All findings were considered significant at a P value threshold of < 0.05. Where results of statistical test are shown; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Table 7.1. Directly conjugated monoclonal antibodies used for cell surface flow cytometry studies

Antibody Specificity	Fluorochrome	Species	Isotype	Clone	Company
CCR4	PerCP/Cy5.5	Mouse	IgG2b, κ	TG6/CCR4	Biolegend
CCR7	PerCP/Cy5.5	Mouse	IgG2a, κ	G043H7	Biolegend
CD103	PE	Mouse	IgG1, κ	Ber-ACT8	Biolegend
CD107a	PE	Mouse	IgG1, κ	H4A3	BD Pharmingen
CD159a (NKG2A)	PE	Mouse	IgG2a, κ	131411	R & D
CD159c (NKG2C)	PE	Mouse	IgG1, κ	134591	R & D
CD27	APC	Mouse	IgG1, κ	M-T271	BD Pharmingen
CD279 (PD-1)	PerCP/Cy5.5	Mouse	IgG1, κ	EH12.2H7	Biolegend
CD28	PE	Mouse	IgG1, κ	CD28.2	Biolegend
CD3	Brilliant Violet 421	Mouse	IgG1, κ	UCHT1	Biolegend
CD3	Brilliant Violet 510	Mouse	IgG2a, κ	OKT3	Biolegend
CD3	PE/Cy7	Mouse	IgG2a	HIT3a	Biolegend
CD3	FITC	Mouse	IgG1, κ	UCHT1	Biolegend
CD314 (NKG2D)	APC	Mouse	IgG1, κ	1D11	eBioscience
CD335 (NKp46)	PerCP/Cy5.5	Mouse	IgG1, κ	9E2	Biolegend
CD336 (NKp44)	APC	Mouse	IgG1, κ	P44-8	Biolegend
CD337 (NKp30)	PE	Mouse	IgG1, κ	P30-15	Biolegend
CD4	Brilliant Violet 421	Mouse	IgG1, κ	RPA-T4	Biolegend
CD45	PE/Cy7	Mouse	IgG1, κ	HI30	Biolegend
CD45RA	PE	Mouse	IgG2b, κ	HI100	Biolegend
CD56	Pacific Blue	Mouse	IgG2a, κ	MEM-188	Biolegend
CD62L (L-selectin)	APC	Mouse	IgG1, κ	DREG-56	Biolegend
CD69	PerCP/Cy5.5	Mouse	IgG1, κ	FN50	Biolegend
CD8a	APC/Cy7	Mouse	IgG1, κ	HIT8a	Biolegend
CD8α	Brilliant Violet 510	Mouse	IgG1, κ	RPA-T8	Biolegend
CLA	eFluor-660	Rat	IgM	HECA-452	eBioscience
MICA	APC	Mouse	IgG2b, κ	159227	R & D
MICA/B	Alexa Fluor 647	Mouse	IgG2a, κ	6D4	Biolegend
MICB	APC	Mouse	IgG2b, κ	236511	R & D
TCR alpha/beta	PE	Mouse	IgG1, κ	IP26	Biolegend
TCR PAN gamma/delta	FITC	Mouse	IgG1, κ	IMMU510	Beckman Coulter
TCR PAN gamma/delta	PE/Cy7	Mouse	IgG1, κ	IMMU510	Beckman Coulter

TCR Vδ1	FITC	Mouse	IgG1	TS8.2	Thermo Scientific
TCR Vδ2	PerCP	Mouse	IgG1, κ	B6	Biolegend
TCR Vδ3	APC	Mouse	IgG1, κ	P11.5B	Beckman Coulter
ULBP1	PE	Mouse	IgG2a, κ	170818	R & D
ULBP2/5/6	PE	Mouse	IgG2a, κ	165903	R & D
ULBP3	PE	Mouse	IgG2a, κ	166510	R & D

Table 7.2. Directly conjugated monoclonal antibodies used as isotype-matched controls for cell surface flow cytometry studies

Antibody Specificity	Fluorochrome	Species	Isotype	Clone	Company
Isotype	Alexa Fluor 647	Mouse	IgG1, κ	MOPC-21	Biolegend
Isotype	APC	Mouse	IgG2b, κ	133303	R & D
Isotype	APC	Mouse	IgG1, κ	MOPC-21	Biolegend
Isotype	PE	Mouse	IgG2a, κ	20102	R & D
Isotype	PE	Mouse	IgG1, κ	MOPC-21	Biolegend
Isotype	PE	Mouse	IgG2b, κ	MG2b-57	Biolegend
Isotype	PerCP/Cy5.5	Mouse	IgG1, κ	MOPC-21	Biolegend

Table 7.3. Directly conjugated monoclonal antibodies used for intracellular cytokine/chemokine production studies

Antibody Specificity	Fluorochrome	Species	Isotype	Clone	Company
CCL4 (MIP-1β)	APC	Mouse	IgG2a, κ	FL34Z3L	eBioscience
GM-CSF	APC	Rat	IgG2a, κ	BVD2-21C11	Biolegend
IFNγ	Brilliant Violet 421	Mouse	IgG1, κ	4S.B3	Biolegend
IFNγ	PE	Mouse	IgG1, κ	B27	Biolegend
IL13	PE	Rat	IgG1, κ	JES10-5A2	Biolegend
IL13	PerCP/Cy5.5	Rat	IgG1, κ	JES10-5A2	Biolegend
IL17A	Alexa Fluor 647	Mouse	IgG1, κ	BL168	Biolegend
IL2	PE	Rat	IgG2a, κ	MQ1-17H12	Biolegend
IL22	PE	Mouse	IgG1, κ	BG/IL22	Biolegend
IL4	Alexa Fluor 647	Mouse	IgG1, κ	8D4-8	Biolegend
TNFα	PE	Mouse	IgG1, κ	MAb11	Biolegend
TNFα	PerCP/Cy5.5	Mouse	IgG1, κ	MAb11	Biolegend
TNFα	APC	Mouse	IgG1, κ	MAb11	Biolegend

Table 7.4. Unconjugated monoclonal antibodies and recombinant proteins used in functional studies

Protein	Species	Isotype	Clone	Company
Biotin anti-human CD3 antibody	Mouse	IgG1, κ	UCHT1	Biolegend
Biotin anti-human CD314 (NKG2D) antibody	Mouse	IgG1, κ	1D11	Biolegend
Anti-human CD3 antibody	Mouse	IgG2a, κ	OKT3	Biolegend
Anti-human CD314 (NKG2D) antibody	Mouse	IgG1, κ	1D11	Biolegend
Polyclonal IgG	Human	IgG	Polyclonal	Sigma
Purified mouse IgG1, κ	Mouse	IgG1, κ	MOPC-21	Biolegend
Purified mouse IgG1, κ	Mouse	IgG2a, κ	MOPC-173	Biolegend
Recombinant MICA-Fc chimera	Human	IgG1, κ		R & D
Recombinant ULBP2-Fc chimera	Human	IgG1, κ		R & D
Recombinant ULBP3-Fc chimera	Human	IgG1, κ		R & D

References

Abeler-Dorner, L., Swamy, M., Williams, G., Hayday, A.C., and Bas, A. (2012). Butyrophilins: an emerging family of immune regulators. Trends Immunol. *33*, 34–41.

Agea, E., Russano, A., Bistoni, O., Mannucci, R., Nicoletti, I., Corazzi, L., Postle, A.D., De Libero, G., Porcelli, S.A., and Spinozzi, F. (2005). Human CD1-restricted T cell recognition of lipids from pollens. J. Exp. Med. *202*, 295–308.

Ahmed, N.N., Grimes, H.L., Bellacosa, A., Chan, T.O., and Tsichlis, P.N. (1997). Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. Proc. Natl. Acad. Sci. U.S.A. *94*, 3627–3632.

Alejenef, A., Pachnio, A., Halawi, M., Christmas, S.E., Moss, P.A.H., and Khan, N. (2014). Cytomegalovirus drives Vδ2-neg γδ T cell inflation in many healthy virus carriers with increasing age. Clin. Exp. Immunol. *176*, 418–428.

Allan, R.S., Smith, C.M., Belz, G.T., van Lint, A.L., Wakim, L.M., Heath, W.R., and carbone, F.R. (2003). Epidermal viral immunity induced by $CD8\alpha^+$ dendritic cells but not by Langerhans cells. Science. *301*, 1925–1928.

Alter, G., Malenfant, J.M., and Altfeld, M. (2004). CD107a as a functional marker for the identification of natural killer cell activity. J. Immunol. Methods *294*, 15–22.

André, P., Castriconi, R., Espéli, M., Anfossi, N., Juarez, T., Hüe, S., Conway, H., Romagné, F., Dondero, A., Nanni, M., et al. (2004). Comparative analysis of human NK cell activation induced by NKG2D and natural cytotoxicity receptors. Eur. J. Immunol. *34*, 961–971.

Appleman, L.J., van Puijenbroek, A.A.F.L., Shu, K.M., Nadler, L.M., and Boussiotis, V.A. (2002). CD28 Costimulation Mediates Down-Regulation of p27kip1 and Cell Cycle Progression by Activation of the PI3K/PKB Signaling Pathway in Primary Human T Cells. J. Immunol. *168*, 2729–2736.

Aragane, Y., Riemann, H., Bhardwaj, R.S., Schwarz, A., Sawada, Y., Yamada, H., Luger, T.A., Kubin, M., Trinchieri, G., and Schwarz, T. (1994). IL-12 is expressed and released by human keratinocytes and epidermoid carcinoma cell lines. J. Immunol. *153*, 5366–5372.

Ariotti, S., Hogenbirk, M.A., Dijkgraaf, F.E., Visser, L.L., Hoekstra, M.E., Song, J.-Y., Jacobs, H., Haanen, J.B., and Schumacher, T.N. (2014). Skin-resident memory $CD8^+$ T cells trigger a state of tissue-wide pathogen alert. Science *346*, 101–105.

Artis, D., and Sonnenberg, G.F. (2015). Innate lymphoid cells in the initiation,regulation and resolution of inflammation. Nat. Med. *21*, 698–708.

Asarnow, D.M., Kuziel, W.A., Bonyhad, M., Tigelaar, R.E., Tucker, P.W., and Allison, J.P. (1988). Limited diversity of γδ antigen receptor genes of $Thy\text{-}1^+$ dendritic epidermal cells. Cell *55*, 837–847.

Autran, B., Triebel, F., Katlama, C., Rozenbaum, W., Hercend, T., and Debre, P. (1989). T cell receptor gamma/delta+ lymphocyte subsets during HIV infection. Clin. Exp. Immunol. *75*, 206–210.

Azuma, M., Phillips, J.H., and Lanier, L.L. (1993). $CD28^-$ T lymphocytes. Antigenic and functional properties. J. Immunol. *150*, 1147–1159.

Bai, L., Picard, D., Anderson, B., Chaudhary, V., Luoma, A., Jabri, B., Adams, E.J., Savage, P.B., and Bendelac, A. (2012). The majority of CD1d-sulfatide-specific T cells in human blood use a semiinvariant Vδ1 TCR. Eur. J. Immunol. *42*, 2505–2510.

Balbi, B., Valle, M.T., Oddera, S., Giunti, D., Manca, F., Rossi, G.A., and Allegra, L. (1993). T-lymphocytes with $\gamma\delta^+$ $V\delta2^+$ antigen receptors are present in increased proportions in a fraction of patients with tuberculosis or with sarcoidois. Am. Rev. Respir. Dis. *148*, 1685–1690.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245–252.

Barbee, S.D., Woodward, M.J., Turchinovich, G., Mention, J.-J., Lewis, J.M., Boyden, L.M., Lifton, R.P., Tigelaar, R., and Hayday, A.C. (2011). Skint-1 is a highly specific, unique selecting component for epidermal T cells. Proc. Natl. Acad. Sci. U.S.A. *108*, 3330–3335.

Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G.J., and Ahmed, R. (2005). Restoring function in exhausted CD8 T cells during chronic viral infection. Nature *439*, 682–687.

Barcy, S., De Rosa, S.C., Vieira, J., Diem, K., Ikoma, M., Casper, C., and Corey, L. (2008). $\gamma\delta^+$ T Cells Involvement in Viral Immune Control of Chronic Human Herpesvirus 8 Infection. J. Immunol. *180*, 3417–3425.

Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J.H., Lanier, L.L., and Spies, T. (1999). Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science *285*, 727–729.

Bedoui, S., Whitney, P.G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R.S., Wojtasiak, M., Shortman, K., Carbone, F.R., et al. (2009). Cross-presentation of viral and self antigens by skin-derived $CD103^+$ dendritic cells. Nat. Immunol. *10*, 488–495.

Bendelac, A. (1995). Positive selection of mouse $NK1^+$ T cells by CD1-expressing cortical thymocytes. J. Exp. Med. *182*, 2091–2096.

Bennett, N.J., Ashiru, O., Morgan, F.J.E., Pang, Y., Okecha, G., Eagle, R.A., Trowsdale, J., Sissons, J.G.P., and Wills, M.R. (2010). Intracellular Sequestration of the NKG2D Ligand ULBP3 by Human Cytomegalovirus. J. Immunol. *185*, 1093–1102.

Berg, E.L., Yoshino, T., Rott, L.S., Robinson, M.K., Warnock, R.A., Kishimoto, T.K., Picker, L.J., and Butcher, E.C. (1991). The cutaneous lymphocyte antigen is a

skin lymphocyte homing receptor for the vascular lectin endothelial cell-leukocyte adhesion molecule 1. J. Exp. Med. *174*, 1461–1466.

Berg, R.E., Cordes, C.J., and Forman, J. (2002). Contribution of $CD8^+$ T cells to innate immunity: IFN-gamma secretion induced by IL-12 and IL-18. Eur. J. Immunol. *32*, 2807–2816.

Berman, B., Chen, V.L., France, D.S., Dotz, W.I., and Petroni, G. (1983). Anatomical mapping of epidermal Langerhans cell densities in adults. Br. J. Dermatol. *109*, 553–558.

Bhushan, M., Bleiker, T.O., Ballsdon, A.E., Allen, M.H., Sopwith, M., Robinson, M.K., Clarke, C., Weller, R.P.J.B., Graham-Brown, R.A.C., Keefe, M., et al. (2002). Anti-E-selectin is ineffective in the treatment of psoriasis: a randomized trial. Br. J. Dermatol. *146*, 824–831.

Billadeau, D.D., Upshaw, J.L., Schoon, R.A., Dick, C.J., and Leibson, P.J. (2003). NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. Nat. Immunol. *4*, 557–564.

Boismenu, R., and Havran, W.L. (1994). Modulation of epithelial cell growth by intraepithelial gamma delta T cells. Science *266*, 1253–1255.

Boismenu, R., Feng, L., Xia, Y.Y., Chang, J.C., and Havran, W.L. (1996a). Chemokine expression by intraepithelial gamma delta T cells. Implications for the recruitment of inflammatory cells to damaged epithelia. J. Immunol. *157*, 985–992.

Boismenu, R., Hobbs, M.V., Boullier, S., and Havran, W.L. (1996b). Molecular and cellular biology of dendritic epidermal T cells. Semin. Immunol. *8*, 323–331.

Borchers, M.T., Harris, N.L., Wesselkamper, S.C., Vitucci, M., and Cosman, D. (2006). NKG2D ligands are expressed on stressed human airway epithelial cells. Am. J. Physiol. Cell. Mol. Physiol. *291*, L222–L231.

Bos, J.D., and Kapsenberg, M.L. (1986). The skin immune system: Its cellular constituents and their interactions. Immunol. Today *7*, 235–240.

Bos, J.D., Teunissen, M.B., Cairo, I., Krieg, S.R., Kapsenberg, M.L., Das, P.K., and Borst, J. (1990). T-cell receptor gamma delta bearing cells in normal human skin. J. Invest. Dermatol. *94*, 37–42.

Bos, J.D., Zonneveld, I., Das, P.K., Krieg, S.R., van der Loos, C.M., and Kapsenberg, M.L. (1987). The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. J. invest. Dermatol. *88*, 569–573.

Boyden, L.M., Lewis, J.M., Barbee, S.D., Bas, A., Girardi, M., Hayday, A.C., Tigelaar, R.E., and Lifton, R.P. (2008). Skint1, the prototype of a newly identified immunoglobulin superfamily gene cluster, positively selects epidermal γδ T cells. Nat. Genet. *40*, 656–662.

Boyman, O., Heft, H.P., Conrad, C., Nickoloff, B.J., Suter, M., and Nestle, F.O.

(2004). Spontaneous Development of Psoriasis in a New Animal Model Shows an Essential Role for Resident T Cells and Tumor Necrosis Factor. J. Exp. Med. *199*, 731–736.

Brewig, N., Kissenpfennig, A., Malissen, B., Veit, A., Bickert, T., Fleischer, B., Mostbock, S., and Ritter, U. (2009). Priming of $CD8^+$ and $CD4^+$ T Cells in Experimental Leishmaniasis Is Initiated by Different Dendritic Cell Subtypes. J. Immunol. *182*, 774–783.

Bryceson, Y.T., March, M.E., Ljunggren, H.-G., and Long, E.O. (2006). Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. Blood *107*, 159–166.

Bucy, R.P., Chen, C.L., and Cooper, M.D. (1989). Tissue localization and CD8 accessory molecule expression of T gamma delta cells in humans. J. Immunol. *142*, 3045–3049.

Bursch, L.S., Wang, L., Igyarto, B., Kissenpfennig, A., Malissen, B., Kaplan, D.H., and Hogquist, K.A. (2007). Identification of a novel population of $Langerin^+$ dendritic cells. J. Exp. Med. *204*, 3147–3156.

Cai, Y., Shen, X., Ding, C., Qi, C., Li, K., Li, X., Jala, V.R., Zhang, H.-G., Wang, T., Zheng, J., et al. (2011). Pivotal Role of Dermal IL-17-Producing γδ T Cells in Skin Inflammation. Immunity *35*, 596–610.

Caligiuri, M.A. (2008). Human natural killer cells. Blood *112*, 461–9.

Campbell, D.J., and Butcher, E.C. (2002). Rapid acquisition of tissue-specific homing phenotypes by $CD4^+$ T cells activated in cutaneous or mucosal lymphoid tissues. J. Exp. Med. *195*, 135–141.

Campbell, J.J., Haraldsen, G., Pan, J., Rottman, J., Qin, S., Ponath, P., Andrew, D.P., Warnke, R., Ruffing, N., and Kassam, N. (1999). The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. Nature *400*, 776–780.

Carding, S.R., and Egan, P.J. (2002). γδ T cells: Functional plasticity and heterogeneity. Nat. Rev. Immunol. *2*, 336–345.

Carmi, Y., Rinott, G., Dotan, S., Elkabets, M., Rider, P., Voronov, E., and Apte, R.N. (2011). Microenvironment-Derived IL-1 and IL-17 Interact in the Control of Lung Metastasis. J. Immunol. *186*, 3462–3471.

Carter, L.L., and Murphy, K.M. (1999). Lineage-specific requirement for signal transducer and activator of transcription (Stat)4 in interferon gamma production from $CD4^{(+)}$ versus $CD8^{(+)}$ T cells. J. Exp. Med. *189*, 1355–1360.

Chan, A., Hong, D.L., Atzberger, A., Kollnberger, S., Filer, A.D., Buckley, C.D., McMichael, A., Enver, T., and Bowness, P. (2007). CD56bright Human NK Cells Differentiate into CD56dim Cells: Role of Contact with Peripheral Fibroblasts. J. Immunol. *179*, 89–94.

Chodaczek, G., Papanna, V., Zal, M.A., and Zal, T. (2012). Body-barrier surveillance by epidermal γδ TCRs. Nat. Immunol. *13*, 272–282.

Chowers, Y., Holtmeier, W., Harwood, J., Morzycka-Wroblewska, E., and Kagnoff, M.F. (1994). The V delta 1 T cell receptor repertoire in human small intestine and colon. J. Exp. Med. *180*, 183–190.

Clark, R.A., Watanabe, R., Teague, J.E., Schlapbach, C., Tawa, M.C., Adams, N., Dorosario, A.A., Chaney, K.S., Cutler, C.S., LeBoeuf, N.R., et al. (2012). Skin Effector Memory T Cells Do Not Recirculate and Provide Immune Protection in Alemtuzumab-Treated CTCL Patients. Sci. trans. Med. *4*, 117ra7–117ra7.

Clark, R.A. (2015). Resident memory T cells in human health and disease. Sci. trans. Med. *7*, 269rv1.

Clark, R.A., and Kupper, T.S. (2007). IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin. Blood *109*, 194–202.

Clark, R.A., Chong, B.F., Mirchandani, N., Yamanaka, K.-I., Murphy, G.F., Dowgiert, R.K., and Kupper, T.S. (2006a). A Novel Method for the Isolation of Skin Resident T Cells from Normal and Diseased Human Skin. J. Invest. Dermatol. *126*, 1059–1070.

Clark, R.A., Chong, B., Mirchandani, N., Brinster, N.K., Yamanaka, K.-I., Dowgiert, R.K., and Kupper, T.S. (2006b). The vast majority of CLA^+ T cells are resident in normal skin. J. Immunol. *176*, 4431–4439.

Cooper, M.A., Fehniger, T.A., Turner, S.C., Chen, K.S., Ghaheri, B.A., Ghayur, T., Carson, W.E., and Caligiuri, M.A. (2001). Human natural killer cells: a unique innate immunoregulatory role for the $CD56^{(bright)}$ subset. Blood *97*, 3146–3151.

Cordova, A., Toia, F., La Mendola, C., Orlando, V., Meraviglia, S., Rinaldi, G., Todaro, M., Cicero, G., Zichichi, L., Donni, P.L., et al. (2012). Characterization of Human γδ T Lymphocytes Infiltrating Primary Malignant Melanomas. PLoS ONE *7*, e49878.

Correia, D.V., Fogli, M., Hudspeth, K., da Silva, M.G., Mavilio, D., and Silva-Santos, B. (2011). Differentiation of human peripheral blood $V\delta1^+$ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells. Blood *118*, 992–1001.

Cosman, D., Müllberg, J., Sutherland, C.L., Chin, W., Armitage, R., Fanslow, W., Kubin, M., and Chalupny, N.J. (2001). ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity *14*, 123–133.

Couzi, L., Levaillant, Y., Jamai, A., Pitard, V., Lassalle, R., Martin, K., Garrigue, I., Hawchar, O., Siberchicot, F., Moore, N., et al. (2010). Cytomegalovirus-Induced γδ T Cells Associate with Reduced Cancer Risk after Kidney Transplantation. J. Am. Soc. Nephrol. *21*, 181–188.

Craiglow, B.G., and King, B.A. (2014). Killing Two Birds with One Stone: Oral Tofacitinib Reverses Alopecia Universalis in a Patient with Plaque Psoriasis. J. Invest. Dermaol. *134*, 2988–2990.

Cumberbatch, M., Dearman, R.J., and Kimber, I. (1997). Langerhans cells require signals from both tumour necrosis factor-alpha and interleukin-1 beta for migration. Immunology *92*, 388–395.

Cumberbatch, M., Dearman, R.J., Antonopoulos, C., Groves, R.W., and Kimber, I. (2001). Interleukin (IL)-18 induces Langerhans cell migration by a tumour necrosis factor-alpha- and IL-1beta-dependent mechanism. Immunology *102*, 323–330.

De Creus, A., Van Beneden, K., Stevenaert, F., Debacker, V., Plum, J., and Leclercq, G. (2002). Developmental and Functional Defects of Thymic and Epidermal Vγ3 Cells in IL-15-Deficient and IFN Regulatory Factor-1-Deficient Mice. J. Immunol. *168*, 6486–6493.

De Paoli, P., Gennari, D., Martelli, P., Cavarzerani, V., Comoretto, R., and Santini, G. (1990). Gamma delta T cell receptor-bearing lymphocytes during Epstein-Barr virus infection. J. Infect. Dis. *161*, 1013–1016.

De Rosa, S.C., Andrus, J.P., Perfetto, S.P., Mantovani, J.J., Herzenberg, L.A., Herzenberg, L.A., and Roederer, M. (2004). Ontogeny of γδ T Cells in Humans. The Journal of Immunology *172*, 1637–1645.

Deniger, D.C., Maiti, S.N., Mi, T., Switzer, K.C., Ramachandran, V., Hurton, L.V., Ang, S., Olivares, S., Rabinovich, B.A., Huls, M.H., et al. (2014). Activating and Propagating Polyclonal Gamma Delta T Cells with Broad Specificity for Malignancies. Clin. Can. Res. *20*, 5708–5719.

Deusch, K., Lüling, F., Reich, K., Classen, M., Wagner, H., and Pfeffer, K. (1991). A major fraction of human intraepithelial lymphocytes simultaneously expresses the gamma/delta T cell receptor, the CD8 accessory molecule and preferentially uses the V delta 1 gene segment. Eur. J. Immunol. *21*, 1053–1059.

Di Meglio, P., Perera, G.K., and Nestle, F.O. (2011). The Multitasking Organ: Recent Insights into Skin Immune Function. Immunity *35*, 857–869.

Diefenbach, A., Tomasello, E., Lucas, M., Jamieson, A.M., Hsia, J.K., Vivier, E., and Raulet, D.H. (2002). Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. Nat. Immunol. *3*, 1142–1149.

Dieli, F., Poccia, F., Lipp, M., Sireci, G., Caccamo, N., Di Sano, C., and Salerno, A. (2003). Differentiation of Effector/Memory Vδ2 T Cells and Migratory Routes in Lymph Nodes or Inflammatory Sites. J. Exp. med. *198*, 391–397.

Dimova, T., Brouwer, M., Gosselin, F., Tassignon, J., Leo, O., Donner, C., Marchant, A., and Vermijlen, D. (2015). Effector Vγ9Vδ2 T cells dominate the human fetal γδ T-cell repertoire. Proc. Natl. Acad. Sci. U.S.A. *112*, E556–E565.

Draghi, M., Pashine, A., Sanjanwala, B., Gendzekhadze, K., Cantoni, C., Cosman, D., Moretta, A., Valiante, N.M., and Parham, P. (2007). NKp46 and NKG2D

recognition of infected dendritic cells is necessary for NK cell activation in the human response to influenza infection. J. Immunol. *178*, 2688–2698.

Dunn, C., Chalupny, N.J., Sutherland, C.L., Dosch, S., Sivakumar, P.V., Johnson, D.C., and Cosman, D. (2003). Human Cytomegalovirus Glycoprotein UL16 Causes Intracellular Sequestration of NKG2D Ligands, Protecting Against Natural Killer Cell Cytotoxicity. J. Exp. Med. *197*, 1427–1439.

Eagle, R.A., and Trowsdale, J. (2007). Promiscuity and the single receptor: NKG2D. Nat. rev. Immunol. *7*, 737–744.

Eberl, M., Hintz, M., Reichenberg, A., Kollas, A.-K., Wiesner, J., and Jomaa, H. (2003). Microbial isoprenoid biosynthesis and human γδ T cell activation. FEBS Lett. *544*, 4–10.

Eberl, M., Jomaa, H., and Hayday, A.C. (2004). Integrated immune responses to infection - cross-talk between human gammadelta T cells and dendritic cells. Immunology *112*, 364–368.

Ebert, L.M., Meuter, S., and Moser, B. (2006). Homing and function of human skin gammadelta T cells and NK cells: relevance for tumor surveillance. J. Immunol. *176*, 4331–4336.

Edelbaum, D., Mohamadzadeh, M., Bergstresser, P.R., Sugamura, K., and Takashima, A. (1995). Interleukin (IL)-15 Promotes the Growth of Murine Epidermal gammadelta T Cells by a Mechanism Involving the beta- and gammac-Chains of the IL-2 Receptor. J. Invest. Dermatol. *105*, 837–843.

Elbe, A., Foster, C.A., and Stingl, G. (1996). T-cell receptor alpha beta and gamma delta T cells in rat and human skin--are they equivalent? Sem. Immunol. *8*, 341–349.

Fehniger, T.A., Shah, M.H., Turner, M.J., VanDeusen, J.B., Whitman, S.P., Cooper, M.A., Suzuki, K., Wechser, M., Goodsaid, F., and Caligiuri, M.A. (1999). Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. J. Immunol. *162*, 4511–4520.

Fisher, J.P.H., Yan, M., Heuijerjans, J., Carter, L., Abolhassani, A., Frosch, J., Wallace, R., Flutter, B., Capsomidis, A., Hubank, M., et al. (2014). Neuroblastoma Killing Properties of Vδ2 and Vδ2-Negative γδ T Cells Following Expansion by Artificial Antigen-Presenting Cells. Clin. Can. Res. *20*, 5720–5732.

Flacher, V., Bouschbacher, M., Verronese, E., Massacrier, C., Sisirak, V., Berthier-Vergnes, O., de Saint-Vis, B., Caux, C., Dezutter-Dambuyant, C., Lebecque, S., et al. (2006). Human Langerhans Cells Express a Specific TLR Profile and Differentially Respond to Viruses and Gram-Positive Bacteria. J. Immunol. *177*, 7959–7967.

Foster, C.A., Yokozeki, H., Rappersberger, K., Koning, F., Volc-Platzer, B., Rieger, A., Coligan, J.E., Wolff, K., and Stingl, G. (1990). Human epidermal T cells predominantly belong to the lineage expressing alpha/beta T cell receptor. J. Exp. Med. *171*, 997–1013.

Freed-Pastor, W.A., Mizuno, H., Zhao, X., Langerød, A., Moon, S.-H., Rodriguez-Barrueco, R., Barsotti, A., Chicas, A., Li, W., Polotskaia, A., et al. (2012). Mutant p53 Disrupts Mammary Tissue Architecture via the Mevalonate Pathway. Cell *148*, 244–258.

Fuhlbrigge, R.C., Kieffer, J.D., Armerding, D., and Kupper, T.S. (1997). Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells. Nature *389*, 978–981.

Fujishima, N., Hirokawa, M., Fujishima, M., Yamashita, J., Saitoh, H., Ichikawa, Y., Horiuchi, T., Kawabata, Y., and Sawada, K.I. (2007). Skewed T cell receptor repertoire of $V\delta1^+$ γδ T lymphocytes after human allogeneic haematopoietic stem cell transplantation and the potential role for Epstein-Barr virus-infected B cells in clonal restriction. Clin. Exp. Immunol. *149*, 70–79.

Fujita, H. (2013). The role of IL-22 and Th22 cells in human skin diseases. J. Dermatol. Sci. *72*, 3–8.

Gaide, O., Emerson, R.O., Jiang, X., Gulati, N., Nizza, S., Desmarais, C., Robins, H., Krueger, J.G., Clark, R.A., and Kupper, T.S. (2015). Common clonal origin of central and resident memory T cells following skin immunization. Nat. Med. *21*, 647–653.

Galkowska, H., Wojewodzka, U., and Olszewski, W.L. (1995). Cytokines and adherence molecules involved in spontaneous dendritic cell-lymphocyte clustering in skin afferent lymph. Scand. J. Immunol. *42*, 324–330.

G.A.O.P.C.T.W.T.C.C.C. 2 (2010). A genome-wide association study identifies new psoriasis susceptibility loci and an interaction between. Nat. Gen. *42*, 985–990.

Gasser, S., Orsulic, S., Brown, E.J., and Raulet, D.H. (2005). The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature *436*, 1186–1190.

Gebhardt, T., Whitney, P.G., Zaid, A., Mackay, L.K., Brooks, A.G., Heath, W.R., Carbone, F.R., and Mueller, S.N. (2012). Different patterns of peripheral migration by memory CD4. Nature *477*, 216–219.

Gentles, A.J., Newman, A.M., Liu, C.L., Bratman, S.V., Feng, W., Kim, D., Nair, V.S., Xu, Y., Khuong, A., Hoang, C.D., et al. (2015). The prognostic landscape of genes and infiltrating immune cells across human cancers. Nat. Med. 1–12.

Giacomelli, R., Matucci-Cerinic, M., Cipriani, P., Ghersetich, I., Lattanzio, R., Pavan, A., Pignone, A., Cagnoni, M.L., and Lotti, T. (1998). Circulating Vδ1+ T cells are activated and accumulate in the skin of systemic sclerosis patients. Arthritis Rheum. *41*, 327–334.

Gilfillan, S., Ho, E.L., Cella, M., Yokoyama, W.M., and Colonna, M. (2002). NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. Nat. Immunol. *3*, 1150–1155.

Ginhoux, F., Collin, M.P., Bogunovic, M., Abel, M., Leboeuf, M., Helft, J.,

Ochando, J., Kissenpfennig, A., Malissen, B., Grisotto, M., et al. (2007). Blood-derived dermal langerin^{+} dendritic cells survey the skin in the steady state. J. Exp. Med. *204*, 3133–3146.

Girardi, M., Glusac, E., Filler, R.B., Roberts, S.J., Propperova, I., Lewis, J., Tigelaar, R.E., and Hayday, A.C. (2003). The Distinct Contributions of Murine T Cell Receptor (TCR)$\gamma\delta^{+}$ and TCR$\alpha\beta^{+}$ T Cells to Different Stages of Chemically Induced Skin Cancer. J. Exp. Med. *198*, 747–755.

Girardi, M., Lewis, J.M., Filler, R.B., Hayday, A.C., and Tigelaar, R.E. (2006). Environmentally Responsive and Reversible Regulation of Epidermal Barrier Function by γδ T Cells. J. Invest. Dermatol. *126*, 808–814.

Girardi, M., Lewis, J., Glusac, E., Filler, R.B., Geng, L., Hayday, A.C., and Tigelaar, R.E. (2002). Resident skin-specific gammadelta T cells provide local, nonredundant regulation of cutaneous inflammation. J. Exp. Med. *195*, 855–867.

Girardi, M., Oppenheim, D.E., Steele, C.R., Lewis, J.M., Glusac, E., Filler, R.B., Hobby, P., Sutton, B., Tigelaar, R.E., and Hayday, A.C. (2001). Regulation of cutaneous malignancy by γδ T cells. Science *294*, 605–609.

Girolomoni, G., Caux, C., Lebecque, S., Dezutter-Dambuyant, C., and Ricciardi-Castagnoli, P. (2002). Langerhans cells: still a fundamental paradigm for studying the immunobiology of dendritic cells. Trends Immunol. *23*, 6–8.

Glatzel, A., Entschladen, F., Zollner, T.M., Kraiczy, P., Brade, V., Kaufmann, R., Janssen, O., Lengl-Janssen, B., Wesch, D., and Kabelitz, D. (2002). The responsiveness of human V delta 1 gamma delta T cells to Borrelia burgdorferi is largely restricted to synovial-fluid cells from patients with Lyme arthritis. J. Infect. Dis. *186*, 1043–1046.

Gober, H.J., Kistowska, M., Angman, L., Jeno, P., Mori, L., and De Libero, G. (2003). Human T Cell Receptor γδ Cells Recognize Endogenous Mevalonate Metabolites in Tumor Cells. J. Exp. Med. *197*, 163–168.

Gober, M.D., Fishelevich, R., Zhao, Y., Unutmaz, D., and Gaspari, A.A. (2007). Human Natural Killer T Cells Infiltrate into the Skin at Elicitation Sites of Allergic Contact Dermatitis. J. Invest. Dermatol. *128*, 1460–1469.

Gottlieb, S.L., Gilleaudeau, P., Johnson, R., Estes, L., Woodworth, T.G., Gottlieb, A.B., and Krueger, J.G. (1995). Response of psoriasis to a lymphocyte-selective toxin (DAB389IL-2) suggests a primary immune, but not keratinocyte, pathogenic basis. Nat. Med. *1*, 442–447.

Gracie, J.A., Robertson, S.E., and McInnes, I.B. (2003). Interleukin-18. J. Leuk. Biol. *73*, 213–224.

Groh, V., Bahram, S., Bauer, S., Herman, A., Beauchamp, M., and Spies, T. (1996). Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc. Natl. Acad. Sci. U.S.A. *93*, 12445–12450.

Groh, V., Porcelli, S., Fabbi, M., Lanier, L.L., Picker, L.J., Anderson, T., Warnke,

R.A., Bhan, A.K., Strominger, J.L., and Brenner, M.B. (1989). Human lymphocytes bearing T cell receptor gamma/delta are phenotypically diverse and evenly distributed throughout the lymphoid system. J. Exp. Med. *169*, 1277–1294.

Groh, V., Rhinehart, R., Secrist, H., Bauer, S., Grabstein, K.H., and Spies, T. (1999). Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. Proc. Natl. Acad. Sci. U.S.A. *96*, 6879–6884.

Groh, V., Steinle, A., Bauer, S., and Spies, T. (1998). Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. Science *279*, 1737–1740.

Groh, V., Rhinehart, R., Randolph-Habecker, J., Topp, M.S., Riddell, S.R., and Spies, T. (2001). Costimulation of CD8αβ T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nat. Immunol. *2*, 255–260.

Gudjonsson, J.E., Johnston, A., Dyson, M., Valdimarsson, H., and Elder, J.T. (2007). Mouse Models of Psoriasis. J. Invest. Dermatol. *127*, 1292–1308.

Halary, F. (2005). Shared reactivity of $V\delta2^{-}$ γδ T cells against cytomegalovirus infected cells and tumor intestinal epithelial cells. J. Exp. Med. *201*, 1567–1578.

Haniffa, M., Gunawan, M., and Jardine, L. (2015). Human skin dendritic cells in health and disease. J. Dermatol. Sci. *77*, 85–92.

Haniffa, M., Shin, A., Bigley, V., McGovern, N., Teo, P., See, P., Wasan, P.S., Wang, X.-N., Malinarich, F., Malleret, B., et al. (2012). Human Tissues Contain CD141. Immunity *37*, 60–73.

Havran, W.L., and Allison, J.P. (1988). Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. Nature *335*, 443–445.

Hayday, A., Theodoridis, E., Ramsburg, E., and Shires, J. (2001). Intraepithelial lymphocytes: exploring the Third Way in immunology. Nat. Immunol. *2*, 997–1003.

Hayday, A., and Vantourout, P. (2013). A Long-Playing CD about the γδ TCR Repertoire. Immunity *39*, 994–996.

Hayday, A.C. (2009). Gammadelta T cells and the lymphoid stress-surveillance response. Immunity *31*, 184–196.

Heilig, J.S., and Tonegawa, S. (1986). Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. Nature *322*, 836–840.

Heinemann, A., Zhao, F., Pechlivanis, S., Eberle, J., Steinle, A., Diederichs, S., Schadendorf, D., and Paschen, A. (2012). Tumor Suppressive MicroRNAs miR-34a/c Control Cancer Cell Expression of ULBP2, a Stress-Induced Ligand of the Natural Killer Cell Receptor NKG2D. Cancer Res. *72*, 460–471.

Henri, S., Poulin, L.F., Tamoutounour, S., Ardouin, L., Guilliams, M., de Bovis, B., Devilard, E., Viret, C., Azukizawa, H., Kissenpfennig, A., et al. (2010). $CD207^{+}$ $CD103^{+}$ dermal dendritic cells cross-present keratinocyte-derived antigens

irrespective of the presence of Langerhans cells. J. Exp. Med. *207*, 189–206.

Hijnen, D., Knol, E.F., Gent, Y.Y., Giovannone, B., Beijn, S.J.P., Kupper, T.S., Bruijnzeel-Koomen, C.A.F.M., and Clark, R.A. (2012). T Cells in the Lesional Skin of Atopic Dermatitis and Psoriasis Patients Are an ImportantSource of IFN. J. Invest. Dermatol. *133*, 973–979.

Hogewoning, A.A., Goettsch, W., van Loveren, H., de Fijter, J.W., Vermeer, B.J., and Bouwes Bavinck, J.N. (2001). Skin infections in renal transplant recipients. Clin. Transplant *15*, 32–38.

Holtmeier, W., Chowers, Y., Lurneng, A., Morzycka-Wroblewska, E., and Kagnoff, M.F. (1995). The delta T cell receptor repertoire in human colon and peripheral blood is oligoclonal irrespective of V region usage. J. Clin. Invest. *96*, 1108–1117.

Holtmeier, W., Pfänder, M., Hennemann, A., Zollner, T.M., Kaufmann, R., and Caspary, W.F. (2001). The TCR-delta repertoire in normal human skin is restricted and distinct from the TCR-delta repertoire in the peripheral blood. J. Invest. Dermatol. *116*, 275–280.

Honjo, M., Elbe, A., Steiner, G., Assmann, I., Wolff, K., and Stingl, G. (1990). Thymus-Independent Generation of Thy-1+ Epidermal Cells from a Pool of Thy-1$^-$ Bone Marrow Precursors. J. Invest. Dermatol. *95*, 562–567.

Hüe, S., Mention, J.-J., Monteiro, R.C., Zhang, S., Cellier, C., Schmitz, J., Verkarre, V., Fodil, N., Bahram, S., Cerf-Bensussan, N., et al. (2004). A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity *21*, 367–377.

Hviid, L., Akanmori, B.D., Loizon, S., Kurtzhals, J.A., Ricke, C.H., Lim, A., Koram, K.A., Nkrumah, F.K., Mercereau-Puijalon, O., and Behr, C. (2000). High frequency of circulating gamma delta T cells with dominance of the V(delta)1 subset in a healthy population. Int. Immunol. *12*, 797–805.

Ibusuki, A., Kawai, K., Yoshida, S., Uchida, Y., Nitahara-Takeuchi, A., Kuroki, K., Kajikawa, M., Ose, T., Maenaka, K., Kasahara, M., et al. (2013). NKG2D Triggers Cytotoxicity in Murine Epidermal γδ T Cells via PI3K-Dependent, Syk/ZAP70-Independent Singaling Pathway. J. Invest. Dermatol. *134*, 396–404.

Igyártó, B.Z., Haley, K., Ortner, D., Bobr, A., Gerami-Nejad, M., Edelson, B.T., Zurawski, S.M., Malissen, B., Zurawski, G., Berman, J., et al. (2011). Skin-Resident Murine Dendritic Cell Subsets Promote Distinct and Opposing Antigen-Specific T Helper Cell Responses. Immunity *35*, 260–272.

Ito, T., Ito, N., Saatoff, M., Hashizume, H., Fukamizu, H., Nickoloff, B.J., Takigawa, M., and Paus, R. (2007). Maintenance of Hair Follicle Immune Privilege Is Linked to Prevention of NK Cell Attack. J. Invest. Dermatol. *128*, 1196–1206.

Itohara, S., Farr, A.G., Lafaille, J.J., Bonneville, M., Takagaki, Y., Haas, W., and Tonegawa, S. (1990). Homing of a gamma delta thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. Nature *343*, 754–757.

Iwasaki, A., and Medzhitov, R. (2015). Control of adaptive immunity by the innate immune system. Nat. Immunol. *16*, 343–353.

Jabbari, A., Dai, Z., Xing, L., Cerise, J.E., Ramot, Y., Berkun, Y., Sanchez, G.A.M., Goldbach-Mansky, R., Christiano, A.M., Clynes, R., et al. (2015). Reversal of alopecia areata following treatment with the JAK1/2 inhibitor Baricitinib. EBioMedicine. *2*, 351–355.

Jakob, T., and Udey, M.C. (1998). Regulation of E-cadherin-mediated adhesion in Langerhans cell-like dendritic cells by inflammatory mediators that mobilize Langerhans cells in vivo. J. Immunol. *160*, 4067–4073.

Jameson, J., Ugarte, K., Chen, N., Yachi, P., Fuchs, E., Boismenu, R., and Havran, W.L. (2002). A role for skin gammadelta T cells in wound repair. Science *296*, 747–749.

Janeway, C.A. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb. Symp. Quant. Biol. *54 Pt 1*, 1–13.

Jensen, K.D.C., Su, X., Shin, S., Li, L., Youssef, S., Yamasaki, S., Steinman, L., Saito, T., Locksley, R.M., Davis, M.M., et al. (2008). Thymic Selection Determines γδ T Cell Effector Fate: Antigen-Naive Cells Make Interleukin-17 and Antigen-Experienced Cells Make Interferon γ. Immunity *29*, 90–100.

Jiang, X., Campbell, J.J., and Kupper, T.S. (2010). Embryonic trafficking of γδ T cells to skin is dependent on E/P selectin ligands and CCR4. Proc. Natl. Acad. Sci. U.S.A. *107*, 7443–7448.

Jiang, X., Clark, R.A., Liu, L., Wagers, A.J., Fuhlbrigge, R.C., and Kupper, T.S. (2012). Skin infection generates non-migratory memory $CD8^+$ T_{RM} cells providing global skin immunity. Nature *483*, 227–231.

Jung, H., Hsiung, B., Pestal, K., Procyk, E., and Raulet, D.H. (2012). RAE-1 ligands for the NKG2D receptor are regulated by E2F transcription factors, which control cell cycle entry. J. Exp. Med. *209*, 2409–2422.

Kalyan, S., and Kabelitz, D. (2012). Defining the nature of human γδ T cells: a biographical sketch of the highly empathetic. Cell. Mol. Immunol. *10*, 21–29.

Kaminski, H., Garrigue, I., Couzi, L., Taton, B., Bachelet, T., Moreau, J.F., Dechanet-Merville, J., Thiebaut, R., and Merville, P. (2015). Surveillance of γδ T Cells Predicts Cytomegalovirus Infection Resolution in Kidney Transplants. J. Am. Soc. Nephrol. ASN.2014100985 [ePub ahead of print].

Kaplan, D.H., Jenison, M.C., Saeland, S., Shlomchik, W.D., and Shlomchik, M.J. (2005). Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. Immunity *23*, 611–620.

Kasiske, B.L., Snyder, J.J., Gilbertson, D.T., and Wang, C. (2004). Cancer after Kidney Transplantation in the United States. Am. J. Transplant. *4*, 905–913.

Kim, B.S., Siracusa, M.C., Saenz, S.A., Noti, M., Monticelli, L.A., Sonnenberg,

G.F., Hepworth, M.R., Van Voorhees, A.S., Comeau, M.R., and Artis, D. (2013). TSLP Elicits IL-33-Independent Innate Lymphoid Cell Responses to Promote Skin Inflammation. Sci. Trans. Med. *5*, 170ra16–170ra16.

Kim, B.S. (2014). Innate Lymphoid Cells in the Skin. J. Invest. Dermatol. *135*, 673–678.

Kissenpfennig, A., Henri, S., Dubois, B., Laplace-Builhé, C., Perrin, P., Romani, N., Tripp, C.H., Douillard, P., Leserman, L., Kaiserlian, D., et al. (2005). Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinctfrom slower migrating Langerhans cells. Immunity *22*, 643–654.

Kistowska, M., Rossy, E., Sansano, S., Gober, H.-J., Landmann, R., Mori, L., and De Libero, G. (2008). Dysregulation of the host mevalonate pathway during early bacterial infection activates human TCR γδ cells. Eur. J. Immunol. *38*, 2200–2209.

Klareskog, L., Tjernlund, U., Forsum, U., and Peterson, P.A. (1977). Epidermal Langerhans cells express Ia antigens. Nature *268*, 248–250.

Klechevsky, E., Morita, R., Liu, M., Cao, Y., Coquery, S., Thompson-Snipes, L., Briere, F., Chaussabel, D., Zurawski, G., Palucka, A.K., et al. (2008). Functional Specializations of Human Epidermal Langerhans Cells and $CD14^+$ Dermal Dendritic Cells. Immunity *29*, 497–510.

Knight, A., Madrigal, A.J., Grace, S., Sivakumaran, J., Kottaridis, P., Mackinnon, S., Travers, P.J., and Lowdell, M.W. (2010). The role of Vδ2-negative γδ T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. Blood *116*, 2164–2172.

Knight, A., Mackinnon, S., and Lowdell, M.W. (2012a). Human Vdelta1 gamma-delta T cells exert potent specific cytotoxicity against primary multiple myeloma cells. Cytotherapy *14*, 1110–1118.

Knight, J., Spain, S.L., Capon, F., Hayday, A., Nestle, F.O., Clop, A., Wellcome Trust Case Control Consortium, Genetic Analysis of Psoriasis Consortium, I-chip for Psoriasis Consortium, Barker, J.N., et al. (2012b). Conditional analysis identifies three novel major histocompatibility complex loci associated with psoriasis. Hum. Mol. Genet. *21*, 5185–5192.

Koning, F., Stingl, G., Yokoyama, W.M., Yamada, H., Maloy, W.L., Tschachler, E., Shevach, E.M., and Coligan, J.E. (1987). Identification of a T3-associated gamma delta T cell receptor on Thy-1^+ dendritic epidermal Cell lines. Science *236*, 834–837.

Krähenbühl, O., Gattesco, S., and Tschopp, J. (1992). Murine Thy-1^+ dendritic epidermal T cell lines express granule-associated perforin and a family of granzyme molecules. Immunobiology *184*, 392–401.

Kubo, A., Nagao, K., Yokouchi, M., Sasaki, H., and Amagai, M. (2009). External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. J. Exp. Med. *206*, 2937–2946.

Kumamoto, Y., Linehan, M., Weinstein, J.S., Laidlaw, B.J., Craft, J.E., and Iwasaki,

A. (2013). Dermal Dendritic Cells Drive T Helper 2 Cell-Mediated Immunity. Immunity *39*, 733–743.

Kuziel, W.A., Takashima, A., Bonyhadi, M., Bergstresser, P.R., Allison, J.P., Tigelaar, R.E., and Tucker, P.W. (1987). Regulation of T-cell receptor gamma-chain RNA expression in murine Thy-1$^+$ dendritic epidermal cells. Nature *328*, 263–266.

Laggner, U., Di Meglio, P., Perera, G.K., Hundhausen, C., Lacy, K.E., Ali, N., Smith, C.H., Hayday, A.C., Nickoloff, B.J., and Nestle, F.O. (2011). Identification of a Novel Proinflammatory Human Skin-Homing Vγ9Vδ2 T Cell Subset with a Potential Role in Psoriasis. J. Immunol. *187*, 2783–2793.

Lamb, L.S., Jr, Gee, A.P., Hazlett, L.J., Musk, P., Parrish, R.S., O'Hanlon, T.P., Geier, S.S., Folk, R.S., Harris, W.G., McPherson, K., et al. (1999). Influence of T Cell Depletion Method on Circulating γδ T Cell Reconstitution and Potential Role in the Graft-Versus-Leukemia Effect. Cytotherapy *1*, 7–19.

Lanca, T., Correia, D.V., Moita, C.F., Raquel, H., Neves-Costa, A., Ferreira, C., Ramalho, J.S., Barata, J.T., Moita, L.F., Gomes, A.Q., et al. (2010). The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to γδ T-cell cytotoxicity. Blood *115*, 2407–2411.

Lanier, L.L. (2005). NK cell recognition. Annu. Rev. Immunol.

Leslie, D.S., Vincent, M.S., Spada, F.M., Das, H., Sugita, M., Morita, C.T., and Brenner, M.B. (2002). CD1-mediated γδ T Cell Maturation of Dendritic Cells. J. Exp. Med. *196*, 1575–1584.

Lewis, J.M., Girardi, M., Roberts, S.J., D Barbee, S., Hayday, A.C., and Tigelaar, R.E. (2006). Selection of the cutaneous intraepithelial γδ$^+$ T cell repertoire by a thymic stromal determinant. Nat. Immunol. *7*, 843–850.

Luoma, A.M., Castro, C.D., Mayassi, T., Bembinster, L.A., Bai, L., Picard, D., Anderson, B., Scharf, L., Kung, J.E., Sibener, L.V., et al. (2013). Crystal Structure of Vδ1 T Cell Receptor in Complex with CD1d-Sulfatide Shows MHC-like Recognition of a Self-Lipid by Human γδ T Cells. Immunity *39*, 1032–1042.

Ma, A., Koka, R., and Burkett, P. (2006). Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. Annu. Rev. Immunol. *24*, 657–679.

Ma, S., Cheng, Q., Cai, Y., Gong, H., Wu, Y., Yu, X., Shi, L., Wu, D., Dong, C., and Liu, H. (2014). IL-17A Produced by γδ T Cells Promotes Tumor Growth in Hepatocellular Carcinoma. Cancer Res. *74*, 1969–1982.

Mabuchi, T., Takekoshi, T., and Hwang, S.T. (2011). Epidermal CCR6$^+$ γδ T Cells Are Major Producers of IL-22 and IL-17 in a Murine Model of Psoriasiform Dermatitis. J. Immunol. *187*, 5026–5031.

Maccalli, C., Nonaka, D., Piris, A., Pende, D., Rivoltini, L., Castelli, C., and Parmiani, G. (2007). NKG2D-Mediated Antitumor Activity by Tumor-Infiltrating Lymphocytes and Antigen-Specific T-Cell Clones Isolated from Melanoma Patients. Clin. Can. Res. *13*, 7459–7468.

Mackay, L.K., Braun, A., Macleod, B.L., Collins, N., Tebartz, C., Bedoui, S., carbone, F.R., and Gebhardt, T. (2015). Cutting Edge: CD69 Interference with Sphingosine-1-Phosphate Receptor Function Regulates Peripheral T Cell Retention. J. Immunol. *194*, 2059–2063.

Mackay, L.K., Stock, A.T., Ma, J.Z., Jones, C.M., Kent, S.J., Mueller, S.N., Heath, W.R., Carbone, F.R., and Gebhardt, T. (2012). Long-lived epithelial immunity by tissue-resident memory T (T_{RM}) cells in the absence of persisting local antigen presentation. Proc. Natl. Acad. Sci. U.S.A. *109*, 7037–7042.

Madison, K.C. (2003). Barrier function of the skin: “la raison d'etre” of the epidermis. J. Invest. Dermatol. *121*, 234–241.

Maeurer, M.J., Martin, D., Walter, W., Liu, K., Zitvogel, L., Halusczcak, K., Rabinowich, H., Duquesnoy, R., Storkus, W., and Lotze, M.T. (1996). Human intestinal Vdelta1^{+} lymphocytes recognize tumor cells of epithelial origin. J. Exp. Med. *183*, 1681–1696.

Malissen, B., Tamoutounour, S., and Henri, S. (2014). The origins and functions of dendritic cells and macrophages in the skin. Nat. Rev. Immunol. *14*, 417–428.

Martin, B., Hirota, K., Cua, D.J., Stockinger, B., and Veldhoen, M. (2009). Interleukin-17-Producing γδ T Cells Selectively Expand in Response to Pathogen Products and Environmental Signals. Immunity *31*, 321–330.

Masopust, D. (2001). Preferential Localization of Effector Memory Cells in Nonlymphoid Tissue. Science *291*, 2413–2417.

Masopust, D., Choo, D., Vezys, V., Wherry, E.J., Duraiswamy, J., Akondy, R., Wang, J., Casey, K.A., Barber, D.L., Kawamura, K.S., et al. (2010). Dynamic T cell migration program provides resident memory within intestinal epithelium. J. Exp. Med. *207*, 553–564.

Masopust, D., Vezys, V., Wherry, E.J., Barber, D.L., and Ahmed, R. (2006). Cutting Edge: Gut Microenvironment Promotes Differentiation of a Unique Memory CD8 T Cell Population. J. Immunol. *176*, 2079–2083.

Matsue, H., Cruz, P.D., Bergstresser, P.R., and Takashima, A. (1993). Profiles of cytokine mRNA expressed by dendritic epidermal T cells in mice. J. Invest. Dermatol. *101*, 537–542.

Mattarollo, S.R., Rahimpour, A., Choyce, A., Godfrey, D.I., Leggatt, G.R., and Frazer, I.H. (2010). Invariant NKT Cells in Hyperplastic Skin Induce a Local Immune Suppressive Environment by IFNγ Production. J. Immunol. *184*, 1242–1250.

McElwee, K.J., Boggess, D., King, L.E., and Sundberg, J.P. (1998). Experimental induction of alopecia areata-like hair loss in C3H/HeJ mice using full-thickness skin grafts. J. Invest. Dermatol. *111*, 797–803.

McVay, L.D., Jaswal, S.S., Kennedy, C., Hayday, A., and Carding, S.R. (1998). The generation of human γδ T cell repertoires during fetal development. J. Immunol. *160*,

5851–5860.

Medzhitov, R., and Janeway, C., Jr (2000). Innate immunity. N. Eng. J. Med. *343*, 338–344.

Meresse, B., Chen, Z., Ciszewski, C., Tretiakova, M., Bhagat, G., Krausz, T.N., Raulet, D.H., Lanier, L.L., Groh, V., and Spies, T. (2004). Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. Immunity *21*, 357–366.

Michel, M.-L., Pang, D.J., Haque, S.F.Y., Potocnik, A.J., Pennington, D.J., and Hayday, A.C. (2012). Interleukin 7 (IL-7) selectively promotes mouse and human IL-17-producing γδ cells. Proc. Natl. Acad. Sci. U.S.A. *109*, 17549–17554.

Mistry, A.R., and O'Callaghan, C.A. (2007). Regulation of ligands for the activating receptor NKG2D. Immunology *121*, 439–447.

Mizukawa, Y., Yamazaki, Y., Teraki, Y., Hayakawa, J., Hayakawa, K., Nuriya, H., Kohara, M., and Shiohara, T. (2002). Direct evidence for interferon-gamma production by effector-memory-type intraepidermal T cells residing at an effector site of immunopathology in fixed drug eruption. Am. J. Pathol. *161*, 1337–1347.

Mohamadzadeh, M., McGuire, M.J., Smith, D.J., Gaspari, A.A., Bergstresser, P.R., and Takashima, A. (1996). Functional roles for granzymes in murine epidermal gamma(delta) T-cell-mediated killing of tumor targets. J. Invest. Dermatol. *107*, 738–742.

Mohamed, R.H., Sutoh, Y., Itoh, Y., Otsuka, N., Miyatake, Y., Ogasawara, K., and Kasahara, M. (2015). The SKINT1-Like Gene Is Inactivated in Hominoids But Not in All Primate Species: Implications for the Origin of Dendritic Epidermal T Cells. PLoS ONE *10*, e0123258.

Moon, J.J., and Nelson, B.H. (2001). Phosphatidylinositol 3-Kinase Potentiates, but Does Not Trigger, T Cell Proliferation Mediated by the IL-2 Receptor. J. Immunol. *167*, 2714–2723.

Moore, T.A., Freeden-Jeffry, von, U., Murray, R., and Zlotnik, A. (1996). Inhibition of gamma delta T cell development and early thymocyte maturation in IL-7$^{-/-}$ mice. J. Immunol. *157*, 2366–2373.

Morita, C.T., Beckman, E.M., Bukowski, J.F., Tanaka, Y., Band, H., Bloom, B.R., Golan, D.E., and Brenner, M.B. (1995). Direct presentation of nonpeptide prenyl pyrophosphate antigens to human γδ T cells. Immunity *3*, 495–507.

Morita, C., Lee, H., Leslie, D., Tanaka, Y., Bukowski, J., and Marker-Hermann, E. (1999). Recognition of nonpeptide prenyl pyrophosphate antigens by human gammadelta T cells. Microbes Infect. *1*, 175–186.

Murakami, R., Denda-Nagai, K., Hashimoto, S.-I., Nagai, S., Hattori, M., and Irimura, T. (2013). A Unique Dermal Dendritic Cell Subset That Skews the Immune Response toward Th2. PLoS ONE *8*, e73270.

Müller, G., Saloga, J., Germann, T., Bellinghausen, I., Mohamadzadeh, M., Knop, J., and Enk, A.H. (1994). Identification and induction of human keratinocyte-derived IL-12. J. Clin. Invest. *94*, 1799–1805.

Naik, S.M., Cannon, G., Burbach, G.J., Singh, S.R., Swerlick, R.A., Wilcox, J.N., Ansel, J.C., and Caughman, S.W. (1999). Human keratinocytes constitutively express interleukin-18 and secrete biologically active interleukin-18 after treatment with pro-inflammatory mediators and dinitrochlorobenzene. J. Invest. Dermatol. *113*, 766–772.

Nakamizo, S., Egawa, G., Tomura, M., Sakai, S., Tsuchiya, S., Kitoh, A., Honda, T., Otsuka, A., Nakajima, S., Dainichi, T., et al. (2015). Dermal Vγ4^{+} T Cells Possess a Migratory Potency to the Draining Lymph Nodes and Modulate CD8+ T-cell Activity through TNF-α Production. J. Invest. Dermatol. *135*, 1007–1015.

Nedellec, S., Sabourin, C., Bonneville, M., and Scotet, E. (2010). NKG2D Costimulates Human Vγ9Vδ2 T Cell Antitumor Cytotoxicity through Protein Kinase C-Dependent Modulation of Early TCR-Induced Calcium and Transduction Signals. J. Immunol. *185*, 55–63.

Nestle, F.O., Di Meglio, P., Qin, J.-Z., and Nickoloff, B.J. (2009a). Skin immune sentinels in health and disease. Nat. Rev. Immunol. *9*, 679–691.

Nestle, F.O., Kaplan, D.H., and Barker, J.N. (2009b). Psoriasis. N. Eng. J. Med. *361*, 496–509.

Ng, L.G., Hsu, A., Mandell, M.A., Roediger, B., Hoeller, C., Mrass, P., Iparraguirre, A., Cavanagh, L.L., Triccas, J.A., Beverley, S.M., et al. (2008). Migratory Dermal Dendritic Cells Act as Rapid Sensors of Protozoan Parasites. PLoS Pathog *4*, e1000222.

Nice, T.J., Coscoy, L., and Raulet, D.H. (2009). Posttranslational regulation of the NKG2D ligand Mult1 in response to cell stress. J. Exp. Med. *206*, 287–298.

Nielsen, M.M., Dyring-Andersen, B., Schmidt, J.D., Witherden, D., Lovato, P., Woetmann, A., dum, N.O., Poulsen, S.S., Havran, W.L., Geisler, C., et al. (2015). NKG2D-Dependent Activation of Dendritic Epidermal T Cells in Contact Hypersensitivity. J. Invest. Dermatol. *135*, 1311–1319.

Nussbaumer, O., Gruenbacher, G., Gander, H., and Thurnher, M. (2011). DC-like cell-dependent activation of human natural killer cells by the bisphosphonate zoledronic acid is regulated by γδ T lymphocytes. Blood *118*, 2743–2751.

O'Reilly Zwald, F., and Brown, M. (2011). Skin cancer in solid organ transplant recipients: Advances in therapy and management. J. Am. Acad. Dermatol. *65*, 253–261.

Okamoto Yoshida, Y., Umemura, M., Yahagi, A., O'Brien, R.L., Ikuta, K., Kishihara, K., Hara, H., Nakae, S., Iwakura, Y., and Matsuzaki, G. (2010). Essential Role of IL-17A in the Formation of a Mycobacterial Infection-Induced Granuloma in the Lung. J. Immunol. *184*, 4414–4422.

Oppenheim, D.E., Roberts, S.J., Clarke, S.L., Filler, R., Lewis, J.M., Tigelaar, R.E., Girardi, M., and Hayday, A.C. (2005). Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. Nat. Immunol. *6*, 928–937.

Orsini, D.L., Res, P.C., Van Laar, J.M., Muller, L.M., Soprano, A.E., Kooy, Y.M., Tak, P.P., and Koning, F. (1993). A subset of V delta 1^+ T cells proliferates in response to Epstein-Barr virus-transformed B cell lines in vitro. Scand. J. Immunol. *38*, 335–340.

Ouchi, T., Kubo, A., Yokouchi, M., Adachi, T., Kobayashi, T., Kitashima, D.Y., Fujii, H., Clausen, B.E., Koyasu, S., Amagai, M., et al. (2011). Langerhans cell antigen capture through tight junctions confers preemptive immunity in experimental staphylococcal scalded skin syndrome. J. Exp. Med. *208*, 2607–2613.

Pantelyushin, S., Haak, S., Ingold, B., Kulig, P., Heppner, F.L., Navarini, A.A., and Becher, B. (2012). Rorγt$^+$ innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. J. Clin. Invest. *122*, 2252–2256.

Paschen, A., Sucker, A., Hill, B., Moll, I., Zapatka, M., Nguyen, X.D., Sim, G.C., Gutmann, I., Hassel, J., Becker, J.C., et al. (2009). Differential Clinical Significance of Individual NKG2D Ligands in Melanoma: Soluble ULBP2 as an Indicator of Poor Prognosis Superior to S100B. Cli. Can. Res. *15*, 5208–5215.

Peiser, M., Koeck, J., Kirschning, C.J., Wittig, B., and Wanner, R. (2008). Human Langerhans cells selectively activated via Toll-like receptor 2 agonists acquire migratory and CD4$^+$ T cell stimulatory capacity. J. Leuk. Biol. *83*, 1118–1127.

Pende, D., Rivera, P., Marcenaro, S., Chang, C.C., Biassoni, R., Conte, R., Kubin, M., Cosman, D., Ferrone, S., Moretta, L., et al. (2002). UL16-Binding Protein Expression on Tumor Cell Lines of Different Histotypes Analysis of Tumor Susceptibility to NKG2D-dependent Natural Killer Cell Cytotoxicity. Cancer Res. *62*, 6178–6186.

Peng, S.L., Gerth, A.J., Ranger, A.M., and Glimcher, L.H. (2001). NFATc1 and NFATc2 together control both T and B cell activation and differentiation. Immunity *14*, 13–20.

Pennington, D.J., Silva-Santos, B., Shires, J., Theodoridis, E., Pollitt, C., Wise, E.L., Tigelaar, R.E., Owen, M.J., and Hayday, A.C. (2003). The inter-relatedness and interdependence of mouse T cell receptor γδ$^+$ and αβ$^+$ cells. Nat. Immunol. *4*, 991–998.

Petukhova, L., Duvic, M., Hordinsky, M., Norris, D., Price, V., Shimomura, Y., Kim, H., Singh, P., Lee, A., Chen, W.V., et al. (2010). Genome-wide association study in alopecia areata implicates both innate and adaptive immunity. Nature *466*, 113–117.

Picker, L.J., Michie, S.A., Rott, L.S., and Butcher, E.C. (1990). A unique phenotype of skin-associated lymphocytes in humans. Preferential expression of the HECA-452 epitope by benign and malignant T cells at cutaneous sites. Am. J. Path. *136*, 1053–

1068.

Pollock, R.A., Chandran, V., Pellett, F.J., Thavaneswaran, A., Eder, L., Barrett, J., Rahman, P., Farewell, V., and Gladman, D.D. (2013). The functional MICA-129 polymorphism is associated with skin but not joint manifestations of psoriatic disease independently of HLA-B and HLA-C. Tissue Antigens *82*, 43–47.

Pollock, R., Chandran, V., Barrett, J., Eder, L., Pellett, F., Yao, C., Lino, M., Shanmugarajah, S., Farewell, V.T., and Gladman, D.D. (2011). Differential major histocompatibility complex class I chain-related A allele associations with skin and joint manifestations of psoriatic disease. Tissue Antigens *77*, 554–561.

Poulin, L.F., Henri, S., de Bovis, B., Devilard, E., Kissenpfennig, A., and Malissen, B. (2007). The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells. J. Exp. Med. *204*, 3119–3131.

Purwar, R., Campbell, J., Murphy, G., Richards, W.G., Clark, R.A., and Kupper, T.S. (2011). Resident Memory T Cells (T_{RM}) Are Abundant in Human Lung: Diversity, Function, and Antigen Specificity. PLoS ONE *6*, e16245.

Raffaghello, L., Prigione, I., Airoldi, I., Camoriano, M., Levreri, I., Gambini, C., Pende, D., Steinle, A., Ferrone, S., and Pistoia, V. (2004). Downregulation and/or Release of NKG2D Ligands as Immune Evasion Strategy of Human Neuroblastoma. Neoplasia *6*, 558–568.

Rakasz, E., Rigby, S., de Andres, B., Mueller, A., Hagen, M., Dailey, M.O., Sandor, M., and Lynch, R.G. (1998). Homing of transgenic gammadelta T cells into murine vaginal epithelium. Int. Immunol. *10*, 1509–1517.

Ramírez-Valle, F., Gray, E.E., and Cyster, J.G. (2015). Inflammation induces dermal $V\gamma4^+$ γδT17 memory-like cells that travel to distant skin and accelerate secondary IL-17–driven responses. Proc. Natl. Acad. Sci. U.S.A. *112*, 8046–8051.

Ramsay, H.M., Fryer, A.A., Hawley, C.M., Smith, A.G., and Harden, P.N. (2002). Non-melanoma skin cancer risk in the Queensland renal transplant population. Br. J. Dermatol. *147*, 950–956.

Raulet, D.H., Gasser, S., Gowen, B.G., Deng, W., and Jung, H. (2013). Regulation of Ligands for the NKG2D Activating Receptor. Annu. Rev. Immunol. *31*, 413–441.

Rei, M., Pennington, D.J., and Silva-Santos, B. (2015). The Emerging Protumor Role of γδ T Lymphocytes: Implications for Cancer Immunotherapy. Cancer Res. *75*, 798–802.

Reif, K., Burgering, B.M., and Cantrell, D.A. (1997). Phosphatidylinositol 3-kinase links the interleukin-2 receptor to protein kinase B and p70 S6 kinase. J. Biol. Chem. *272*, 14426–14433.

Rhodes, D.A., Chen, H.-C., Price, A.J., Keeble, A.H., Davey, M.S., James, L.C., Eberl, M., and Trowsdale, J. (2015). Activation of human γδ T cells by cytosolic interactions of BTN3A1 with soluble phosphoantigens and the cytoskeletal adaptor periplakin. J. Immunol. *194*, 2390–2398.

Rincon-Orozco, B., Kunzmann, V., Wrobel, P., Kabelitz, D., Steinle, A., and Herrmann, T. (2005). Activation of Vγ9Vδ2 T cells by NKG2D. J. Immunol. *175*, 2144–2151.

Roberts, A.I., Lee, L., Schwarz, E., and Groh, V. (2001). Cutting edge: NKG2D receptors induced by IL-15 costimulate CD28-negative effector CTL in the tissue microenvironment. J. Immunol. *167*, 5527–5530.

Rolle, A., Mousavi-Jazi, M., Eriksson, M., Odeberg, J., Soderberg-Naucler, C., Cosman, D., Karre, K., and Cerboni, C. (2003). Effects of Human Cytomegalovirus Infection on Ligands for the Activating NKG2D Receptor of NK Cells: Up-Regulation of UL16-Binding Protein (ULBP)1 and ULBP2 Is Counteracted by the Viral UL16 Protein. J. Immunol. *171*, 902–908.

Romani, N., Koide, S., Crowley, M., Witmer-Pack, M., Livingstone, A.M., Fathman, C.G., Inaba, K., and Steinman, R.M. (1989). Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells. J. Exp. Med. *169*, 1169–1178.

Rooney, J.W., Sun, Y.L., and Glimcher, L.H. (1995). Novel NFAT sites that mediate activation of the interleukin-2 promoter in response to T-cell receptor stimulation. Mol. Cell. Biol. *15*, 6299–6310

Rossol, R., Dobmeyer, J.M., Dobmeyer, T.S., Klein, S.A., Rossol, S., Wesch, D., Hoelzer, D., Kabelitz, D., and Helm, E.B. (1998). Increase in $V\delta1^+$ γδ T cells in the peripheral blood and bone marrow as a selective feature of HIV-1 but not other virus infections. Br. J. Haematol. *100*, 728–734.

Rowden, G., Lewis, M.G., and Sullivan, A.K. (1977). Ia antigen expression on human epidermal Langerhans cells. Nature *268*, 247–248.

Ruckert, R., Asadullah, K., Seifert, M., Budagian, V.M., Arnold, R., Trombotto, C., Paus, R., and Bulfone-Paus, S. (2000). Inhibition of Keratinocyte Apoptosis by IL-15: A New Parameter in the Pathogenesis of Psoriasis? J. Immunol. *165*, 2240–2250.

Rusnak, F., and Mertz, P. (2000). Calcineurin: form and function. Physiol. Rev. *80*, 1483–1521.

Russano, A.M., Bassotti, G., Agea, E., Bistoni, O., Mazzocchi, A., Morelli, A., Porcelli, S.A., and Spinozzi, F. (2007). CD1-Restricted Recognition of Exogenous and Self-Lipid Antigens by Duodenal $\gamma\delta^+$ T Lymphocytes. J. Immunol. *178*, 3620–3626.

Russano, A.M., Agea, E., Corazzi, L., Postle, A.D., De Libero, G., Porcelli, S., de Benedictis, F.M., and Spinozzi, F. (2006). Recognition of pollen-derived phosphatidyl-ethanolamine by human CD1d-restricted γδ T cells. J. Allergy Clin. Immunol. *117*, 1178–1184.

Saeki, H., Moore, A.M., Brown, M.J., and Hwang, S.T. (1999). Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to

regional lymph nodes. J. Immunol. *162*, 2472–2475.

Salimi, M., Barlow, J.L., Saunders, S.P., Xue, L., Gutowska-Owsiak, D., Wang, X., Huang, L.C., Johnson, D., Scanlon, S.T., McKenzie, A.N.J., et al. (2013). A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. J. Exp. Med. *210*, 2939–2950.

Sallusto, F., Lenig, D., Förster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature *401*, 708–712.

Sandberg, Y., Almeida, J., Gonzalez, M., Lima, M., Bárcena, P., Szczepañski, T., van Gastel-Mol, E.J., Wind, H., Balanzategui, A., van Dongen, J.J.M., et al. (2006). TCR$\gamma\delta^+$ large granular lymphocyte leukemias reflect the spectrum of normal antigen-selected TCR$\gamma\delta^+$ T-cells. Leukemia *20*, 505–513.

Sandstrom, A., Peigné, C.-M., Léger, A., Crooks, J.E., Konczak, F., Gesnel, M.-C., Breathnach, R., Bonneville, M., Scotet, E., and Adams, E.J. (2014). The Intracellular B30.2 Domain of Butyrophilin 3A1 Binds Phosphoantigens to Mediate Activation of Human Vγ9Vδ2 T Cells. Immunity *40*, 490–500.

Sathaliyawala, T., Kubota, M., Yudanin, N., Turner, D., Camp, P., Thome, J.J.C., Bickham, K.L., Lerner, H., Goldstein, M., Sykes, M., et al. (2013). Distribution and Compartmentalization of Human Circulating and Tissue-Resident Memory T Cell Subsets. Immunity *38*, 187–197.

Schenkel, J.M., and Masopust, D. (2014). Tissue-Resident Memory T Cells. Immunity *41*, 886–897.

Schenkel, J.M., Fraser, K.A., Beura, L.K., Pauken, K.E., Vezys, V., and Masopust, D. (2014). Resident memory CD8 T cells trigger protective innate and adaptive immune responses. Science *346*, 98–101.

Schenkel, J.M., Fraser, K.A., Vezys, V., and Masopust, D. (2013). Sensing and alarm function of resident memory CD8+ T cells. Nat. Immunol. *14*, 509–513.

Schön, M.P., Schön, M., Parker, C.M., and Williams, I.R. (2002). Dendritic epidermal T cells (DETC) are diminished in integrin alphaE(CD103)-deficient mice. J. Invest. Dermatol. *119*, 190–193.

Schuler, G., and Steinman, R.M. (1985). Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. J. Exp. Med. *161*, 526–546.

Schwartz, E., Shapiro, R., Shina, S., and Bank, I. (1996). Delayed expansion of V delta 2^+ and V delta 1^+ gamma delta T cells after acute Plasmodium falciparum and Plasmodium vivax malaria. J. Allergy Clin. Immunol. *97*, 1387–1392.

Shafi, S., Vantourout, P., Wallace, G., Antoun, A., Vaughan, R., Stanford, M., and Hayday, A. (2011). An NKG2D-Mediated Human Lymphoid Stress Surveillance Response with High Interindividual Variation. Sci. Trans. Med. *3*, 113ra124–113ra124.

Sharp, L.L., Jameson, J.M., Cauvi, G., and Havran, W.L. (2004). Dendritic epidermal T cells regulate skin homeostasis through local production of insulin-like growth factor 1. Nat. Immunol. *6*, 73–79.

Shiow, L.R., Rosen, D.B., Brdičková, N., Xu, Y., An, J., Lanier, L.L., Cyster, J.G., and Matloubian, M. (2006). CD69 acts downstream of interferon-α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature *440*, 540–544.

Shires, J., Theodoridis, E., and Hayday, A.C. (2001). Biological insights into TCRgammadelta$^+$ and TCRalphabeta$^+$ intraepithelial lymphocytes provided by serial analysis of gene expression (SAGE). Immunity *15*, 419–434.

Shklovskaya, E., Roediger, B., and Fazekas de St Groth, B. (2008). Epidermal and Dermal Dendritic Cells Display Differential Activation and Migratory Behavior While Sharing the Ability to Stimulate CD4$^+$ T Cell Proliferation In Vivo. J. Immunol. *181*, 418–430.

Siegers, G.M., Dhamko, H., Wang, X.-H., Mathieson, A.M., Kosaka, Y., Felizardo, T.C., Medin, J.A., Tohda, S., Schueler, J., Fisch, P., et al. (2011). Human Vδ1 γδ T cells expanded from peripheral blood exhibit specific cytotoxicity against B-cell chronic lymphocytic leukemia-derived cells. Cytotherapy *13*, 753–764.

Silva-Santos, B., Pennington, D.J., and Hayday, A.C. (2005). Lymphotoxin-mediated regulation of gammadelta cell differentiation by alphabeta T cell progenitors. Science *307*, 925–928.

Song, G.G., Kim, J.-H., and Lee, Y.H. (2013). Associations between the major histocompatibility complex class I chain-related gene A transmembrane (MICA-TM) polymorphism and susceptibility to psoriasis and psoriatic arthritis: a meta-analysis. Rheumatol. Int. *34*, 117–123.

Spada, F.M., Grant, E.P., Peters, P.J., Sugita, M., Melián, A., Leslie, D.S., Lee, H.K., van Donselaar, E., Hanson, D.A., Krensky, A.M., et al. (2000). Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. J. Exp. Med. *191*, 937–948.

Spencer, J., and Isaacson, P.G. (1989). Human T-cell receptor expression. Nature *337*, 416.

Spetz, A.L., Strominger, J., and Groh-Spies, V. (1996). T cell subsets in normal human epidermis. Am. J. Pathol. *149*, 665–674.

Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J.P., Eberl, G., Koyasu, S., Locksley, R.M., McKenzie, A.N.J., Mebius, R.E., et al. (2013). Innate lymphoid cells – a proposal for uniform nomenclature. Nat. Rev. Immunol. *13*, 145–149.

Sprent, C.D.S.J. (2008). Homeostasis of Naive and Memory T Cells. Immunity *29*, 848–862.

Steinert, E.M., Schenkel, J.M., Fraser, K.A., Beura, L.K., Manlove, L.S., Igyártó, B.Z., Southern, P.J., and Masopust, D. (2015). Quantifying Memory CD8 T Cells Reveals Regionalization of Immunosurveillance. Cell *161*, 737–749.

Steinman, R.M., Hawiger, D., and Nussenzweig, M.C. (2003). Tolerogenic Dendritic Cells. Annu. Rev. Immunol. *21*, 685–711.

Stern-Ginossar, N., Gur, C., Biton, M., Horwitz, E., Elboim, M., Stanietsky, N., Mandelboim, M., and Mandelboim, O. (2008). Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. Nat. Immunol. *9*, 1065–1073.

Stingl, G., Gunter, K.C., Tschachler, E., Yamada, H., Lechler, R.I., Yokoyama, W.M., Steiner, G., Germain, R.N., and Shevach, E.M. (1987a). Thy-1^+ dendritic epidermal cells belong to the T-cell lineage. Proc. Natl. Acad. Sci. U.S.A. *84*, 2430–2434.

Stingl, G., Katz, S.I., Clement, L., Green, I., and Shevach, E.M. (1978). Immunologic functions of Ia-bearing epidermal Langerhans cells. J. Immunol. *121*, 2005–2013.

Stingl, G., Koning, F., Yamada, H., Yokoyama, W.M., Tschachler, E., Bluestone, J.A., Steiner, G., Samelson, L.E., Lew, A.M., and Coligan, J.E. (1987b). Thy-1^+ dendritic epidermal cells express T3 antigen and the T-cell receptor gamma chain. Proc. Natl. Acad. Sci. U.S.A. *84*, 4586–4590.

Stingl, G., Wolff-Schreiner, E.C., Pichler, W.J., Gschnait, F., Knapp, W., and Wolff, K. (1977). Epidermal Langerhans cells bear Fc and C3 receptors. Nature *268*, 245–246.

Stoitzner, P., Zanella, M., Ortner, U., Lukas, M., Tagwerker, A., Janke, K., Lutz, M.B., Schuler, G., Echtenacher, B., Ryffel, B., et al. (1999). Migration of langerhans cells and dermal dendritic cells in skin organ cultures: augmentation by TNF-alpha and IL-1beta. J. Leuk. Biol. *66*, 462–470.

Stoitzner, P., Holzmann, S., McLellan, A.D., Ivarsson, L., Stössel, H., Kapp, M., Kämmerer, U., Douillard, P., Kämpgen, E., Koch, F., et al. (2003). Visualization and characterization of migratory Langerhans cells in murine skin and lymph nodes by antibodies against Langerin/CD207. J. Invest. Dermatol. *120*, 266–274.

Stoitzner, P., Tripp, C.H., Eberhart, A., Price, K.M., Jung, J.Y., Bursch, L., Ronchese, F., and Romani, N. (2006). Langerhans cells cross-present antigen derived from skin. Proc. Natl. Acad. Sci. U.S.A. *103*, 7783–7788.

Stoll, S., Jonuleit, H., Schmitt, E., Müller, G., Yamauchi, H., Kurimoto, M., Knop, J., and Enk, A.H. (1998). Production of functional IL-18 by different subtypes of murine and human dendritic cells (DC): DC-derived IL-18 enhances IL-12-dependent Th1 development. Eur. J. Immunol. *28*, 3231–3239.

Streilein, J.W. (1983). Skin-associated lymphoid tissues (SALT): origins and functions. J. Invest. Dermatol. *80*, 12s–16s.

Strid, J., Sobolev, O., Zafirova, B., Polic, B., and Hayday, A. (2011). The Intraepithelial T Cell Response to NKG2D-Ligands Links Lymphoid Stress Surveillance to Atopy. Science *334*, 1293–1297.

Strid, J., Hourihane, J., Kimber, I., Callard, R., and Strobel, S. (2004). Disruption of the stratum corneum allows potent epicutaneous immunization with protein antigens resulting in a dominant systemic Th2 response. Eur. J. Immunol. *34*, 2100–2109.

Strid, J., Roberts, S.J., Filler, R.B., Lewis, J.M., Kwong, B.Y., Schpero, W., Kaplan, D.H., Hayday, A.C., and Girardi, M. (2008). Acute upregulation of an NKG2D ligand promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis. Nat. Immunol. *9*, 146–154.

Suárez-Fariñas, M., Fuentes-Duculan, J., Lowes, M.A., and Krueger, J.G. (2010). Resolved Psoriasis Lesions Retain Expression of a Subset of Disease-Related Genes. J. Invest. Dermatol. *131*, 391–400.

Sugaya, M., Nakamura, K., and Tamaki, K. (1999). Interleukins 18 and 12 synergistically upregulate interferon-gamma production by murine dendritic epidermal T cells. J. Invest. Dermatol. *113*, 350–354.

Sumaria, N., Roediger, B., Ng, L.G., Qin, J., Pinto, R., Cavanagh, L.L., Shklovskaya, E., Fazekas de St Groth, B., Triccas, J.A., and Weninger, W. (2011). Cutaneous immunosurveillance by self-renewing dermal γδ T cells. J. Exp. Med. *208*, 505–518.

Sutherland, C.L., Chalupny, N.J., Schooley, K., VandenBos, T., Kubin, M., and Cosman, D. (2002). UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. J. Immunol. *168*, 671–679.

Sutton, C.E., Lalor, S.J., Sweeney, C.M., Brereton, C.F., Lavelle, E.C., and Mills, K.H.G. (2009). Interleukin-1 and IL-23 InduceInnate IL-17 Production from γδ T Cells, Amplifying Th17 Responses and Autoimmunity. Immunity *31*, 331–341.

Tanaka, Y., Morita, C.T., Tanaka, Y., Nieves, E., Brenner, M.B., and Bloom, B.R. (1995). Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. Nature *375*, 155–158.

Tang, F., Sally, B., Ciszewski, C., Abadie, V., Curran, S.A., Groh, V., FitzGerald, O., Winchester, R.J., and Jabri, B. (2013). Interleukin 15 Primes Natural Killer Cells to Kill via NKG2D and cPLA2 and This Pathway Is Active in Psoriatic Arthritis. PLoS ONE *8*, e76292.

Tay, S.S., Roediger, B., Tong, P.L., Tikoo, S., and Weninger, W. (2013). The Skin-Resident Immune Network. Curr. Derm. Rep. *3*, 13–22.

Teunissen, M.B.M., Munneke, J.M., Bernink, J.H., Spuls, P.I., Res, P.C.M., Velde, te, A., Cheuk, S., Brouwer, M.W.D., Menting, S.P., Eidsmo, L., et al. (2014a). Composition of Innate Lymphoid Cell Subsets in the Human Skin: Enrichment of NCR. J. Invest. Dermatol. *134*, 2351–2360.

Teunissen, M.B.M., Yeremenko, N.G., Baeten, D.L.P., Chielie, S., Spuls, P.I., de Rie, M.A., Lantz, O., and Res, P.C.M. (2014b). The IL-17A-producing CD8+ T-cell population in psoriatic lesional skin comprises mucosa-associated invariant T cells and conventional T cells. J. Invest. Dermatol. *134*, 2898–2907.

Tietz, W., Allemand, Y., Borges, E., Laer, von, D., Hallmann, R., Vestweber, D., and Hamann, A. (1998). CD4+ T cells migrate into inflamed skin only if they express ligands for E- and P-selectin. J. Immunol. *161*, 963–970.

Toulon, A., Breton, L., Taylor, K.R., Tenenhaus, M., Bhavsar, D., Lanigan, C., Rudolph, R., Jameson, J., and Havran, W.L. (2009). A role for human skin-resident T cells in wound healing. J. Exp. Med. *206*, 743–750.

Trautinger, F., Knobler, R., Willemze, R., Peris, K., Stadler, R., Laroche, L., D'Incan, M., Ranki, A., Pimpinelli, N., Ortiz-Romero, P., et al. (2006). EORTC consensus recommendations for the treatment of mycosis fungoides/Sézary syndrome. Eur. J. Cancer. *42*, 1014–1030.

Trifari, S., Kaplan, C.D., Tran, E.H., Crellin, N.K., and Spits, H. (2009). Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)17, T(H)1 and T(H)2 cells. Nat. Immunol. *10*, 864–871.

Turchinovich, G., and Hayday, A.C. (2011). Skint-1 Identifies a Common Molecular Mechanism for the Development of Interferon-g-Secreting versus Interleukin-17-Secreting gd T Cells. Immunity *35*, 59–68.

Uldrich, A.P., Le Nours, J., Pellicci, D.G., Gherardin, N.A., McPherson, K.G., Lim, R.T., Patel, O., Beddoe, T., Gras, S., Rossjohn, J., et al. (2013). CD1d-lipid antigen recognition by the γδ TCR. Nat. Immunol. *14*, 1137–1145.

Upshaw, J.L., Arneson, L.N., Schoon, R.A., Dick, C.J., Billadeau, D.D., and Leibson, P.J. (2006). NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. Nat. Immunol. *7*, 524–532.

Utzschneider, D.T., Legat, A., Fuertes Marraco, S.A., Carrié, L., Luescher, I., Speiser, D.E., and Zehn, D. (2013). T cells maintain an exhausted phenotype after antigen withdrawal and population reexpansion. Nat. Immunol. *14*, 603–610.

Van Kaer, L., Parekh, V.V., and Wu, L. (2015). The Response of CD1d-Restricted Invariant NKT Cells to Microbial Pathogens and Their Products. Front. Immunol. *6*.

van Oers, N.S., Killeen, N., and Weiss, A. (1996). Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. J. Exp. Med. *183*, 1053–1062.

Vantourout, P., Willcox, C., Turner, A., Swanson, C.M., Haque, Y., Sobolev, O., Grigoriadis, A., Tutt, A., and Hayday, A. (2014). Immunological Visibility: Posttranscriptional Regulation of Human NKG2D Ligands by the EGF Receptor Pathway. Sc. Trans. Med. *6*, 231ra49–231ra49.

Vantourout, P., and Hayday, A. (2013). Six-of-the-best: unique contributions of γδ T cells to immunology. Nat. Rev. Immunol. *13*, 88–100.

Vavassori, S., Kumar, A., Wan, G.S., Ramanjaneyulu, G.S., Cavallari, M., Daker, El, S., Beddoe, T., Theodossis, A., Williams, N.K., Gostick, E., et al. (2013).

Butyrophilin 3A1 binds phosphorylated antigens and stimulates human γδ T cells. Nat. Immunol. *14*, 908–916.

Verneris, M.R., Karimi, M., Karami, M., Baker, J., Jayaswal, A., and Negrin, R.S. (2004). Role of NKG2D signaling in the cytotoxicity of activated and expanded $CD8^+$ T cells. Blood *103*, 3065–3072.

Vocanson, M., Hennino, A., Rozi res, A., Poyet, G., and Nicolas, J.F. (2009). Effector and regulatory mechanisms in allergic contact dermatitis. Allergy *64*, 1699–1714.

Vroom, T.M., Scholte, G., Ossendorp, F., and Borst, J. (1991). Tissue distribution of human gamma delta T cells: no evidence for general epithelial tropism. J. Clin. Pathol. *44*, 1012–1017.

Wakim, L.M., Woodward-Davis, A., and Bevan, M.J. (2010). Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. Proc. Natl. Acad. Sci. U.S.A. *107*, 17872–17879.

Wakita, D., Sumida, K., Iwakura, Y., Nishikawa, H., Ohkuri, T., Chamoto, K., Kitamura, H., and Nishimura, T. (2010). Tumor-infiltrating IL-17-producing γδ T cells support the progression of tumor by promoting angiogenesis. Eur. J. Immunol. *40*, 1927–1937.

Wang, H., Henry, O., Distefano, M.D., Wang, Y.C., Raikkonen, J., Monkkonen, J., Tanaka, Y., and Morita, C.T. (2013). Butyrophilin 3A1 Plays an Essential Role in Prenyl Pyrophosphate Stimulation of Human Vγ9Vδ2 T Cells. J. Immunol. *191*, 1029–1042.

Wang, H., Sarikonda, G., Puan, K.J., Tanaka, Y., Feng, J., Giner, J.L., Cao, R., Monkkonen, J., Oldfield, E., and Morita, C.T. (2011). Indirect Stimulation of Human Vγ9Vδ2 T Cells through Alterations in Isoprenoid Metabolism. J. Immunol. *187*, 5099–5113.

Wang, R., Natarajan, K., Revilleza, M., Boyd, L.F., Zhi, L., Zhao, H., Robinson, H., and Margulies, D.H. (2012). Structural basis of mouse cytomegalovirus m152/gp40 interaction with RAE1γ reveals a paradigm for MHC/MHC interaction in immune evasion. Proc. Natl. Acad. Sci. U.S.A. *109*, E3578–E3587.

Wang, X.-N., McGovern, N., Gunawan, M., Richardson, C., Windebank, M., Siah, T.-W., Lim, H.-Y., Fink, K., Li, J.L.Y., Ng, L.G., et al. (2014). A Three-Dimensional Atlas of Human Dermal Leukocytes, Lymphatics, and Blood Vessels. J. Invest. Dermatol. *134*, 965–974.

Watanabe, R., Gehad, A., Yang, C., Scott, L.L., Teague, J.E., Schlapbach, C., Elco, C.P., Huang, V., Matos, T.R., Kupper, T.S., et al. (2015). Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. Sci. Trans. Med. *7*, 279ra39.

Weiss, A., Imboden, J., Shoback, D., and Stobo, J. (1984). Role of T3 surface molecules in human T-cell activation: T3-dependent activation results in an increase in cytoplasmic free calcium. Proc. Natl. Acad. Sci. U.S.A. *81*, 4169–4173.

Wencker, M., Turchinovich, G., Di Marco Barros, R., Deban, L., Jandke, A., Cope, A., and Hayday, A.C. (2013). Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor responsiveness. Nat. Immunol. *15*, 80–87.

Willberg, C.B. (2014). Mucosal-associated invariant T-cells: new players in anti-bacterial immunity. Front. Immunol. *5*, 480.

Willcox, C.R., Pitard, V., Netzer, S., Couzi, L., Salim, M., Silberzahn, T., Moreau, J.-F., Hayday, A.C., Willcox, B.E., and Déchanet-Merville, J. (2012). Cytomegalovirus and tumor stress surveillance by binding of a human γδ T cell antigen receptor to endothelial protein C receptor. Nat. Immunol. *13*, 872–879.

Williamson, P., Merida, I., and Gaulton, G. (1993). Protein and lipid kinase activation cascades in interleukin-2 receptor signalling. Sem. Immunol. *5*, 337–344.

Witmer-Pack, M.D., Olivier, W., Valinsky, J., Schuler, G., and Steinman, R.M. (1987). Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. J. Exp. Med. *166*, 1484–1498.

Wu, J., Cherwinski, H., Spies, T., Phillips, J.H., and Lanier, L.L. (2000). DAP10 and DAP12 form distinct, but functionally cooperative, receptor complexes in natural killer cells. J. Exp. Med. *192*, 1059–1068.

Wu, J., Groh, V., and Spies, T. (2002). T Cell Antigen Receptor Engagement and Specificity in the Recognition of Stress-Inducible MHC Class I-Related Chains by Human Epithelial γδ T Cells. J. Immunol. *169*, 1236–1240.

Xing, L., Dai, Z., Jabbari, A., Cerise, J.E., Higgins, C.A., Gong, W., de Jong, A., Harel, S., DeStefano, G.M., Rothman, L., et al. (2014). Alopecia areata is driven by cytotoxic T lymphocytes and is reversed by JAK inhibition. Nat. Med. *20*, 1043–1049.

Xu, B., Pizarro, J.C., Holmes, M.A., McBeth, C., Groh, V., Spies, T., and Strong, R.K. (2011). Crystal structure of a gammadelta T-cell receptor specific for the human MHC class I homolog MICA. Proc. Natl. Acad. Sci. U.S.A. *108*, 2414–2419.

Yoshiki, R., Kabashima, K., Honda, T., Nakamizo, S., Sawada, Y., Sugita, K., Yoshioka, H., Ohmori, S., Malissen, B., Tokura, Y., et al. (2014). IL-23 from Langerhans Cells Is Required for the Development of Imiquimod-Induced Psoriasis-Like Dermatitis by Induction of IL-17A-Producing. J. Invest. Dermatol. *134*, 1912–1921.

Zhao, J., Huang, J., Chen, H., Cui, L., and He, W. (2006). Vδ1 T cell receptor binds specifically to MHC I chain related A: Molecular and biochemical evidences. Biochem. Biophys. Res. Comm. *339*, 232–240.

Zheng, Y., Danilenko, D.M., Valdez, P., Kasman, I., Eastham-Anderson, J., Wu, J., and Ouyang, W. (2006). Interleukin-22, a T_H17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature *445*, 648–651.

Zhu, J., Koelle, D.M., Cao, J., Vazquez, J., Huang, M.L., Hladik, F., Wald, A., and

Corey, L. (2007). Virus-specific $CD8^+$ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation. J. Exp. Med. *204*, 595–603.

Zhu, J., Peng, T., Johnston, C., Phasouk, K., Kask, A.S., Klock, A., Jin, L., Diem, K., Koelle, D.M., Wald, A., et al. (2013). Immune surveillance by $CD8aa^+$ skin-resident T ells in human herpes virus infection. Nature 497, 494–497.