TRINITY COLLEGE DUBLIN
COLÁISTE NA TRÍONÓIDE
THE UNIVERSITY OF DUBLIN

Functional studies on human γδ T cells and their interactions with dendritic cells and B cells

A dissertation submitted to Trinity College Dublin, University of Dublin,
as requirement for the degree of Doctor in Philosophy (PhD)
in Clinical Medicine

25/09/2015

Andreea Petrasca, B.Sc (Hons), M.Sc

Student number 10264277

Work carried out between September 2011 and September 2015

Supervisor: Dr. Derek Doherty
Head of Department of Immunology
School of Medicine
Trinity College Dublin

sfi
science foundation ireland
fondúireacht eolaíochta éireann

Declaration

I, Andreea Petrasca, the undersigned declare that this thesis is the result of my own work. It has not previously been submitted to this university or any other, and any assistance received by way of borrowing from the work of others has been cited and acknowledged. I have read and understood the college's regulations on plagiarism and I make this declaration in the knowledge that a breach of the rules pertaining to submission may carry serious consequences.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

Signed: Andreea Petrasca 25 September 2015

Acknowledgements

First and foremost I would like to thank the best and most wonderful parents anyone could have, Ivan and Angela, without whom, I would have never gotten this far. For their endless love and support and providing me with the best education I could have ever dreamed of, hvala!

I would also like to express my deepest gratitude to my supervisor, Derek Doherty, for offering me the opportunity to carry out such exciting research. For all his help, encouragement and wisdom throughout the past 4 years and his complete dedication and constant support which were key in completing this PhD. I would also like to thank Derek for allowing me to be independent. His positivity has been truly motivating. Moreover, I am very grateful for all the collaborations he has allowed me to get involved in.

To my fiancé James, who fully understands first-handedly the pressure and dedication that is required in doing a PhD and for forgiving me for all those late nights spent on tissue culture and flow cytometry.

My sincerest thanks also go out to some key mentors that have helped shape the scientist I am today. To Vinny O'Reilly, for introducing me to tissue culture and flow cytometry and for setting a great example. To Ann Atzberger for her invaluable teachings, and whose passion for flow cytometry sparked an interest in me for flow cytometry. To Padraic Dunne, for all his guidance, and for allowing me to ask lots of "silly" questions. To professors John Jackson, Con Feighery and Jacinta Kelly for being an integral part in our lab meetings and whose wisdom, insight and enthusiasm inspired me to excel at immunology.

I would also like to express my sincerest thanks to my younger sister, Alina, for being understanding and putting up with me after coming home following long and tiring days. Also, to my friend Ciara for all the fun activities we enjoyed after many productive days. Last, but not least I would like to thank my partners in crime and wonderful friends Yasmeen Ghnewa, Éilis Dockry and Serena Arduini for lifting my spirits on those not-so-productive days.

Academic Outputs

Publications

- "Human Vδ2^{+} γδ T cells differentially induce maturation, cytokine production, and alloreactive T cell stimulation by dendritic cells and B cells". Andreea Petrasca, Derek G Doherty. Research Article, Frontiers in Immunology, 5: 650; December 19, 2014.

- "Vδ2 T cells differentially stimulate the production of proinflammatory cytokines by dendritic cells and B cells". Andreea Petrasca, Derek G Doherty. In: Proceedings of the 15th International Congress of Immunology (22-27 August 2013, Milan, Italy), Medimond, Bologna, Italy pp 21-24, 2014.

- "HIV-1 Tat clade-specific cytokine induction in monocytes/macrophages is not evidenced in total or Vγ9Vδ2 T lymphocytes". Alan Kennedy, Andreea Petrasca, Derek G Doherty, Martina Hennessy, Paul J Spiers. Research letter, AIDS, 28:131-133, January 2, 2014.

- "Synthesis and immunostimulatory activity of two α-S-galactosyl phenyl-capped ceramides". Niamh Murphy, Andreea Petrasca, Niamh M. Murphy, Vincent O'Reilly, Paul Evans, Derek G. Doherty, Xiangming Zhu. Research article, Arkivoc, 2013 (ii) 363-377, February 8, 2013

Manuscripts in submission

- "Human Natural Killer cell expression of ULBP2 is associated with a mature functional phenotype" Kiva Brennan, Sinéad Keating, Andreea Petrasca, Vincent P O'Reilly, Joseph Keane, Derek G. Doherty, Clair M. Gardiner.

- "Synthesis of N-Acetylglucosamine glycolipid mimetics and evaluation of their ability to stimulate human invariant natural killer T cells". Trinidad Velasco-

Torrijos, Carol Buggy, Andreea Petrasca, Niamh M. Murphy, Vincent O'Reilly, Derek G. Doherty.

- Serum Adipokines and Vascular Phenotype in Juvenile-onset Systemic Lupus Erythematosus. Catherine Quinlan, Andreea Petrasca, John Deanfield, Michael Riordan, Rukshana Shroff, Pilkington Clarissa, Stephen Marks, Kjell Tullus.

Collaborations

- "The role of Vγ9Vδ2 T cells in immunity against *Clostridium difficile*". Micheál MacAogáin, Andreea Petrasca, Derek Doherty, Thomas Rogers.

- "Investigating the potential of humanised mice as a pre-clinical model for Vδ2 T cell based immunotherapy". Eoin O'Brien, Vincent O'Reilly, Andreea Petrasca, Margaret Dunne, Cathal Harmon, Fionnuala Hickey, Derek Doherty, Cliona O'Farrelly, Mark Little.

- "The effect of physical activity on cells of the immune system". Grace Lavelle, Andreea Petrasca, Derek Doherty.

- "The role of *Staphylococcus aureus* in activating Vγ9Vδ2 T cells". Stephen Lalor, Andreea Petrasca, Derek Doherty, Rachel McLoughlin.

Oral presentations

"Human Vγ9Vδ2 T cells induce B cell and DC maturation - applications for cancer immunotherapy". *Institute of Molecular Medicine Annual Meeting*, Trinity College Dublin, Nov 2014.

"Human Vγ9Vδ2 T cells can selectively promote T_H1 or T_H2 responses via interactions with dendritic cells or B cells in vitro". *Institute of Molecular Medicine Annual Meeting*, Trinity College Dublin, Nov 2013.

"Reciprocal activating interactions between Vγ9Vδ2 T cells, B cells and dendritic cells". *School of Medicine Postgraduate Research Day*, Trinity College Dublin, Sept 2012.

Poster presentations

"Human Vδ2$^+$ γδ T cells promote differentiation of B cells into effector cells that release antibody and IL-4 and stimulate alloreactive T cell proliferation". British Society for Immunology Congress, Brighton, UK, December 2014.

"Human Vδ2$^+$ γδ T cells promote differentiation of B cells into effector cells that release antibody and IL-4 and stimulate alloreactive T cell proliferation". Irish Society of Immunology Meeting, Dublin, Ireland, September 2014.

"Human Vδ3 T cells induce expression of co-stimulatory molecules by B cells *in vitro*". International Gamma Delta T-cell conference, Chicago, USA, May 2014.

"Human Vγ9Vδ2 T cells can selectively promote T_H1 or T_H2 responses via interactions with dendritic cells or B cells *in vitro*". Andreea Petrasca and Derek G. Doherty, Irish Cytometry Society Conference, Dublin, February 2014.

"Human Vγ9Vδ2 T cells can selectively promote T_H1 or T_H2 responses via interactions with dendritic cells or B cells *in vitro*". Andreea Petrasca and Derek G. Doherty. British Society for Immunology Annual Meeting, Liverpool, United Kingdom, December 2013.

"Human Vγ9Vδ2 T cells can selectively promote T_H1 or T_H2 responses via interactions with dendritic cells or B cells *in vitro*". International Congress of Immunology, Milan, Italy, August 2013.

"Vγ9Vδ2 T cells induce maturation of dendritic cells but not B cells into antigen-presenting cells". International Gamma-delta T-cell conference, Freiburg, Germany, June 2012.

Table of Contents

List of abbreviations used

ADCC	antibody-dependent cell-mediated cytotoxicity
αGalCer	alpha galactosylceramide
ANOVA	analysis of variance
APC	antigen presenting cells
APC	allophycocyanin fluorophore, used in flow cytometry
βGluCer	beta glucosyl ceramide
BHIS	brain heart infusion medium
BSA	bovine serum albumin
BTN	Butyrophilin
CD	cluster of differentiation
cDMEM	complete Dulbecco's modified Eagle's medium
CDT	cytolethal distending toxins
cRPMI	complete Roswell Park Memorial Institute medium
Cy5/7	CyChrome 5/ CyChrome 7
DC	dendritic cells
DMAPP	dimethylallyl diphosphate
DMSO	dimethylsulphoxide
DN	double negative
EBAO	ethidium bromide and acridine orange
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay

FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one control
FSC	forward scatter
γδ-T-APC	γδ T cell with APC function
GM-CSF	granulocyte monocyte-colony stimulating factor
HeLa	Henrietta Lacks cervical cell line
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
iDC	immature dendritic cells
IEL	intraepithelial lymphocytes
IFN	interferon
IL	interleukin
iNKT	invariant natural killer T
IPP	isopentenyl pyrophosphate
IU	international units
LF	limes of flocculation
LPS	lipopolysaccharide
mAb	monoclonal antibodies

MACS	magnetic-activated cell sorting
MAIT	mucosal-associated invariant T
MEP	methylerythritol 4-phosphate
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MICA/MICB	MHC class I-related chains A or B
NK	natural killer
NKT	natural killer T
NKG2	natural killer group 2
pAg	phosphoantigen (phosphorylated pyrophosphate)
PAMP	pathogen associated molecular pattern
PBA	PBS supplemented with BSA and azide
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PFA	paraformaldehyde
PerCP	peridinin chlorophyll protein complex
PHA	phytohaemagglutinin
PMA	phorbol myristate acetate
PPD	purified protein derivative
PRR	pattern recognition receptors
RT	ribotype

SLP	surface layer protein
SSC	side scatter
T_C	cytotoxic T cell
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EM}	effector memory T cell
T_{EMRA}	terminally differentiated effector memory T cell
T_H	T helper
T_{FH}	follicular helper T
TGF	transforming growth factor
TLR	toll-like receptor
TMB	3,3,5,5-tetramethylbenzidine
TNF	tumour necrosis factor
Treg	regulatory T
TT	tetanus toxoid
ULBP	UL-16-binding proteins

Abstract

γδ T cells are innate T cells that play central roles in protection against microorganisms and cellular stress. There are three main subsets in humans: Vδ1, Vδ2 and Vδ3 T cells. The most abundant of these, Vδ2 T cells, recognises phosphoantigens produced in one of two pathways of isoprenoid synthesis, a cellular metabolic pathway employed by all eukaryotes and many bacteria. This allows Vδ2 T cells to monitor for infection and tumour transformation which result in altered cellular concentration of phosphoantigens. Vδ1 and Vδ3 T cells are predominantly found in epithelial tissues and play roles in homeostasis, tissue integrity and lipid surveillance and are found at increased frequencies in some patients with tumours and viral infections. Upon activation, γδ T cells can kill target cells and rapidly promote adaptive immune responses through physical interactions with other immune cells, and rapid and selective secretion of T helper type 1 (T_H1), T_H2, T_H17 and regulatory cytokines.

In the present study we carried out a phenotypic and functional comparison of the three γδ T cell subsets in human peripheral blood and examined methods for generating lines of Vδ2 and Vδ3 T cell for functional characterisation. We found that stimulation with the phosphoantigen (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) and interleukin-2 yielded highly pure populations of Vδ2 T cells capable of producing T_H1 and T_H2 cytokines upon re-stimulation. In contrast, treatment with the aminobisphosphonate zoledronate, which promotes isoprenoid synthesis, resulted in expansion of Vδ2 T cells that produced T_H2 cytokines only. In the absence of a known ligand for Vδ3 T cells, we used the T cell mitogen phytohaemagglutinin to stimulate sorted $V\delta3^+$ cells which resulted in up to 1,000-fold expansion within 3-4 weeks.

We next examined the reciprocal activating interactions between Vδ2 T cells, dendritic cells (DC) and B cells in co-culture experiments and defined the resulting cytokine profiles, cell phenotypes and antibody responses and the molecular interactions involved. We found that Vδ2 T cells promoted maturation of DC into antigen-presenting cells capable of stimulating T_H1 cell responses. In contrast, Vδ2 T cells promoted differentiation of B cells into antibody-secreting plasma cells with

phenotypes of antigen-presenting cells, but which produced T_H2 cytokines. While co-stimulatory molecules, T_H1 cytokines and cell contact were required for DC maturation by Vδ2 T cells, they did not play major roles in B cell maturation.

The present study investigated, for the first time, the relationship between Vδ3 T cells and B cells and revealed that while Vδ3 T cells induced co-stimulatory marker expression by B cells, they failed to induce significant cytokine or antibody secretion. However, activated B cells were able to induce IL-17 secretion by Vδ3 T cells. We also assessed the ability of Vδ3 T cells to recognise CD1 molecules, but found that freshly-isolated or expanded Vδ3 T cells showed no reactivity against CD1a, CD1b, CD1c or CD1d molecules in the presence or absence of a number of glycolipids.

Since *Clostridium difficile* appears to utilise the non-mevalonate pathway of isoprenoid biosynthesis, suggesting that it can produce HMB-PP, we assessed the ability of HMB-PP to stimulate proliferation and cytokine secretion by Vδ2 T cells. We found, in spite of great inter-donor variability, *C. difficile* secreted a Vδ2-stimulating agent which induced T cell proliferation and cytokine production in most donors and was comparable to the stimulating capabilities of HMB-PP. However, the identity of this secreted factor remains to be elucidated.

These findings highlight the role of γδ T cells in immunosurveillance, innate immunity, antigen presentation and activation of adaptive immunity. Their ability to act as a bridge between innate and adaptive immune responses places these cells as attractive candidates for immunotherapy for infectious and immune-mediated diseases and cancer.

Chapter 1

Introduction

1.1 Introduction

1.2 The immune system

The role of the immune system is to provide protection against infectious agents. There are two main branches of the immune system: the innate and the adaptive immune systems.

1.2.1 Innate immunity

The innate immune system, which provides the first line of defense, is equipped with the ability to rapidly respond to invading pathogens, and is triggered when pathogen-associated molecular patterns (PAMP) are recognised through pattern recognition receptors (PRR), which can also recognise damaged or injured cells. The innate system is non-specific and short-lived and does not result in immunological memory. It also includes physical, chemical and microbiological barriers to prevent pathogen entry. There are many components that play roles including complement, clotting factors, antimicrobial protein and secreted molecules which induce highly regulated cascade pathways developed for pathogen elimination (Janeway, 2010).

The main cellular components of the innate system are monocytes, macrophages, granulocytes, natural killer (NK) cells and innate, unconventional T cells, which display immediate effector function upon pathogen detection. Phagocytes such as macrophages and neutrophils engulf and eliminate pathogens, but can also release cytokines, prostaglandins and other factors that drive innate immune responses. The functions of each of these cell types are described in detail in section 1.3.

1.2.2 Adaptive immunity

Adaptive or acquired immunity refers to pathogen-specific defense mechanisms, which can take several days to mount. These responses improve upon pathogen re-encounter due to immune memory, a feature unique to adaptive immunity. This form of immunity develops throughout life and is shaped by the host's exposure to pathogens. Adaptive immunity is mediated by T and B lymphocytes which bear unique receptors. Each lymphocyte bears a single type of receptor with unique antigen

specificity. This diversity is generated by random genetic rearrangements and recombinations that take place between gene segments that encode adaptive immune receptors. This process is exclusive to cells of the adaptive immune system and allows for generation of a diverse repertoire of antigen-specific receptors. B cells can further undergo somatic mutation to increase affinity for antigen binding. It is estimated that as many as 10^{11} specificities may be generated in this manner (Tonegawa, 1983).

Although the adaptive and innate immune systems comprise two distinct arms of the body's defense, interaction between them is key to successfully defending against harmful pathogens.

1.2.3 Chemical messengers of immunity

Cytokines are chemical mediators of immune signalling, secreted mainly by cells of the immune system, but can also be secreted from epithelial cells, endothelial cells and fibroblasts. They include interleukins, interferons, lymphokines, chemokines and tumour necrosis factors. Cytokines are involved in orchestrating complex immune process and play key roles in infection, inflammation and cancer. Cytokines can also be subdivided into pro-inflammatory or anti-inflammatory cytokines. IL-1, IFN-γ, IL-12, IL-18 and TNF-α are characterised as pro-inflammatory cytokines, whereas IL-4, IL-10 IFN-α and TGF-β are often recognised as anti-inflammatory cytokines and IL-6 exhibits features of both (Cavaillon, 2001). Some of the main cytokines and their functions in the immune system are summarised in table 1.

Chemokines are chemoattractant cytokines which can stimulate the activation and migration of cells to other sites. They also play roles in cell adhesion, proliferation, differentiation, apoptosis and malignant transformation of cells (Rossi and Zlotnik, 2000). Chemokines are subdivided into CXC or CC subgroups, and their receptors are designated CXCR or CCR, respectively.

Table 1.1. The effects and cellular source of some major cytokines discussed in this study

Cytokine	Main source	Acts on	Function
IFN-γ	CD4 and CD8 T cells; NK cells	macrophages NK cells	Immunity against viruses, bacteria, parasites and tumours
IL-1	Monocytes macrophages B cells, DC	T cells macrophages	Inflammation T cell co-stimulation chemoattractant
IL-2	CD4 T cells	T cells B cells	Anti-microbial infection Discrimination between foreign and self antigens; T cell development, survival & maintenance
IL-4	CD4 T cells Basophils Mast cells	T cells, B cells mast cells basophils	Humoral immunity Allergy Differentiation into T_H2
IL-6	Monocytes Fibroblasts Eosinophils T cells	T cells	Regulating immune responses IL-1 and TNFα inhibition and IL-10 activation
IL-10	CD4 T cells monocytes macrophages DC	Inhibition of T_H1 cells, monocytes, NK cells	Regulating immune responses Tolerance Antibody production
IL-12	DC macrophages monocytes neutrophils	NK cells T cells	Differentiation into T_H1 Angiogenic
IL-13	TH2 cells Mast cells NK cells DC	B cells monocytes	Antibody production Inhibits pro-inflammatory cytokines IL-1, TNF, IL-6
IL-17	T_H17 cells	neutrophils	Allergic responses Immune-mediated inflammatory diseases; Immunity against extracellular bacteria and fungi
IL-21	Activated CD4 T cells; NKT cells	NK cells T cells B cells	Immunity against virus-infected cells and tumour cells
TGF-β	T cells Monocytes	T cells	Immunosuppression

		B cells neutrophils	Differentiation into T_H17
TNF-α	Macrophages NK cells Neutrophils Monocytes CD4 T cells	DC hepatocytes	Inflammation DC activation Cytokine release

1.3 Cells of the immune system

The innate and adaptive immune responses rely on the actions of leukocytes. These originate in the bone marrow as haematopoietic stem cells, which give rise to myeloid and lymphoid progenitor cells. Cells of myeloid origin include macrophages, dendritic cells (DC) and granulocytes, while cells of lymphoid origins include T lymphocytes, B lymphocytes and NK cells (Janeway, 2001). A summary of these cells and their ontological lineages is illustrated below (Fig. 1.1).

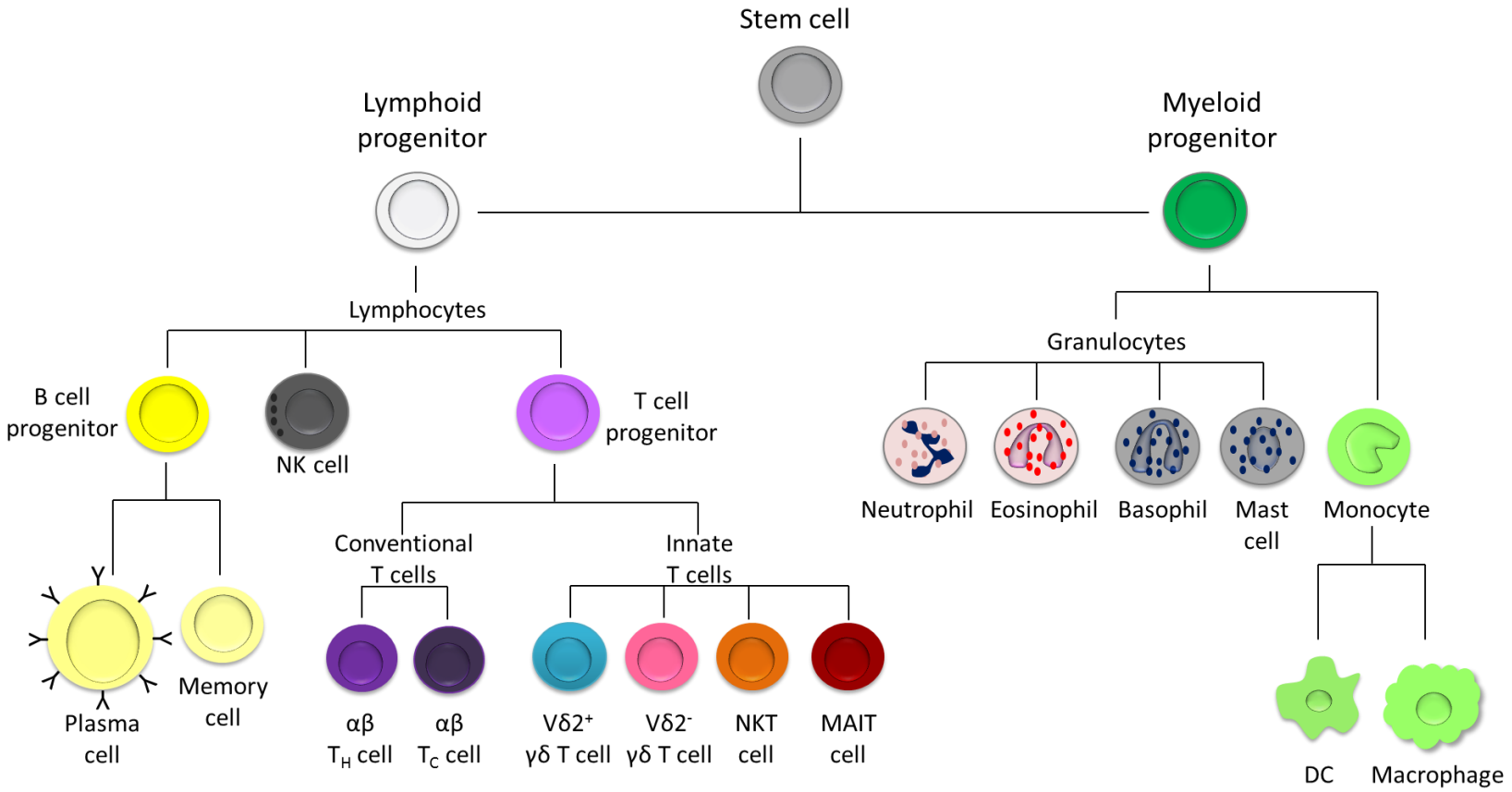

Figure 1.1. Cells of the immune system with lymphoid or myeloid origins. Lymphoid progenitor cells branch into B cell progenitor cells (which differentiate into plasma or memory cells), T cell progenitor cells and NK cells. T cell progenitor cells can develop into conventional T cells which include helper T (T_H) and cytotoxic T (T_C) cells, or innate T cells which include γδ T cells, NKT cells and MAIT cells. Myeloid progenitor cells include monocytes (which differentiate into dendritic cells (DC) or macrophages) and granulocytes such as neutrophils, eosinophils, basophilis and mast cells.

1.3.1 Myeloid cells

Myeloid cells originate in the bone marrow and they give rise to monocytes, granulocytes, erythrocytes and platelets.

Monocytes, which constitute 2-10% of human peripheral blood leukocytes, are normally found in the periphery, where they maintain numbers of resident macrophages, but during inflammation, they migrate to sites of infection where they enter the tissues and differentiate into macrophages or DC. They can directly provide defense against microbial pathogens by secreting antimicrobial factors. Other functions include phagocytosis, antigen presentation and cytokine production (Leon, et al., 2005, Ziegler-Heitbrock, 2014).

Macrophages, which are monocyte-derived tissue resident cells, play important roles in immune surveillance and defense. They express PRR such as toll-like receptors (TLR), receptor kinases, C-type lectin receptors and NOD-like receptors and are major sensors of pathogens in the tissues (Taylor, et al., 2005). Macrophages are highly specialised in removing dead cellular debris via phagocytosis. They are also capable of antigen presentation to T cells and thus are able to stimulate adaptive immune responses.

Granulocytes include neutrophils, eosinophils, basophils and mast cells. Neutrophils are the most abundant phagocyte and comprise 50-60% of circulating leukocytes and are involved in defense against microbes which they mediate via phagocytosis, neutrophil extracellular traps and secretion of granules. Eosinophils are involved in killing parasites via toxic granules. Basophils, which are one of the rarest subtypes in blood, are involved in immunity against parasites and allergens and thus their granules contain various substances including histamine, heparin and prostaglandins. Mast cells are also particularly rich in histamine and heparin, and thus play important roles in allergy and anaphylaxis, but unlike basophils, they are tissue resident.

1.3.1.1 Dendritic cells

DC are the most potent professional antigen presenting cells (APC), specialised for recognising foreign organisms through antigen uptake and processing which results in migration to lymphoid organs and presentation of antigens to T cells (Steinman, 1991). They express PRR such as TLR, NLR and RLR and are well equipped to internalise antigens by phagocytosis, pinocytosis and endocytosis (Doherty, 2015). DC can potently activate naïve T cells and promote expansion and effector T cell differentiation needed for protection, but also play key roles in self-tolerance (Tisch, 2010). The ability of monocytes to differentiate into DC was first described by Sallusto and Lanzavecchia and since then this method of generating myeloid DC has been widely implemented in studies on human DC (Leon, et al., 2005, Sallusto and Lanzavecchia, 1994). DC are distributed throughout tissues to maximise antigen capture. They can be found in two distinct functional states. They can be immature, whereby they induce tolerance to self, or mature DC, which confer protection against foreign antigens. Progression from immature DC (iDC) to mature DC upon activation induces changes in cytokine profiles, morphology, phenotype, cell surface markers and adhesion molecules (Banchereau, et al., 2000). DC maturation results in upregulation of major histocompatibility complex (MHC) and co-stimulatory molecules and an increase in IL-12 secretion, which is critical in development of helper T 1 (T_H1) responses. In addition, they can also secrete IL-18, IL-21 and IL-23 which promote differentiation of naïve T cells. Lipopolysaccharide (LPS) and proinflammatory cytokines are capable of activating DC, as are certain T cells. DC can also be classed as tolerogenic or immunogenic owing to the capacity of DC which are not fully mature to induce T cell tolerance *in vivo*. Antigens can be delivered to specific DC populations without the need for maturation stimuli (Steinman, et al., 2003b). In contrast, mature DC are thought to be immunogenic, a role which was first described in the context of transplantation and were termed “nature’s adjuvants”. This was demonstrated by exposing immature DC to antigens and injecting these into mice, which resulted in T cells restricted to the antigens the DC were exposed to (Steinman, 2007).

Plasmacytoid DC, which derive their name from their resemblance to plasma cells, appear to also be lymphoid-derived (Olweus, et al., 1997). They play a role in

mediating antiviral immunity by producing high amounts of IFN-α in response to viral infection. In contrast to myeloid DC, they respond to IL-13 rather than GM-CSF and although they are capable of endocytosis and antigen presentation they are weak at capturing soluble and particulate antigens (Steinman, 2003).

1.3.1.2 Antigen presentation

Antigen presentation is an essential step in initiating adaptive immune responses, and it involves MHC molecules class I (MHC-I) and class II (MHC-II), which are involved in binding to peptide fragments and displaying them on the cell surface for recognition by T cells. Presentation of foreign peptides to CD4 T cells via MHC-II mediates adaptive immune responses, while presentation of self peptides to CD8 T cells via MHC-I is involved in destruction of malignant or infected host cells.

APC, which include DC, B cells or macrophages present peptide fragments within MHC molecules to T cells. Recognition of a peptide fragment by the T cell receptor (TCR) constitutes the first signal in T cell activation. Recognition of costimulatory molecules such as CD80 and CD86 by the T cell, as well as interaction between CD40 and CD40L provides the second signal in T cell activation. Receipt of these two signals by $CD4^+$ T cells results in proliferation of naïve T cells which can differentiate into T_H cells or regulatory T (T_{reg}) cells. Cytokine secretion by the APC determines the fate of the naïve T cell which in turn determines the nature of the immune response. Depending on the cytokines secreted, naïve CD4 T cells can differentiate into T_H1, T_H2, T_H17, T_{reg} or follicular helper T cells (T_{FH}) cells (Fig. 1.2). In contrast, naïve CD8 T cells, which recognise intracellular peptides coupled to MHC-I molecules, differentiate into cytotoxic T cells (T_C).

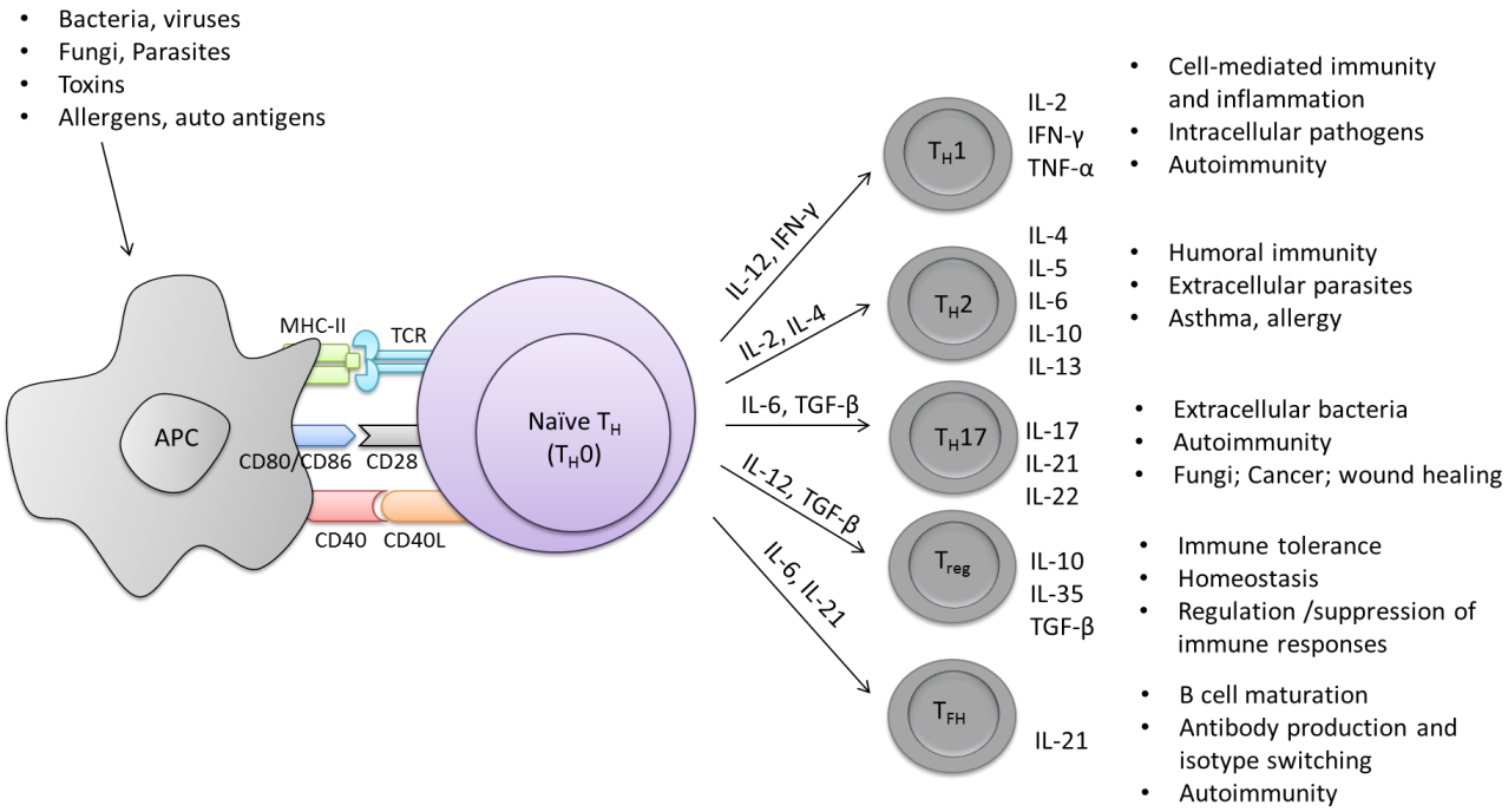

Figure. 1.2 Antigen presentation and the resulting T helper profiles. Antigen presentation is the first event in an adaptive immune response. Antigen presenting cells (APC) such as DC, B cells or macrophages present peptide fragments within an MHC molecule to the T cell. Recognition of this peptide fragment by the TCR constitutes the first signal in T cell activation. The second signal is provided by the recognition of costimulatory molecules such as CD80 and CD86 by the T cell, as well as interaction between CD40 and CD40L. Receipt of these two signals results in naïve T cell proliferation and differentiation. Cytokine secretion by the APC determines the fate of the naïve T cell which in turn determines the nature of the immune response. Depending on the cytokines secreted, naïve T cells can differentiate into T_H1, T_H2, T_H17, T_{reg} or T_{FH} cells. Other T_H cells include T_H3 and T_H9 cells.

1.3.2 Lymphoid cells

1.3.2.1 NK cells

NK cells are cytotoxic lymphocytes with critical roles in the innate immune system. They express CD56 and NKp46 on the cell surface and are $CD3^-$ (Walzer, et al., 2007). They also express activating receptors, NKG2C, NKG2D, natural cytotoxicity receptors (NCR) and CD16 and the inhibitory receptors killer-cell immunoglobulin-like receptors (KIR), which are found only in humans and leukocyte inhibitor receptors (LIR). NK cells can effectively kill tumour cells via perforin and granzyme secretion or receptor-mediated cytotoxicity (Kim, et al., 2000), and they can also secrete cytokines and chemokines (Poli, et al., 2009). They are a major source of IFN-γ (Yao, et al., 1999) but they can also secrete TNF-α and IL-10. NK activation results in activation of macrophages, neutrophils and DC, which subsequently induces antigen-specific T and B cell responses. Furthermore, NK cells are also implicated in virus-infected cell clearance (Guo, et al., 2011) and in antimicrobial immune responses.

The role of lymphocytes is to distinguish self from non-self antigens and promote inflammatory responses against foreign invaders. This is mediated by receptors of structural similarity, pertaining to two distinct classes of lymphocytes: the T cell receptor (TCR) found on T lymphocytes and the B cell receptor (BCR) on B lymphocytes. Receptor diversity is achieved through recombination of variable region genes (Appleman 2003).

1.3.2.2 B cells

B lymphocytes are an essential part of the humoral immune system. They are derived from the bone marrow where they differentiate from lymphoid progenitor cells. They then migrate to the spleen and secondary lymphoid tissues where they mature and differentiate. B cell activation is triggered by antigen binding to the BCR.

The principal and unique function of B cells is antibody production in response to antigens, but they can also secrete cytokines and act as APC, presenting antigen to T cells. Recognition of antigen by the B cell receptor results in antigen internalisation

and cell surface presentation on MHC molecules. These are presented to T cells, which in turn upregulate the co-stimulatory marker CD40L, which is required for B cell activation. CD40L then engages CD40 on the B cells, which results in differentiation of naïve B cells into short-lived plasmablasts, long-lived antibody-secreting plasma cells, germinal centre B cells or memory B cells (Lanzavecchia, et al., 2006, MacLennan, et al., 2003). Protective antibodies secreted by long-lived plasma cells provide a first line of defense against re-infection. If antibody levels are not sufficiently high, a second line of defense consisting of pathogen-experienced memory B cells is triggered to produce antibodies. Memory B cells have a wider repertoire of antigen specificity than long-lived plasma cells, thus playing a crucial role in host immunity upon infection with a novel pathogen (Kurosaki, et al., 2015).

T_{FH}, also known as follicular B helper T cells, are antigen-experienced CD4 T cells expressing the B cell follicle homing receptor CXCR5 and are found in B cell follicles or secondary lymphoid tissues. Through expression of CD40L and IL-4 and IL-21 secretion, they trigger formation and maintenance of germinal centres where they play crucial roles in mediating selection and survival of B cells (Glatman Zaretsky, et al., 2009, Seo, et al., 2009).

There are 5 different immunoglobulin isotypes secreted by B cells: IgM, IgA, IgD, IgG and IgE, and they can be distinguished by their C regions. They have the same antigen specificity, but activate different effector mechanisms. IgM antibody, which is expressed as a monomer on the B cell surface, or secreted as a pentamer, is involved in early stages of humoral immunity. IgA antibodies, which are found as dimers, are found mainly at mucosal sites and are involved in preventing colonisation by pathogens (Underdown and Schiff, 1986). The monomeric IgD plays a role as an antigen receptor on B cells that have not been exposed to antigens, and can activate basophils, mast cells and secrete antimicrobial factors (Chen, et al., 2009, Geisberger, et al., 2006). IgE, which is also a monomer, is involved in allergic responses as it triggers histamine release by mast cells and basophils upon allergen encounter. Furthermore, it plays a protective role against parasitic worms. IgG, which is subcategorised into 4 different monomers, provides the main role in antibody-based immunity against invading pathogens, and can even cross the placenta to provide

passive immunity to the growing foetus. Immature B cells express only IgM prior to antigen exposure. Mature (naïve) B cells express both IgM and IgD, thus allowing them to respond to antigens (Goding, 1978). B cell activation results in differentiation into plasma cells thus facilitating antibody secretion. Furthermore, a B cell can undergo isotype switching, thus altering the class of antibody it produces over the course of an immune response and thus can secrete IgE, IgA or IgG (Janeway, 2010).

1.3.3 T cells

T lymphocytes, referred to as αβ T cells, as they express a TCR composed of α and β chains, are central orchestrators of immune responses, and play a vital role in the adaptive immune system. They comprise the majority of peripheral blood T lymphocytes. T cell development begins in the thymus where bone marrow-derived progenitor cells undergo stringent positive and negative selection resulting in naïve T cell populations exhibiting unique TCR. This diverse antigen specificity allows T cells to recognise a broad array of complex protein antigens presented on the surface of APC by MHC molecules (den Haan, et al., 2014).

CD4 T cells, also known as T_H cells release cytokines and make contact-dependent interactions with other cells, thereby stimulating and regulating adaptive immune responses. They are capable of promoting B cell maturation, activation of macrophages and cytotoxic T cells, to name but a few. CD4 T cells are activated when presented with peptide antigens by APC via MHC-II molecules. Once activated, they proliferate and differentiate into effector T cells that can secrete T_H1, T_H2, T_H17, T_{reg} or T_{FH} cytokines. These are summarised in Fig.1.2.

CD8 T cells, also known as T_C, are involved in killing tumour and virus-infected cells. CD8 T cells recognise antigens presented within MHC-I molecules and are tightly regulated by T_{reg} cells to prevent autoimmunity. T_{reg}, which represent up to 10% of $CD4^+$ T cells are known to mediate antigen-specific suppression and prevention of autoreactive T cells, which they mediate through direct contact, as suggested by *in vitro* studies, and through secretion of regulatory cytokines IL-10 and TGF-β (Longhi, et al., 2006, Mills, 2004).

Memory T cells, which usually express CD45RO, can persist long after an infection has resolved. They quickly expand into effector T cells upon re-exposure to their cognate antigen, and thus providing immune "memory" against previously encountered pathogens. Memory T cells can be further subdivided depending on homing potential and effector functions into central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory (T_{EMRA}) T cells. In contrast to naïve T cells, antigen-specific memory T cells do not require TCR signals, but require IL-7 and IL-15 for their long-term survival (den Haan, et al., 2014).

Regulatory T cells can undergo T cell anergy, which is a tolerance mechanism that can occur whereby the T cell is functionally inactivated following antigen encounter but is maintained for an extended period of time in an "unresponsive state". This serves to preserve immune responses from premature inactivation by T_{reg}. Clonal anergy describes unresponsiveness at the cellular level, where T cells do not proliferate or secrete IL-2 upon antigen stimulation. Exogenous IL-2 can however reverse anergy by stimulating the IL-2 receptor on these anergic T cells (Appleman and Boussiotis, 2003, Schwartz, 2003).

A small proportion of αβ T cells express TCR that have low variation. These semi-invariant populations include invariant Natural Killer T cells (iNKT) and Mucosal Associated Invariant T (MAIT) cells (Adams, et al., 2015) and these will be described in further detail below.

1.3.4 NKT cells

NKT cells comprise T cells with properties of both NK cells (including NKG2C and NKG2D) and T lymphocytes (Bendelac, et al., 2007, Brigl and Brenner, 2004). They are categorised into type I, also known as invariant NKT (iNKT), or type II NKT cells.

Type I NKT cells are characterised by expression of a semi-invariant TCR consisting of Vα24Jα18 paired with Vβ11 (Bendelac, et al., 2007) and a similar subset is found in mice. They are strongly reactive to marine sponge-derived glycolipid alpha galactosylceramide (αGalCer) and can also recognise bacterial-derived lipids and self-lipids such as β-D-glucopyranosylceramide (Brennan et al., 2011) and

isoglobotrihexosyl ceramide (Arrenberg, et al., 2010). They express high levels of the activation markers CD69, CD44, CD122 and low expression of CD62L, a marker expressed by LN-homing naïve T cells (Matsuda, et al., 2000, Bendelac, et al., 1992). Type I NKT cells play important roles in antimicrobial and local and systemic immune responses and in controlling tumour development. However, they can also have negative effects in the pathogenesis of autoimmune and allergic disorders (Macho-Fernandez and Brigl, 2015). They can generate large amounts of IFN-γ, IL-2, IL-4, IL-9, IL-10, IL-13, IL-17, IL-21 and GM-CSF (Coquet, et al., 2008, Gumperz, et al., 2002, Stetson, et al., 2003) and cytotoxic molecules such as perforin and granzyme (Gumperz, et al., 2002, Lee, et al., 2002) and can interact with various other immune cells (Brigl and Brenner, 2004, Brennan, et al., 2013). Greater proportions of $CD4^+$ type I NKT cells produce T_H2 cytokines, while all NKT cells can produce both T_H1- and T_H2-type cytokines (O'Reilly, et al., 2011).

Human type II NKT cells, also termed non-invariant NKT cells (Godfrey, et al., 2000), express a diverse range of TCR (Behar, et al., 1999) and are CD1d-restricted cells but do not recognise αGalCer and do not express the invariant Vα14-Jα18 TCR. Although they have distinct antigen specificities, they share many features with type I NKT cells such as rapid secretion of IFN-γ, IL-2, IL-4, IL-10, IL-17, GM-CSF and perforin (Weng, et al., 2014, Zhao, et al., 2014) and high autoreactivity (Gumperz, et al., 2000). Some type II NKT cells have been shown to recognise the self-glycolipid sulfatide and upon sulfatide stimulation they result in inhibition of iNKT cell function, while mediating protection against liver disease and type I diabetes in mouse models (Arrenberg, et al., 2011, Halder, et al., 2007, Subramanian, et al., 2012).

Cytokine production by NKT cells can result in activation of T cells, NK cells (Lin, et al., 2006, Carnaud, et al., 1999) and macrophages (Zeng, et al., 2013) but can also suppress functions of neutrophils (De Santo, et al., 2008, Hwang, et al., 2006).

1.3.5 MAIT cells

Mucosal associated invariant T (MAIT) cells express a semi-invariant TCR composed of an α chain (Vα7.2/Jα33) paired with an oligoclonal β chain (Porcelli, et al., 1993, Tilloy, et al., 1999) and they recognise antigen complexes via the MHC-I-like molecule MR1.

The TCR of MAIT cells recognises riboflavin and folic acid metabolites bound to MR1 in a conserved docking mode, thus acting like a PRR (Birkinshaw, et al., 2014, Lepore, et al., 2014, Treiner, et al., 2003). These cells are abundant in humans and are predominantly found in the gut *lamina propria* (Kjer-Nielsen, et al., 2012). MAIT cells are activated by bacteria and are capable of producing IL-2, IFN-γ and IL-17 and granzymes (Dusseaux, et al., 2011, Guo, et al., 2015). MAIT cells have been shown to be involved in defense against various pathogens such as *Escherichia coli*, *enterobacteria*, *staphylococci* and *mycobacterium*, and yeasts that contain the riboflavin synthetic pathway, but not viruses (Gold, et al., 2013, Le Bourhis, et al., 2010). This indicates that they recognise an antigen that is conserved among a range of intracellular microbes.

1.4 γδ T cells

In contrast to αβ T cells, γδ T cells, which account for 0.5-5% of total blood T cells and higher proportions in tissues (Morita, et al., 2000), recognise unconventional antigens such as phosphorylated microbial metabolites and lipid antigens in an MHC-unrestricted manner. γδ T cells are rarely found in the spleen, lymph nodes, the thymus or Peyer's patches but they are localised in tissues (Hein and Mackay, 1991, Bucy, et al., 1988) such as the gut and liver (Rajoriya, et al., 2014, Brandes, et al., 2003, Li, et al., 1996, McCarthy, et al., 2013). In the intestine, γδ T cells are found as intraepithelial lymphocytes (IEL) (Hein and Mackay, 1991, Bucy, et al., 1988, Goodman and Lefrancois, 1988, Deusch, et al., 1991). There are 5 times as many αβ as γδ T cells among intestinal IEL, while in blood αβ T cells outnumber γδ T cells 50 to 1 (Hayday, 2000).

1.4.1 The human γδ T cell receptor

The αβ TCR has a diverse repertoire due to the presence of over 50 Vα and Vβ gene segments available for TCR gene rearrangement (Kabelitz and He, 2012) and although γδ T cells have a rather small repertoire of Vγ and Vδ segments to select from, their diversity is just as large as that of the αβ TCR. This is owing to non-germline encoded mechanisms such as insertion of N-nucleotides during gene rearrangement which contribute to its diversity (Kabelitz and He, 2012). Additionally, it was discovered that

the δ chain rearrangement allows for the incorporation of multiple Dδ segments, thus further increasing diversity (Elliott, et al., 1988, Hata, et al., 1988).

In γδ TCR rearrangement, the three most frequently used δ chains are Vδ1, Vδ2 and Vδ3, while the less common δ segments include Vδ4, Vδ5, Vδ5 and Vδ7, named according to their locations on the δ locus (Thedrez, et al., 2007). The δ chains can pair with one of seven functional Vγ gene segments, namely Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, Vγ9 and Vγ11 (Adams, et al., 2015, Porcelli, et al., 1991, Hinz, et al., 1997).

It is hypothesised that γδ T cells have developed to respond to unique stress antigens that are markers of cell infection or transformation, rather than directly recognising a diversity of microbial antigens and thus serving as a "first line of defense" (Hayday, 2000, Janeway, et al., 1988).

1.4.2 Human γδ T cells subsets

Human γδ T cells fall into one of two categories: Vγ9Vδ2 T cells or non-Vδ2 T cells with the former being the predominant subset in blood, while the latter is more common in tissues. The two groups show distinct functions, migratory patterns and homing capabilities (Zheng, et al., 2013).

1.4.2.1 Non-Vδ2 T cells

Non-Vδ2 T cells, which are functionally distinct from Vγ9Vδ2 T cells, include Vδ1, Vδ3 and Vδ5 T cells and these are found to be expanded in cytomegalovirus infection in kidney transplant patients and are thought to play protective roles against tumours and infection (Halary, et al., 2005).

$V\delta1^+$ γδ T cells are mainly tissue resident and are the most frequent subset among human intraepithelial cells in the skin (Ebert, et al., 2006) and small intestine (Holtmeier, et al., 2001, Hayday, et al., 2001) and they represent over 50% of foetal blood γδ T cells at birth (Dimova, et al., 2015). They can also be found in lymph nodes (Brandes, et al., 2003) and certain tumours (Maeurer, et al., 1996). Vδ1 T cells have been reported to recognise stress-inducible MHC class I-related molecules MICA and MICB (Groh, et al., 1998) and lipid antigens presented by CD1 molecules (Russano, et

al., 2007, Bai, et al., 2012, Hayday and Vantourout, 2013, Luoma, et al., 2013) and UL-16 binding proteins (ULBP) which bind to cytomegalovirus-infected cells (Cosman, et al., 2001). They have also been reported to respond to epithelial tumours (Maeurer, et al., 1996, Groh, et al., 1998, Coscas, et al., 2004) and lymphomas (Catellani, et al., 2007, Hacker, et al., 1992) and can recognise different members of the MHC superfamily (Vantourout and Hayday, 2013). Vδ1 T cells have also been reported to be expanded in HIV patients (Poles, et al., 2003, Rossol, et al., 1998, Wesch and Kabelitz, 2003), cytomegalovirus (Dechanet, et al., 1999) and *Candida* (Fenoglio, et al., 2009, Maher, et al., 2015) infection and B cell chronic lymphocytic leukaemia (Siegers and Lamb, 2014) and have been shown to produce IFN-y and IL-17.

Vδ3 T cells represent the third most common subset in peripheral blood, and little is known about this subset. Its ligand specificities are unknown, but they are reported to be expanded in renal and stem cell transplant patients and patients with leukaemia and chronic viral infection (Halary, et al., 2005, Dechanet, et al., 1999, Knight, et al., 2010, Couzi, et al., 2010) and have been shown in one study to recognise $CD1d^{+}$ cells (Mangan, et al., 2013).

1.4.2.2 Vγ9Vδ2 T cells

γδ T cells consisting of Vγ9 paired with Vδ2 account for 50-95% of γδ T cells in healthy individuals (Porcelli, et al., 1991, Hinz, et al., 1997, Kabelitz, et al., 1999) and are unique to humans and primates. These are found in very small numbers in newborns, and exposure to environmental microorganisms stimulates their expansion (Parker, et al., 1990). Furthermore, they change with age and differ between genders (Caccamo, et al., 2006b). Vγ9Vδ2 recognise organic-based pyrophosphate molecules termed phosphoantigens (pAg) which are intermediates of isoprenoid metabolism (Fig. 1.3), a pathway essential for cell survival (Morita, et al., 2000, Constant, et al., 1994, Hintz, et al., 2001, Puan, et al., 2007, Tanaka, et al., 1995). There are two distinct pathways of isoprenoid biosynthesis, with the methylerythritol 4 phosphate (MEP) pathway being used by most bacteria and protozoa (Chen and Letvin, 2003) but not eukaryotes. In contrast, all eukaryotic and some prokaryotic cells use the mevalonate pathway. Both pathways result in isopentenyl pyrophosphate (IPP) required as a precursor for many

cellular components. A table of pathways employed by common pathogens is illustrated below (Table 2).

The most potent pAg, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), which is an intermediate of the non-mevalonate MEP pathway (Fig. 1.3, Fig. 1.4) specifically activates Vγ9Vδ2 T cells at pico- to nanomolar concentrations (Altincicek, et al., 2001) and thus allow Vγ9Vδ2 T cells to recognise foreign pathogens that use this pathway. The mevalonate pathway does not produce HMB-PP but results in production of IPP, which requires up to 10,000-fold higher concentrations for activating Vγ9Vδ2 T cells (Fig. 1.4) than intermediates of the microbial non-mevalonate pathway (Puan, et al., 2007). However, the IPP levels normally found in healthy cells are not sufficient to trigger Vγ9Vδ2 T cell activation, but cell stress and other triggers can result in dysregulated metabolism which could be indicative of infection or cancerous cells, overproduce the metabolite IPP, and therefore allow Vγ9Vδ2 T cells to recognise IPP as a self-antigen in conditions of disease.

Aminobisphosphonates, which are compounds in clinical use for the treatment of osteoporosis and bone metastasis can also activate Vδ2 T cells *in vivo* and *in vitro*. They have been shown to result in accumulation of IPP through inhibition of the IPP-processing enzyme farnesyl pyrophosphate synthase (Fig. 1.3) (Gober, et al., 2003, Kunzmann, et al., 1999, Thompson, et al., 2006). Aminobisphosphonates have also been shown to activate Vγ9Vδ2 T cells *in vitro* (Das, et al., 2001, Kabelitz, et al., 2004).

As Vγ9Vδ2 T cells frequencies are found to be decreased in many diseases, therapies involving *in vivo* expansion of Vγ9Vδ2 T cell using aminobisphosphonates are currently in place to increase the numbers of circulating Vγ9Vδ2 T cells. However, Wang and colleagues (Wang, et al., 2011) have discovered toxicity when various aminobisphosphonates were used continuously to expand Vγ9Vδ2 T cells from peripheral blood mononuclear cells (PBMC), thus suggesting that prolonged exposure to aminobisphosphonates inhibits their proliferation due to inhibition of the isoprenoid pathway. To overcome this, they suggest pulsing them to limit toxicity when expanding *ex vivo*. Pulsing allows monocytes to take up the

aminobisphosphonates through fluid phase endocytosis, which simulates the rapid clearance through renal excretion *in vivo* (Wang, et al., 2011).

A class of mycobacterial nonphosphorylated alkyl amines are also known to stimulate Vγ9Vδ2 T cells, but require much higher (milimolar) concentrations which are 10^6 -10^8 fold higher than those of HMB-PP (Bukowski, et al., 1999). These include isobutylamine and isoamylamine, which when used to stimulate PBMC, in the presence of IL-2 were able to induce more than 10-fold expansion of Vγ9Vδ2 T cells (Hayday, et al., 2001).

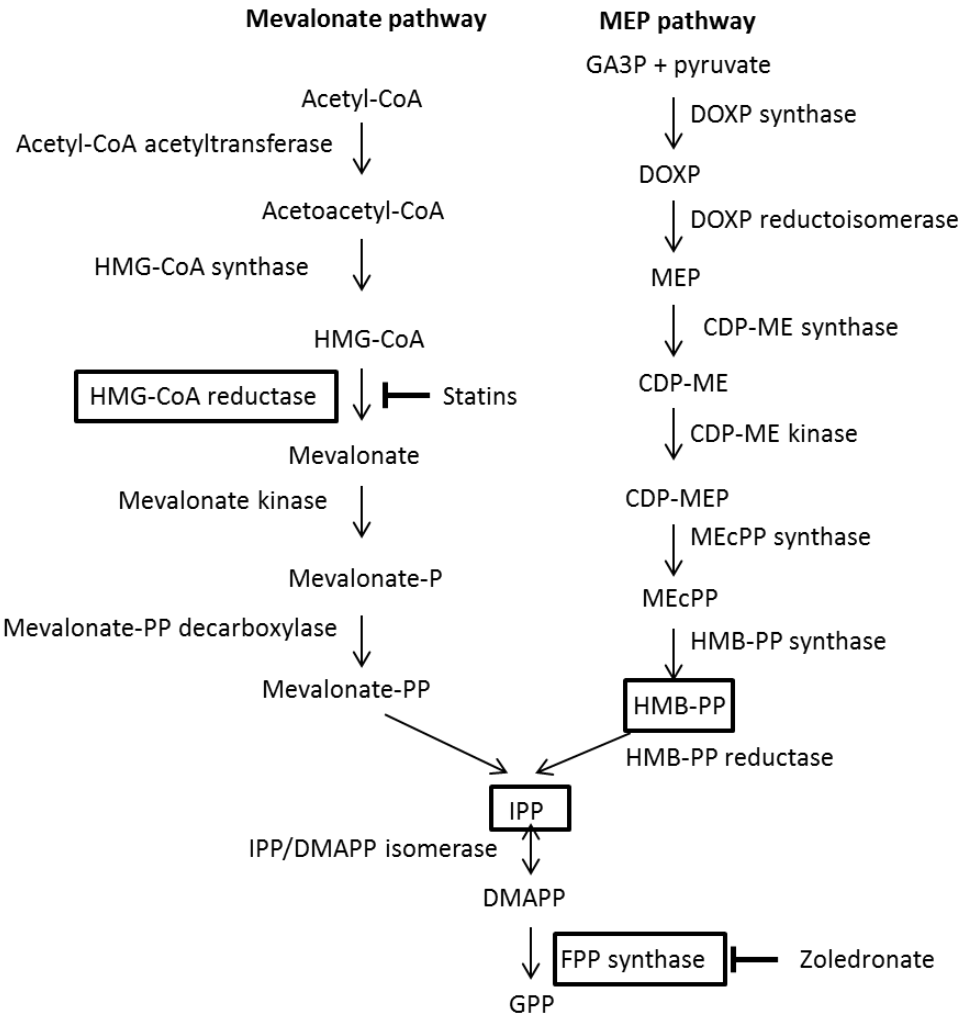

Figure 1.3 Representation of the MEP and mevalonate pathways for isopentenyl pyrophophosphate (IPP) biosynthesis. Statins inhibit HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway. HMB-PP is produced through the MEP pathway, and both pathways result in IPP synthesis. Aminobisphosphonates such as zoledronate block the enzyme responsible for breaking down IPP, thus resulting in IPP accumulation. Adapted from Heuston et al (2012).

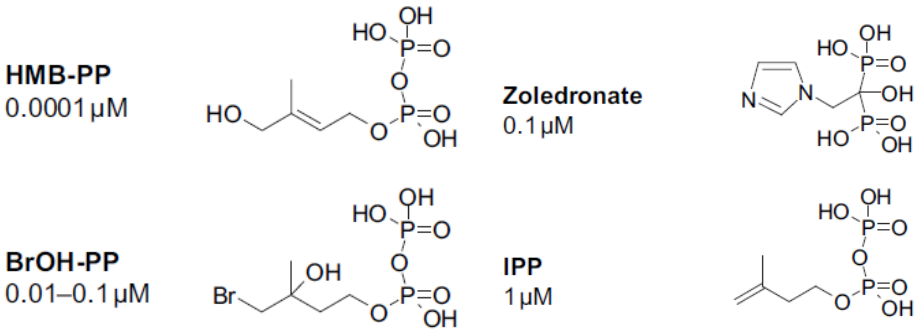

Figure 1.4 Molecular structures of non-peptide compounds and their in vitro bioactivities on Vγ9Vδ2 T cells. HMB-PP, (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate. BrOH-PP, bromohydrin pyrophosphate. IPP, isopentenyl pyrophosphate. Zoledronate, also known as Zometa or zoledronic acid. Adapted from Moser and Eberl (2007).

Table 1.2. Distribution of the MEP and mevalonate pathways amongst Gram-positive and Gram-negative pathogens (adapted from Heuston et al., 2012).

Gram + pathogen	MEP	Mevalo-nate	Gram - pathogen	MEP	Mevalo-nate
Bacillus anthracis	+	-	*B. abortus*	+	-
Bacillus subtilis	+	-	*Borrelia burgdorferi*	-	+
Clostridium difficile	+	-	*Chlamydia trachomatis*	+	-
Clostridium botulinum	+	-	*Chlamydia pneumonia*	+	-
Clostridium perfringens	+	-	*S. enterica*	+	-
Entercoccus faecalis	-	+	*Escherichia coli*	+	-
L. monocytogenes	+	+	*F. tularensis*	+	-
L. innocua	-	+	*Legionella pneumophila*	-	+
Listeria seeligeri	-	+	*P. aeruginosa*	+	-
Nocardia terpenica	+	-	*V. cholera*	+	-
Stahylococcus aureus	-	+	*K. pneumonia*	+	-
Streptomyces pyogenes	-	+	*Bordetella pertussis*	+	-
S. pneumonia	-	+	*Haemophilus influenza*	+	-
			Helicobacter pylori	+	-
			Shigella dysenteriae	+	-
			Neisseria gonorrhoeae	+	-
			Neisseria meningitides	+	-
			C. jejuni	+	-
			Y. enterocolitica	+	-

1.4.3 Vγ9Vδ2 T cell activation

The recent discovery of butyrophilin 3A1 (BTN3A1) and its role in Vγ9Vδ2 T cell activation has provided a major breakthrough towards understanding the process of pAg-induced Vγ9Vδ2 T cell activation (Adams, et al., 2015). BTN3A1 belongs to the butyrophilin family, also known as CD277, which encompasses proteins with diverse roles in host homeostasis (Abeler-Dorner, et al., 2012, Arnett and Viney, 2014). The three members found in humans are BTN3A1, BTN3A2 and BTN3A3 (Rhodes, et al., 2001) which are structurally homologous to the B7 superfamily of proteins. BTN3A1 and BTN3A3 also possess an intracellular "B30.2" domain which is thought to be involved in pAg-mediated activation of Vγ9Vδ2 T cells (Adams, et al., 2015). The precise mechanism by which BTN3A1 contributes to Vγ9Vδ2 T cell activation has been controversial. One model proposes that the BTN3A molecule acts as an antigen-presenting molecule which captures and presents pAg on the cell surface to Vγ9Vδ2 T cells which recognise it directly through their TCR (Vavassori, et al., 2013). However, while one study demonstrated the requirement of all three isoforms (Rhodes, et al., 2015), a different study suggested that only BTN3A1 is capable of mediating pAg-induced activation of Vγ9Vδ2 T cells and that this activity was pinpointed to the B30.2 domain of BTN3A1 (Harly, et al., 2012). In support of this, direct binding between HMB-PP and the B30.2 domain was shown via nuclear magnetic resonance studies (Hsiao, et al., 2014) and affinity studies (Altincicek, et al., 2001, Sandstrom, et al., 2014). Furthermore, it was revealed that the difference between BTN3A1 and BTN3A3 was confined to a single amino acid difference within the binding pocket of B30.2 at position 351 where BTN3A1 contained a histidine, while BTN3A3, an arginine. Swapping of this histidine residue onto BTN3A3 provided BTN3A3 with the ability to bind pAg and mediate Vγ9Vδ2 T cell activation, while introduction of arginine onto BTN3A1 abrogated its activity (Sandstrom, et al., 2014), thus confirming that the histidine is important in Vγ9Vδ2 T cell activation.

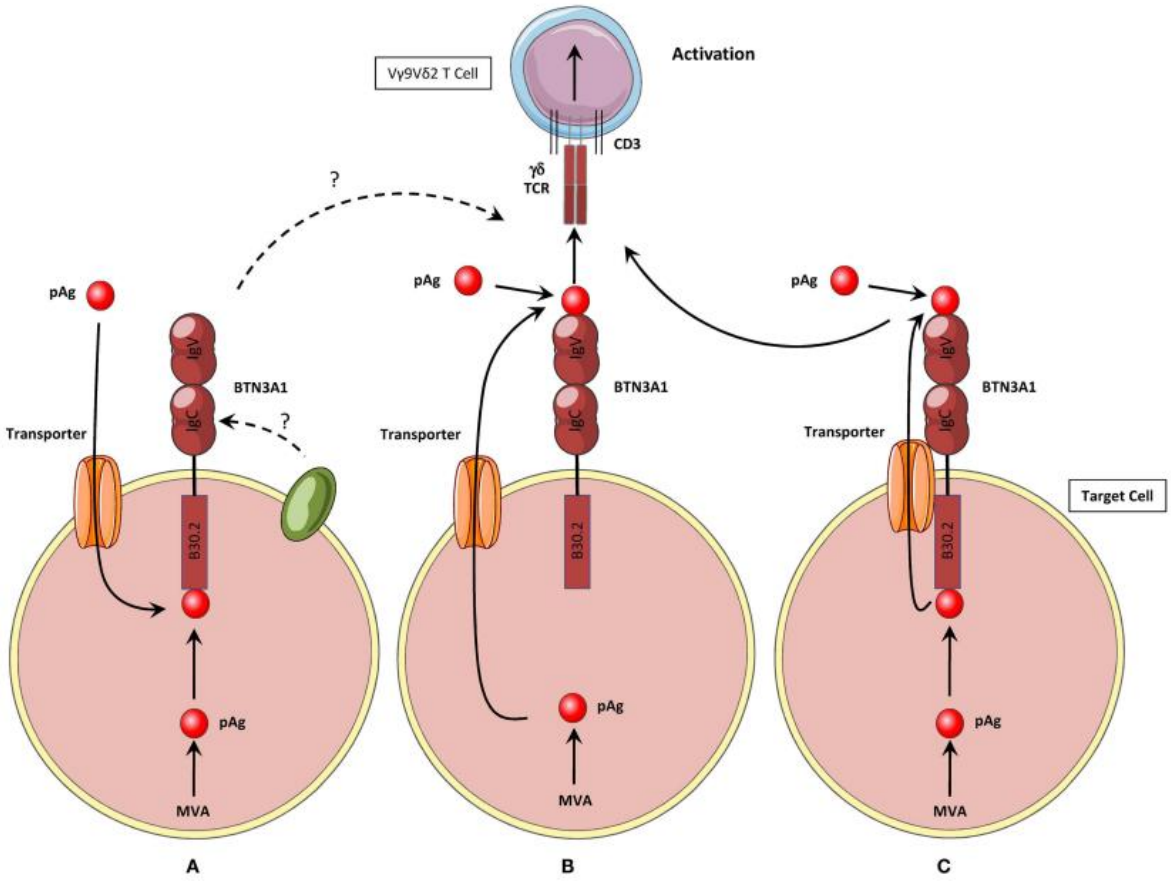

Figure 1.5. Three hypothetical activation mechanisms for activation of Vγ9Vδ2 T cells by phosphoantigens A, pAg are produced intracellularly or internalised by a transporter; they interact with the intracellular (ic) B30.2 domain of BTN3A1 which induces conformational changes and recruitment/exclusion of molecular partners. These changes are sensed by Vγ9Vδ2 T cells, resulting in activation. **B**, ic pAg are exported from the cell via a transporter and the extracellular (ec) and ic pAg interacts with the ec part of CD277/BTN3A1. pAg presented by the ec IgV domain, as antigenic complexes to the Vγ9Vδ2 TCR, which results in activation. **C**, ic pAg interacts with ic B30.2 domain of BTN3A1; they are then exported from the cell by BTN3A1 or a membrane transporter associated with BTN3A1. Both ec or ic pAg can then interact with the ec IgV domain of BTN3A1 and trigger Vγ9Vδ2 T cell activation. Taken from Harly et al (2014).

A model has been proposed whereby binding of the pAg intracellularly is transitioned to the extracellular side of the membrane. The pAg binding to B30.2 would induce a conformational change which could modulate the structure of the extracellular domains of BTN3A. It is not known however whether Vγ9Vδ2 T cells can directly recognise BTN3A (Adams, et al., 2015, Sandstrom, et al., 2014, Wang, et al., 2013). The potential mechanisms of activation are illustrated in Fig. 1.5 (Harly, et al., 2014).

1.4.4 Phenotypes of γδ T cells

1.4.4.1 CD4 and CD8 expression

Most peripheral γδ T cells lack CD4 and CD8 expression, which is not surprising since CD4 and CD8 are components of MHC recognition, and as mentioned above, γδ T cells do not rely on MHC molecules (Hayday, 2000, Kabelitz, et al., 2000).

1.4.4.2 Differentiation status

γδ T cells express molecules associated with different stages of differentiation which can be classified through CD27 and CD45RA expression, as shown by Dieli et al (2003), while αβ T cells classification also requires CCR7 expression (Okada, et al., 2008). Naïve cells are defined as $CD45RA^{+}CD27^{+}$, T_{CM} are $CD45RA^{-}CD27^{+}$, while T_{EMRA} are $CD45RA^{+}CD27^{-}$ and T_{EM} are negative for both markers ($CD45RA^{-}CD27^{-}$). These are summarised in Fig. 1.6.

1.4.4.3 Chemokine receptors

Vγ9Vδ2 T cells have distinct migration properties compared to αβ T cells (Brandes, et al., 2003). Since Vγ9Vδ2 T cells recognise antigens independently of MHC molecules, therefore they do not migrate to secondary lymphoid tissues where antigen presentation by professional APC takes place (Moser and Brandes, 2006). In line with this, most Vγ9Vδ2 T cells (>80%) lack expression of the lymph node (LN)-homing receptor CCR7, whereas most αβ T cells express the receptor. Instead, Vγ9Vδ2 T cells express receptors for a range of inflammatory chemokines (Chen and Letvin, 2003). Some γδ T cells express chemokine receptor CCR5, which binds inflammatory chemokines RANTES and MIP-1β. γδ T cells also express the skin homing chemokine

receptor CCR6 (Dieli, et al., 2003, Glatzel, et al., 2002, Laggner, et al., 2011). However, Moser & Eberl showed that TCR activation resulted in a switch in Vγ9Vδ2 T cells from inflammatory to LN-homing, as evidenced by downregulation of CCR5 and induction of CCR7 (Brandes, et al., 2003, Moser and Eberl, 2007). Furthermore, there was a *de novo* responsiveness to CCL19 and CCL21, thus providing the ability to migrate to where initiation of adaptive immunity takes place (Moser and Eberl, 2007). CCR7 expression, however, was transient and returned to baseline after prolonged culture (O'Sullivan and Thomas, 2003). A subset of Vγ9Vδ2 T cells were also shown to express CXCR5, which release IL-4 and IL-10 which provide help to B cells (Caccamo, et al., 2006). In contrast to αβ T cells, it was found that Vγ9Vδ2 T cells lack receptors for chemokines responsible in homing to peripheral tissues (Moser and Brandes, 2006). Thus, Vγ9Vδ2 T cells can rapidly respond to tissue infection and inflammation (Moser and Eberl, 2007) and resemble effector memory αβ T cells, which like γδ T cells, lack CCR7 and respond to inflammatory chemokines (Moser and Eberl, 2007, Sallusto and Mackay, 2004).

1.4.4.4 Other stimulating markers

Vγ9Vδ2 T cells can express TLR such as TLR2, which is readily directed on the cell surface, and TL3 which is found intracellularly. Activation of these provides potent co-stimulation to activated Vγ9Vδ2 T cells (Pietschmann, et al., 2009, Wesch, et al., 2006, Wesch, et al., 2011).

Vγ9Vδ2 T cells also frequently express NKG2D, an NK-type receptor which delivers an activating signal following specific binding of corresponding ligands such as MICA and MICB or ULBP (Gonzalez, et al., 2006, Biassoni, et al., 2001) and is also expressed by cytolytic CD8 αβ T cells and NK cells (Bauer, et al., 1999). NKG2D can serve as a co-stimulatory signal (Nedellec, et al., 2010). NKG2A, a HLA-E-specific inhibitory receptor, is present on most γδ T cells (Angelini, et al., 2011, Halary, et al., 1997), while NKG2C is present in only some individuals (Angelini, et al., 2011). NKG2A and NKG2C both dimerise with CD94, which recognises HLA-E.

Another marker frequently expressed by γδ T cells is CD56, also known as neural cell adhesion molecule and is normally found on NK and NKT cells (Cremer, et al., 1994,

Lanier, et al., 1991), has also been found to be upregulated by γδ T cells upon pAg stimulation. Zheng et al found that $CD56^+$ Vγ9Vδ2 T cells have higher cytolytic effects against cells infected by influenza virus than $CD56^-$ Vγ9Vδ2 T cells. Thus, CD56 expression may identify cells that mediate protection against infectious agents (Zheng, et al., 2013).

T_{EMRA} γδ T cells can express CD16, a receptor for the Fc portion of IgG, which mediates ADCC activity in the presence of tumour-targeting mAb (Tokuyama, et al., 2008).

γδ T cells, like αβ T cells, can express CD27, which upon binding to its ligand CD70, provides survival and proliferative signals (DeBarros, et al., 2011). The inducible co-stimulatory receptor (ICOS) and programmed cell death 1 (PD-1) are absent in Vγ9Vδ2 T cells but can be induced upon activation (Caccamo, et al., 2006, Ribot, et al., 2011, Iwasaki, et al., 2011).

1.4.5 Cytokine and chemokine secretion by γδ T cells

γδ T cells express receptors for inflammatory cytokines produced by granulocytes, monocytes, iDC and NK cells. Within hours of stimulation, Vγ9Vδ2 T cells produce large amounts of inflammatory cytokines IFN-γ and TNF-α (Garcia, et al., 1997, Wang, et al., 2001) but can also be induced to produce T_H2 cytokines such as IL-4 under specific conditions (Spinozzi, et al., 1995, Wesch, et al., 2001). A combination of IL-1β, IL-6, TGF-β and IL-23 has been shown to result in IL-17 production by Vγ9Vδ2 T cells *in vitro* (Ness-Schwickerath, et al., 2010, Caccamo, et al., 2011). In addition, IL-23 together with γδ T cell stimulation can also induce IL-17 production by Vγ9Vδ2 T cells in newborns (Moens, et al., 2011).

Thus, γδ T cells are predominantly T_H1 producers, which is in line with the fact that CD4 expression strongly correlates with T_H2 cytokines, and Vγ9Vδ2 T cells rarely express CD4 (Fowell, et al., 1997, Wen, et al., 1998).

Vγ9Vδ2 T cells have been shown to secrete chemokines CCL2, CCL3, CCL4 (Hoq, et al., 1997) and MIP-1α and RANTES (Glatzel, et al., 2002).

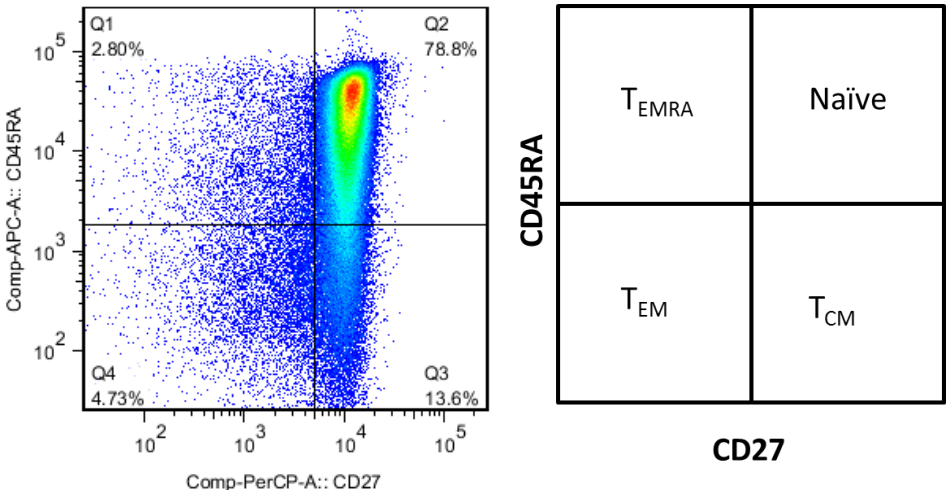

Figure 1.6 Stages of differentiation of γδ T cells according to CD45RA and CD27 expression. Left panel, flow cytometric dot plot of CD45RA vs CD27 in CD3 T cells. Right panel, gating strategy for differentiation status of γδ T cells. $CD45RA^{+}CD27^{+}$ cells are naïve, $CD45RA^{+}CD27^{-}$ are T_{EMRA}, $CD45RA^{-}CD27^{+}$ are T_{CM}, while $CD45RA^{-}CD27^{-}$ are T_{EM}.

1.4.6 γδ T cells in disease

1.4.6.1 Cytotoxicity by γδ T cells

Upon activation, γδ T cells can exert cytotoxicity against viral- and bacterial-infected cells (Oliaro, et al., 2005, Martino, et al., 2007, Qin, et al., 2009) and tumour cells (Alexander, et al., 2008, D'Asaro, et al., 2010) via Fas/FasL- and granulysin-dependent activity (Oliaro, et al., 2005, Koizumi, et al., 1991, Dieli, et al., 2001). They can eliminate Fas^+ and $TRAILR^+$ tumours by upregulating expression of FasL and TRAIL. They also mediate cytotoxicity via perforin and granzyme secretion and NK-mediated cytotoxicity via the CD137 pathway (Qin, et al., 2009, de Koning, et al., 2010). Furthermore they can also recognise stress-induced MICA, MICB and UBLP1 via NKG2D. CD16 expression on Vγ9Vδ2 T cells allowed them to kill opsonised cells and microorganisms via ADCC (Braakman, et al., 1992, Braza and Klein, 2013). Cytotoxicity of Vγ9Vδ2 T cells against influenza and human cytomegalovirus infected cells is independent of ADCC, unlike NK cells. Interestingly, Vγ9Vδ2 T cells can mediate cytotoxicity against various lymphomas, leukemia and carcinomas, while sparing the normal untransformed cells (Fournie et al., 2013).

1.4.6.2 γδ T cells in anti-tumour immunity

γδ T cells provide an advantage over conventional T cells owing to their ability to directly recognise molecules on cancer cells without the need for antigen processing and presentation. They also exhibit the important feature of discriminating between self- and non-self antigens, as certain tumour cells exhibit an increase in IPP levels due to an increase in the activity of 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase (see Fig. 1.3). This is a rate-limiting step in IPP synthesis and results in an increased susceptibility of these tumour cells to γδ T cell-mediated lysis (Gober, et al., 2003, Uchida, et al., 2007, Wrobel, et al., 2007). Statins, which inhibit the function of this enzyme, have been shown to inhibit aminobisphosphonate and alkylamine activation of Vγ9Vδ2 T cells (Gober, et al., 2003, Thompson, et al., 2006, Thompson and Rogers, 2004). Since many human epithelial tumour cells express NKG2D ligands, Vγ9Vδ2 T cells can target and kill these directly (Wrobel, et al., 2007). As a results, Vγ9Vδ2 T cells have been implicated in several clinical trials for cancer treatment

including patients with multiple myeloma, follicular lymphoma, myeloid leukemia, renal cell carcinoma, breast carcinoma and prostate carcinoma. Furthermore, Vγ9Vδ2 T cells are capable of infiltrating solid tumours *in vivo*, which was linked to decreased tumour size and correlated with increased survival. However, it was reported that there is a significant decrease in circulating Vγ9Vδ2 T cell numbers and their ability to produce IFN-γ and TNF-α, but this was restored with zoledronate administration. In line with this, numerous studies are examining the potential of pAg or aminobisphosphonate administration in cancer immunotherapy. This has been found to increase numbers of circulating γδ T cells and consequently tumour reduction (Braza, et al., 2013). Thus, Vγ9Vδ2 T cells clearly play an important role in tumour surveillance and killing of cancer cells.

1.4.6.3 γδ T cells in immunity against viral infection

Vγ9Vδ2 T cells can mediate antiviral activity and in most cases this is enhanced by pAg stimulation. They have been shown to kill cells infected with different influenza strains including H1N1, H5N1 and H9N2 (Qin, et al., 2009). In addition, they can provide adaptive immunity against West Nile virus through activation of DC (Fang, et al., 2010) and they also have a beneficial role in controlling human immunodeficiency virus (HIV) infection (Sciammas and Bluestone, 1999). The antiviral activity decreased as the disease progressed in the HIV patients, but was found to be improved with pAg stimulation.

However, some viruses can have inhibitory effects on Vγ9Vδ2 T cells, and these include herpes simplex virus (Puttur, et al., 2010) and respiratory syncytial virus (Aoyagi, et al., 2003). Furthermore they are found to be expanded in human cytomegalovirus infection and they can help control infection caused by Epstein-Barr virus (De Paoli, et al., 1990) and hepatitis C virus (Tseng, et al., 2001). In hepatitis B infection, Vδ1 and Vγ9Vδ2 T cell frequencies were shown to be increased and Vγ9Vδ2 T cells produced higher IFN-γ levels than in healthy controls (Conroy, et al., 2015).

1.4.6.4 γδ T cells in bacterial infection

In addition to mediating anti-viral immune responses, Vγ9Vδ2 T cells are expanded in various bacterial diseases (Chen and Letvin, 2003, Oliaro, et al., 2005, Barnes, et al., 1992, Perera, et al., 1994). They respond potently to pathogens that utilise the non-mevalonate pathway *in vitro*, including *Mycobacterium tuberculosis*, *Mycobacterium leprae* (Chen, 2013) and *Plasmodium falciparum* (Kabelitz, et al., 2000, Behr, et al., 1996, Kabelitz, et al., 1990). Vγ9Vδ2 T cells have been implicated in immune responses during bacterial infections such as salmonellosis, brucellosis, legionellosis, tularemia, listeriolisis and infections by *Escherichia coli*, *Leishmania* and *Toxoplasma gondii* (Zheng, et al., 2013, Chen and Letvin, 2003), thus making them of great interest for immunotherapeutic manipulation. Vγ9Vδ2 T cells also recognise *Staphylococcus* enterotoxin A via the γδ TCR (Rust, et al., 1990) and canarypox antigens (Morita, et al., 2000, Worku, et al., 2001) and tetanus toxoid (Kozbor, et al., 1989).

1.4.7 APC function by γδ T cells

The interesting discovery of MHC-II and CD80 and CD86 expression by a high proportion of tonsillar γδ T cells has sparked an investigation of potential APC phenotype by γδ T cells. Moser & Brandes found that fresh peripheral blood Vγ9Vδ2 T cells lacked APC molecule expression and had low levels of adhesion molecules (Moser and Brandes, 2006). However, following pAg stimulation, they expressed high levels of APC molecules such as CD40, CD54, CD80, CD83, CD86 and HLA-DR at levels similar to those seen in LPS-treated monocyte-derived DC (Moser and Brandes, 2006, Moser and Eberl, 2007). Expression of these maturation molecules (except CD40 and CD83) was maintained in activated Vγ9Vδ2 T cells during prolonged *in vitro* proliferation, which is in stark contrast to DC for which the effects are transient (Lanzavecchia and Sallusto, 2001). Thus, these Vγ9Vδ2 T cells were termed γδ-T-APC (Moser and Brandes, 2006, Moser and Eberl, 2007). In addition, IPP stimulation of Vγ9Vδ2 T cells also induced clustering with naïve αβ T cells, indicative of APC-responder cell interaction (Moser and Brandes, 2006). Vγ9Vδ2 T cells also had efficient antigen uptake and processing machinery, as illustrated by the ability to present PPD and tetanus toxoid protein antigens to CD4 T cells (Moser and Brandes, 2006, Brandes, et al., 2005, Meuter, et al.,

2010). More importantly, activated Vγ9Vδ2 T cells were capable of processing exogenous soluble viral and tumour proteins and cross-presenting peptide-HLA class I complexes to antigen-specific CD8 αβ T cells (Brandes, et al., 2009) which is characteristic of professional APC (Kabelitz and He, 2012). Meuter and colleagues demonstrated that activated Vγ9Vδ2 T cells can translocate soluble antigen into the cytosol for processing via the proteasomal degradation pathway (Meuter, et al., 2010). Professional APC have the ability to stimulate naïve αβ T cells and present peptide-MHC complexes (Banchereau, et al., 2000). The ability of Vγ9Vδ2 T cells to induce proliferation, T_H activity and cytotoxic T cell generation in αβ T cells and present peptide antigens to $CD4^+$ and $CD8^+$ T cells, qualifies them as professional APC (Meuter, et al., 2010, Brandes, et al., 2009). Thus Vγ9Vδ2 T cells are markedly distinct from monocytes and αβ T cells, which do not qualify as professional APC (Moser and Brandes, 2006). And although αβ T cells can exhibit characteristics of APC, they are not efficient APC (Barnaba, et al., 1994, Lanzavecchia, et al., 1988). In conclusion, γδ-T-APC are as efficient as DC in processing soluble proteins, suggesting they may be useful immunotherapeutic agents in cancers and infectious diseases (Moser and Eberl, 2007).

1.4.8 Interaction between Vγ9Vδ2 and other immune cells

Various innate T cells have gained attention for their roles in DC maturation. T cells expressing the Vδ2 TCR have been shown to induce differentiation of DC (Dunne, et al., 2010, Conti, et al., 2005, Devilder, et al., 2006, Ismaili, et al., 2002, Petrasca and Doherty, 2014) and this was also observed with Vδ1 (Collins, et al., 2005, Leslie, et al., 2002) and Vδ3 T cells (Mangan, et al., 2013). Another innate T cell subset, iNKT cells, can also induce maturation of DC into APC (Fujii, et al., 2003, Kitamura, et al., 1999, Vincent, et al., 2002). NK cells can also interact with DC through cell contact and surface or secreted ligands and can result in DC maturation (Harizi, 2013). In addition to conventional T cells, innate T cells such as iNKT cells (Galli, et al., 2003) and γδ T cells (Brandes, et al., 2003, Caccamo, et al., 2006) are also able to induce differentiation of B cells into antibody-secreting plasma cells but can also induce cytokine secretion and co-stimulatory molecule expression by B cells (O'Reilly, et al., 2011, Petrasca and Doherty, 2014, Kitamura, et al., 1999, Vincent, et al., 2002, Zeng, et al., 2013, Russano, et al., 2006). Vγ9Vδ2 T cells also play a role in survival and

activation of monocytes (Tyler, et al., 2015) and neutrophils and can induce APC markers by neutrophils (Davey, et al., 2014).

1.5 Aims & Objectives

The ability of DC to stimulate both adaptive and innate antitumour immune responses makes DC great candidates for cancer immunotherapy (Anguille, et al., 2015). Vγ9Vδ2 T cells could be used as adjuvants to direct the desired immune responses. However, it is crucial to fully understand the repercussions of utilising Vγ9Vδ2 T cells due to their ability to influence immune responses through interaction with innate and adaptive immune cells. Thus, our overall aim is to further investigate the relationship between Vγ9Vδ2 T cells, DC and B cells, and the mechanisms involved. The results may identify better adjuvants for DC-based therapies and lead to better understanding of the mechanisms by which Vγ9Vδ2 T cells contribute to adaptive immunity. In addition, we seek to examine whether Vδ3 T cells can induce B cell differentiation and the potential role of Vγ9Vδ2 T cells in *Clostridium difficile* infection.

1. To phenotype Vδ1, Vδ2 and Vδ3 T cell subsets in peripheral blood and define and compare the cytokine profiles of expanded Vδ2 and Vδ3 T cell lines.

3. To compare the ability of Vγ9Vδ2 T cells in inducing differentiation, cytokine production by DC and B cells and antibody secretion by B cells.

4. To compare the ability of Vγ9Vδ2 T cells in inducing T cell activation by DC and B cells and examine the resulting cytokine profiles.

5. To investigate the mechanisms involved in activation of DC and B cells by Vγ9Vδ2 T cells and to examine the impact of LPS on DC and B cell maturation.

6. To characterise the relationship between Vδ3 T cells and B cells and the resulting APC phenotype, cytokine profile and antibody production and to assess whether Vδ3 T cells can recognise CD1 molecules.

7. To analyse the potential role of Vγ9Vδ2 T cells in immunity against *Clostridium difficile*.

Chapter 2

Materials and Methods

2.1 Materials & Methods

Equipment, laboratory consumables, reagents and kits used in this investigation are listed in tables 2.1-2.8.

Table 2.1. Equipment

Equipment	Model	Company
Balance	Adventurer Pro	Ohaus, NJ, USA
Cell sorter	Moflo XDP	Beckman Coulter, CA, USA
Centrifuge	Eppendorf 5810	Eppendorf, Hamburg, Germany
Centrifuge	Eppendorf 5415 D	Eppendorf, Hamburg, Germany
Haemocytometer	Neubauer Improved	Marienfeld Superior, Germany
Haemocytometer	Kova slides	Hycor Biomedical, IN, USA
Counter	Tally hand-held counter	Thermo Fisher Scientific, MA, USA
CO_2 Incubator	Galaxy S	RS Biotech, Irvine, UK
CO_2 Incubator	Heracell 150i	Thermo Fisher Scientific, MA, USA
Flow cytometer	CyAn ADP	Beckman Coulter, CA, USA
Flow cytometer	FACS Canto II	Becton Dickinson, Oxford
Freezing container	Nalgene Cryo	Thermo Fisher Scientific, MA, USA
Hot plate and stirrer	AGB 1000	Jenway, UK
Laminar air flow Class II safety cabinet	CleanAir MSC BSS6-2	Thermo Fisher Scientific, MA, US
Laminar air flow Class II safety cabinet	MSC 1.2	Thermo Electron LED Gmbh, Germany
Magnet	LS magnet	Miltenyi Biotech, Bergisch-Gladbach, Germany
Magnet stand	MACS Multi Stand	Miltenyi Biotech, Bergisch-Gladbach, Germany
Magnet	The Big Easy EasySep	Stemcell Tenchnologies, France
Microscope	Inverted; NAO 30	Olympus Corporation, Japan
Multichannel pipette	Finnpipette F2	Thermo Fisher Scientific, MA, USA
PH meter	Benchtop pH20-01	Hanna Instruments, RI, USA
Pipettors p20, p100, p1000	Eppendorf Research plus	Thermo Fisher Scientific, MA, USA
Pipette filler	Motorised, Fisherbrand	Thermo Fisher Scientific, MA, USA
Plate reader	Sunrise	Tecan, Switzerland
Plate shaker	PMS-1000	Grant-bio, UK
UV microscope	E200 Eclipse	Nikon, USA

Vortex	Vortex Genie	Scientific Industries, NY, USA
Water bath	YCW-010E	Gemmy Industrial Corp., Taiwan
-80°C Freezer	Ultra low temp freezer	New Brunswick Scientific, CT, USA

Table 2.2. Consumables and plasticware

Item	Source	Catalogue #
Autoclave tape	Fisher Scientific	11-889-5
Biohazard bin liners	Fisher Scientific	BAJ-560-050U
Cardboard freezer boxes 10x10	Sarstedt	95.064.997
Cell strainers, 40 um	Fisher Scientific	FB35180
CellTrics disposable filters 30uM	Partec	04-0042-2316
Containers, 60 ml	Fisher Scientific	FB51806
Containers, 120 ml	Fisher Scientific	FB51808
Containers, 180 ml	Fisher Scientific	FB51810
Cryovials (Nalgene)	Fisher Scientific	CRY-100-025U
Flow cytometry tubes – FACS Canto	Fisher Scientific	12650366
Flow cytometry tubes – CyAn ADP	Unitech	352063
Flow cytometry for cell sorting, sterile	Unitech	352053
Gloves, medium	Fisher Scientific	SAR-246-050X
Gloves, nitrile, medium	Fisher Scientific	FB69264
LS columns	Miltenyi Biotec	130-042-401
P10 pipettor tips	Sarstedt	70.1130
P200 pipettor tips	Fisher Scientific	FB78044
P1000 pipettor tips	Fisher Scientific	FB78084
Parafilm 75 m 100 mm	Fisher Scientific	SEL-400-060G
Petri Dishes, nonvented, sterile	Fisher Scientific	PDS-140-025E
Plastic pipettes	Sarstedt	86.1171.010
Pipettes, 5 ml	Corning	PN5E1
Pipettes, 10 ml	Fisher Scientific	TKV-670-071L
Pipettes, 25 ml	Fisher Scientific	TKV-670-091F
Plate sealing tape for ELISA	Fisher Scientific	DPS-115-515N
Pre-separation filters, sterile, 30 uM	Miltenyi Biotec	130-041-407
Sharps bins	Fisher Scientific	SAT-641-060M
Support HTS 96 well transwell system	Fisher Scientific	TKT-555-010U
Syringes, 50 ml	Fisher Scientific	SZR-205-160T
Syringe filters, 0.2 uM	Fisher Scientific	FDP-635-010M
Tissue culture flasks, 25 cm2	Sarstedt	83.1810.002
Tissue culture flasks, 75 cm2	Fisher Scientific	TKT-130-210T
Tissue culture plates, 6 well	Fisher Scientific	TKT-520-030T
Tissue culture plates, 24 well	Fisher Scientific	TKT-520-090B
Tissue culture plates round bottom, 96 well	Fisher Scientific	TKT-521-170u

Tubes, 0.5 ml	Sarstedt	72.730.006
Tubes, 2 ml	Sarstedt	72.694.006
Tubes, 5 ml	Sarstedt	62.558.201
Tubes, 15 ml	Sarstedt	62.554.502
Tubes, 30 ml	Fisher Scientific	FB55151
Tubes, 50 ml	Fisher Scientific	62.547.254
Tissue paper	Fisher Scientific	CMC-716-021F
Weighboats polystyrene 100 ml	Fisher Scientific	FB61504
Waterbath treatment	Sigma-Aldrich	S5525

Table 2.3. General reagents & kits

Reagent	Source	Catalogue #
Acridine orange (AO)	Sigma-Aldrich	A6014
αGalCer	Funokoshi co ltd	
Amino acids, essential	Gibco	11130036
Amino acids, non-essential	Gibco	11140035
β-mercaptoethanol	Gibco	31350010
rh BAFF	Immunotools	11343436
Bovine Serum Albumin (BSA)	Fisher Scientific	BPE-9703-100
Cardiolipin	Avanti polar lipids	
Cell proliferation CellTrace Violet kit	Invitrogen	C34557
rh CD40L soluble	Immunotools	11343345
Conjugation kit lightning-link APC (for Vδ3 conjugation)	Innova Biosciences	705-0010
CpG ODN 2006-G5	Invivogen	tlr1-2006g5
Cytometer setup & tracking research beads	BD Biosciences	655050
DMEM medium	Gibco	61965026
Dimetyhl sulphoxide (DMSO)	Sigma-Aldrich	494429
Dimethyl sulfoxide (DMSO)	Fisher Scientific	BP231-100
EasySep Buffer	Stemcell Technologies	20144
Ethidium Bromide (EB)	Alfa Aesar	1239-45-8
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	EN884
Fc block	Biolegend	422302
Fixable viability dye efluor 506	Ebioscience	65-0866-18
FluoroFix Buffer for tandem dyes	Biolegend	422101
Foetal Bovine Serum, Biosera Biowest	Biosciences	51900500
Foetal Bovine serum, Hyclone	Thermo scientific	SH3 071.03
FoxP3 staining buffer set	Ebioscience	00-5523
Fungizone	Gibco	15290026
Ganglioside	Avanti polar lipids	

Hela cells (CD1a-, CD1b-, CD1c-, CD1d- or mock-transfected)	Gift from Dr. Steven Porcelli	New York
Hepes	Gibco	15630056
(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP)	Gift from H Jomaa A Reichenberg	Germany
(E)-1-hydroxy-2-methyl-2butenyl-4-pyrophosphate lithium salt (HMB-PP)	Sigma-Aldrich	95098
Immunoglobulin Master Buffer Kit	BD Biosciences	558683
Immunoglobulin soluble protein Master Buffer kit	BD Biosciences	558264
IgG (total) Cytometric Bead Array Flex kit	BD Biosciences	558679
IgA Cytometric Bead Array Flex kit	BD Biosciences	558681
IgM Cytometric Bead Array Flex kit	BD Biosciences	558680
IgE Cytometric Bead Array Flex kit	BD Biosciences	559682
Industrial methylated spirits	Fisher Scientific	M/4450/17
Ionomycin	Sigma-Aldrich	IO634
Isopropanol	Sigma-Aldrich	34486
Lipopolysaccharide(LPS)	Sigma-Aldrich	L5293
Lymphoprep	Biosciences	1114547
Monensin	Biolegend	420701
Mycoplasma-Off	Minerva Biolabs	15-5000
OneComp beads	Ebioscience	01-1111-42
Paraformaldehyde (PFA, 4% in PBS)	Santa Cruz, USA	sc-281692
Penicillin/Streptomycin	Gibco	15140122
Phytohaemagglutinin (PHA-P)	Sigma-Aldrich	L-9132
Phosphate buffered saline, sterile	Gibco	14190094
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	P1535
RPMI with Glutamax	Gibco	61870010
Saponin	Sigma-Aldrich	S7900
Sodium azide	Sigma-Aldrich	S8032
Sodium pyruvate	Gibco	11360039
Sodium chloride	Fisher Scientific	S/3120/65
Sodium dihydrogen orthophosphate 1-hydrate	BDH Lab. Supplies	102454R
di-Sodium hydrogen orthophosphate anhydrous	Fisher Scientific	S/4480/60
Sulfatide	Avanti polar lipids	
Sulphuric acid (H2SO4)	Sigma-Aldrich	339741
Tetanus Toxoid (from Clostridium tetani)	Calbiochem	CA, USA
Tetramethyl benzidine (TMB)	Sigma-Aldrich	T0440-1L
Trypsin 0.05% EDTA	Gibco	25300054
Tuberculin purified protein derivative RT 23 SSI	Statens Serum Institut	Denmark
Tween 20, for ELISA	Fisher Scientific	BP337-500
Virkon	Fisher Scientific	HYG-205-010P
Zoledronic acid Mylan 4mg/5ml	Pharmacy	

Table 2.4. Cell isolation kits

Cell separation kit	Company	Catalogue#
CD3 Microbeads (positive selection)	Miltenyi Biotec	130-050-101
CD14 Microbeads (positive selection)	Miltenyi Biotec	130-050-201
CD19 Microbeads (positive selection)	Miltenyi Biotec	130-050-301
Anti-γδ TCR Microbead kit (positive selection)	Miltenyi Biotec	130-050-701
B cell enrichment kit (negative selection)	Stemcell Technologies	19054

Table 2.5. Recombinant human cytokines

Cytokine	Company	Catalogue #
GM-CSF	Immunotools	11343125
IL-4	Immunotools	11340045
IL-2	Peprotech	200-02
IL-2	Miltenyi Biotec	130-097-748

Table 2.6. ELISA kits

ELISA kit	Company	Catalogue#
IFN-γ Duoset	R&D Systems	DY285
IFN-γ Deluxe	Biolegend	430105
IL-2 Duoset	R&D Systems	DY202
IL-4 Duoset	R&D Systems	DY204
IL-4 Deluxe	Biolegend	430305
IL-6 Duoset	R&D Systems	DY206
IL-10 Duoset	R&D Systems	DY217B
IL-10 Deluxe	Biolegend	430605
IL-12p40 Deluxe	Biolegend	431705
IL-12p70 Duoset	R&D Systems	DY1270
IL-12p70 Deluxe	Biolegend	431705
IL-13 Duoset	R&D Systems	DY213
IL-17 Duoset	R&D Systems	DY317
TNF-a Deluxe	Biolegend	430204

Table 2.7. Functional grade and low endotoxin azide-free (LEAF) antibodies

Antibody	Clone	Company	Catalogue #
IgG1 isotype LEAF-purified	MG1-45	Biolegend	401405
Anti-human CD86 LEAF-purified	IT2.2	Biolegend	305410

Anti-human CD119 (IFN-γ receptor) LEAF purified	GIR-208	Biolegend	308604
Anti-human CD124 (IL-4 receptor) functional grade purified	X2/45-12	Ebioscience	16-1249-82
Anti-human CD154 (CD40L) functional grade purified	24-31	Ebioscience	16-1548-82
Anti-human IFN-γ functional grade purified	NIB42	Ebioscience	16-7318-85
Anti-human IL-4	MP4-25D2	Ebioscience	16-7048-85
Anti-human TNF-α functional grade purified	MAb1	Ebioscience	16-7348-85

Table 2.8. Fluorochrome-conjugated monoclonal antibodies for flow cytometry

Antibody	Fluorochrome	Clone	Company	Catalogue #
CD1a	PE	HI149	Biolegend	300105
CD1b	FITC	SN13 (K5-1B8)	Biolegend	329105
CD1c	FITC	L161	Biolegend	331517
CD1d	PE	51.1	Ebioscience	12-0016-42
CD3	FITC	UCHT1	Biolegend	300406
CD3	Pacific Blue	HIT3α	Biolegend	300330
CD3	PerCP	UCHT1	Biolegend	300428
CD3	PE-Cy5	HIT3α	Biolegend	300310
CD3	PE-Cy7	HIT3α	Biolegend	300316
CD4	APC	-	Immunotools	21278046S
CD4	FITC	OKT4	Ebioscience	11-0048-42
CD4	PE	SK3	Biolegend	344606
CD4	PE-Cy7	SK3	Biolegend	344612
CD5	APC	UCHT2	Miltenyi Biotec	130-096-577
CD8a	APC	HIT8α	Biolegend	300912
CD8a	APC-Cy7	HIT8α	Biolegend	300925
CD8a	FITC	HIT8α	Biolegend	300906
CD8a	PerCP	HIT8α	Biolegend	300922
CD11c	APC	-	Immunotools	21487116
CD11c	FITC	-	Immunotools	21487113
CD11c	PE-Cy7	3.9	Biolegend	301608
CD14	PE	-	Immunotools	21620144S
CD19	Pacific Blue	HIB19	Biolegend	302224
CD19	PE-Cy7	HIB19	Biolegend	302216
CD19	PE-Cy7	HIB19	Ebiosciences	25-0199-42
CD20	FITC	-	Immunotools	21279203
CD20	PE	-	Immunotools	21279204S
CD20	PE	2H7	Biolegend	302305
CD24	FITC	32D12	Miltenyi Biotec	130-095-952

CD25	PE-Cy7	BC96	Ebioscience	25-0259-42
CD27	FITC	-	Immunotools	21270273
CD27	PerCP-Cy5.5	O323	Biolegend	302820
CD28	APC	CD28.2	Biolegend	302912
CD38	PE-Cy7	HB-7	Biolegend	356608
CD40	PE	-	Immunotools	21270404
CD45RA	APC	HI100	Biolegend	304112
CD45RA	FITC	-	Immunotools	21279453
CD56	FITC	HCD56	Biolegend	318304
CD69	PE	FN50	Biolegend	310906
CD80	APC	-	Immunotools	21270806
CD86	FITC	-	Immunotools	21480863
CD86	PE-Cy7	IT2.2	Biolegend	305421
CD107a	PE-Cy7	H4A3	Biolegend	328618
CD127	APC	eBioRDR5	Ebioscience	17-1278-42
CD161	PerCP-Cy5.5	HP-3G10	Biolegend	339908
FoxP3	PE	236A/E7	Ebioscience	12-4777-42
γδ TCR	FITC	B1	Biolegend	331208
HLA-DR	Pacific Blue	L243	Biolegend	307633
HLA-DR	PerCP-Cy5.5	L243	Biolegend	307630
IFN-γ	APC	4S.B3	Biolegend	502512
IFN-γ	FITC	4S.B3	Biolegend	502506
IL-4	FITC	MP4-25D2	Biolegend	500806
IL-4	PE	8D4-8	Biolegend	500704
IL-6	APC	MQ2-13A5	Biolegend	501112
IL-6	PerCP	-	Immunotools	21670065
IL-10	APC	JES3-19F1	Biolegend	506807
IL-12/IL-23 p40	APC	C11.5	Biolegend	501809
IL-13	APC	JES10-5A2	Biolegend	501907
IL-17a	PE	BL168	Biolegend	512306
IL-17a	PerCP-Cy5.5	BL168	Biolegend	512314
IL-23 p19	PE	23dcdp	Ebioscience	12-7823-41
NKG2D	PE	1D11	Biolegend	320806
TLR2	PE	TL2.1	Biolegend	309708
TGF-β1 (LAP)	PE	FNLAP	Ebioscience	12-9829-42
TNF-α	FITC	Mab11	Biolegend	502906
Vα24	FITC		BD Bioscience	
Vβ11	PE		BD Bioscience	
Vδ1	FITC	TS1	ThermoScientific	TCR2055
Vδ2	PerCP	B6	Biolegend	331410
Vδ2	PE	B6	Biolegend	331408
Vδ3 TCR	Unconjugated	-	Beckman Coulter	Custom Design

2.2 Tissue culture

Tissue culture was carried out under aseptic conditions in a class II laminar airflow unit using tissue culture grade plasticware and reagents. Cultures were maintained in corresponding serum-supplemented media within parafilm-wrapped tissue culture plates or flasks incubated at 37°C in a humidified 5% CO_2 incubator. The serum was filtered and heat inactivated at 56°C for 30 min in order to inactivate complement components and was stored at -80°C before use.

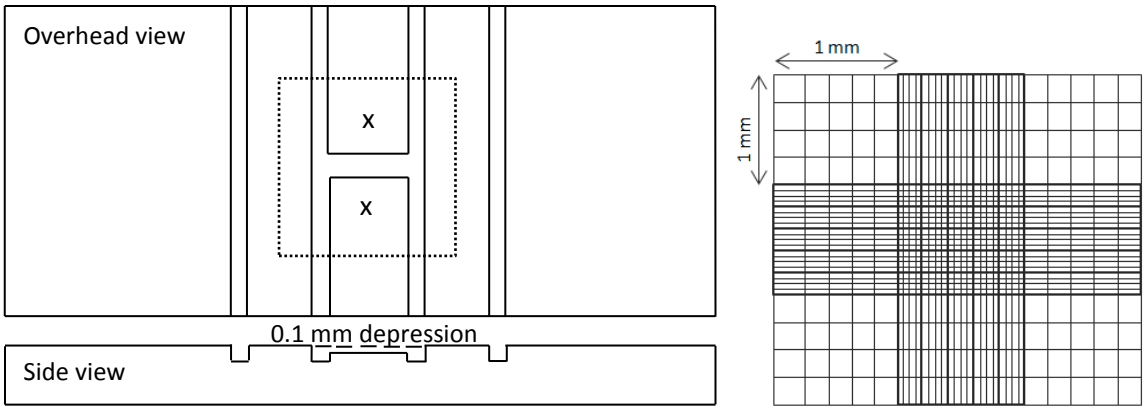

Figure 2.1. Schematic diagram of a Neubauer Haemocytometer. A glass coverslip was placed in the centre of the Haemocytometer. The "x" marks the counting area under the coverslip, where 10 µl of the EB/AO-stained cell sample was placed. **A**, the dotted line marks the position of the coverslip. **B**, enlarged layout of "x" is shown. 10 µl of the EB/AO-stained cell sample was placed in the counting area which holds a volume of 1 mm x 1 mm x 0.1 mm, i.e. 0.1mm^3 or 10^{-4} ml. The number of cells per ml of suspension was calculated using the following equation: cell concentration = (no. of cells counted per center square x dilution factor) x 10^4 cells ml^{-1}. Since viable cells stained bright green and dead cells stained orange, percentage viability was calculated by dividing the number of live cells per ml by the number of total cells per ml and multiplying by 100.

2.3 Cell viability and enumeration

Cell counting and viability was carried out by staining with a solution of ethidium bromide and acridine orange (EB/AO). Ethidium bromide is an intercalating agent that binds to DNA and fluoresces orange under UV light. Acridine orange is a fluorescent cationic dye which can interact with DNA and RNA, and when bound to DNA and exposed to UV light, AO appears green. By this method, viable cells fluoresce green,

while dead cells appear orange. A working solution of EB/AO was made up by diluting a 100 X solution (15 mg AO, 50 mg EB, 1 mL 95% ethanol and 49 mL dH_2O) 1 in 100 in PBS (9 g NaCl, 21.09 g Na_2HPO_4, 0.184 g NaH_2PO_4, 1 L dH_2O, pH 7). Cell samples were diluted in 1 X EB/AO solution: 10 µl of EB/AO-stained cell mixture was pipetted to fill the counting chamber (Fig. 2.1) of a Neubauer haemocytometer (Marienfeld Superior, Germany) or of a disposable haemocytometer (Kova slides, Una Health Ltd, UK). Cells were visualised microscopically under UV light and counted using a hand-held counter.

2.4 Cryopreservation of cells

Cryogenic freezing allows for long-term storage of cells and tissues by stopping all biochemical processes including cell death. To avoid cellular damage, a cryoprotectant vitrification solution is needed to increase viscosity and lower the freezing point of the cell suspension and prevent water loss from cells and subsequent ice formation and crystallisation which would otherwise damage the cells. Dimethyl sulphoxide (DMSO) mixed with foetal bovine serum (FBS) at a ratio of 1:10 was used as the vitrification solution. Cells were centrifuged at 500 g for 7 min and re-suspended in FBS (10-50 x 10^6 cells per ml). The vitrification solution was freshly prepared (20% DMSO and 80% FBS) and cooled to 4°C before adding to the cell suspension at a ratio of 1:1. The cell suspension was placed in cryovials and frozen to -80°C within the freezing container (Nalgene) which cools the samples at a reduced rate of 1°C per minute. For long-term storage, the cells were transferred to liquid Nitrogen (-196°C).

When thawing, the cells were quickly warmed by immersing the cryovials into a hot waterbath (37°C) and swirling to speed up thawing. Pre-warmed complete RPMI (cRPMI) medium (RPMI 1640 supplemented with 10% heat inactivated FBS, 1% penicillin-streptomycin, 1% fungizone, 50 µM L-glutamine, 50 µM β-mercaptoethanol, 1% non-essential amino acid mixture, 1% essential amino acid mixture, 1 mM sodium pyruvate and 25 mM HEPES; Gibco-BRL, Paisley, UK and Thermo-Scientific, Logan, UT) was added dropwise to the cells and the cells were pelleted by centrifugation for 5 min at 800 g. The supernatant was discarded to eliminate any traces of DMSO which would be damaging to the cells. The cells were then washed once more with cRPMI medium before use.

2.5 Flow cytometry

2.5.1 Principles of flow cytometry

Flow cytometry is a powerful tool for the analysis of multiple parameters of individual cells within heterogeneous populations. The cell sample is injected into a flowing stream containing sheath fluid and is passed through a laser beam at the rate of thousands of cells per second. The cells scatter and refract light which is captured as each cell passes through. The magnitude of light scattered in the forward direction at low angles, is proportional to the cell size. Light scattering at larger angles, such as to the side, is caused by granularity and structural complexity inside the cell. Light is quantified by two detectors which convert light intensity into voltage. The use of fluorochrome-conjugated monoclonal antibodies is one of the most common ways to study cellular characteristics using flow cytometry. The antibody will bind to a specific molecule on the cell. When a laser of a particular wavelength strikes the fluorophore, a fluorescent signal is emitted and detected. A series of filters and mirrors is in place so that the particular wavelengths are delivered to the appropriate detectors. This information is then translated into a voltage pulse proportional to the amount of fluorescence emitted and can be presented graphically as distinct populations (see Fig. 2.2).

The Cyan ADP (Beckman Coulter, High Wycombe, UK) and FACS Canto-II (Becton Dickinson, UK) flow cytometers are each equipped with three lasers which emit light at different wavelengths. The violet laser emits at 405 nm, the blue laser emits light at 488 nm and the red laser emits at 640 nm. Collectively, the 3 lasers are capable of detecting 10 distinct parameters, including forward scatter and side scatter (Fig. 2.2), as well as 8 fluorochromes. A table of fluorochromes used is depicted below (Table 2.9). Tandem dyes, which are created by combining a fluorochrome with cyanine molecules to alter the emission spectrum, were analysed within 4 hours of fixation in PFA, or transferred to a PFA-free buffer overnight. Fluorofix buffer was used for samples containing tandem dyes that needed to be stored for more than one day (Biolegend). FL channels are unique to each flow cytometer and they indicate which fluorochromes can be used together. Only one fluorochrome can be used from each FL

channel. FL3 and FL5 can be difficult to resolve from each other due to the close emission spectrum.

Table 2.9. Fluorochromes used in this study and their specifications

Laser	Channel	Fluoro-Chrome	Excitation Max	Emission Max	Description
Blue 488 nm	FL1	FITC	494 nm	520 nm	Fluorescein isothiocyanate
	FL2	PE	496 nm	578 nm	Phycoerythrin
	FL3	PerCP	482 nm	678 nm	Peridin Chlorophyll protein
	FL3	PE-Cy5	496 nm	667 nm	PE-cyanin 5 tandem dye
	FL3	PerCP-Cy5.5	482 nm	695 nm	PerCP-cyanin 5.5 tandem dye
	FL4	PE-Cy7	496 nm	785 nm	PE-cyanin 7 tandem dye
Red 640 nm	FL5	APC	650 nm	660 nm	Allophycocyanin
	FL6	APC-Cy7	650 nm	785 nm	APC-cyanin 7 tandem dye
Violet 405 nm	FL7	Pacific Blue	410 nm	455 nm	Biolegend™
	FL8	Efluor 506	405 nm	506 nm	Ebioscience™

2.5.2 Labeling of cells for flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAb) were obtained from Biolegend (San Diego, CA), Immunotools (Friesoythe, Germany) or eBioscience (Hatfield, UK) (Table 2.8). Cells were stained with mAb in PBA buffer (PBS containing 1% BSA and 0.02% sodium azide; Gibco-BRL, Paisley, UK and Sigma-Aldrich, Dublin, Ireland) and analysed using CyAn ADP or BD FACS Canto-II flow cytometers and Summit 4.3 (Dako), FACS Diva (Becton Dickinson) or FlowJo software (Treestar, Ashland, OR).

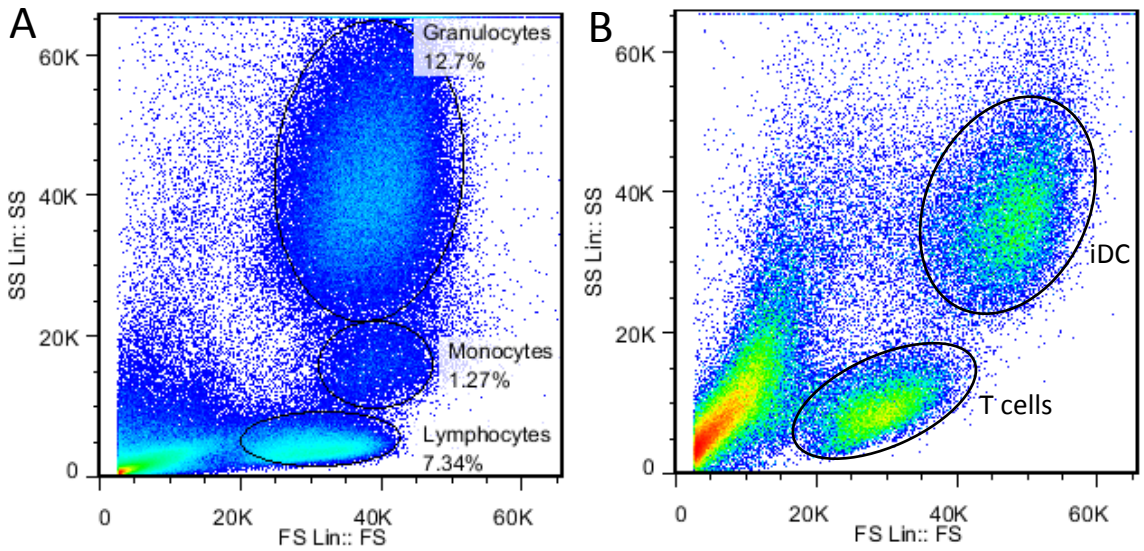

Figure 2.2. Flow cytometry dot plots showing physical properties of peripheral blood and cell lines. Fresh blood (A) or a mixture of DC and γδ T cells (B) were suspended in PBA buffer and acquired on a flow cytometer. Cells pass by laser beams which collect information on their size depending on how the light is scattered. Forward scatter (FS) depicts size, while side scatter (SS) depicts granularity. Each dot on a dot plot represents one cell. Distinct cellular populations can be identified based on knowledge of size and granularity of common cell types. Lymphocytes are the smallest and least granular cell type in peripheral blood, while granulocytes are very large and granular, and monocytes class in between the two. **A**, whole blood sample containing a mixture of cell populations typically found in PBMC**. B**, sample containing DC and γδ T cells. Dead cells and debris are smaller than lymphocytes and they appear on the far left of the dot plot. Cell death is confirmed using a viability dye (FL8).

2.5.3 Antibody -fluorochrome conjugation

Custom made, purified anti-Vδ3 antibody for flow cytometry was purchased (Beckman Coulter) and labelled using a 100 µg APC conjugation kit (Innova Biosciences, UK). The kit recommended using 100-150 µg of antibody for 100 µg of APC. 25 µl of the anti-Vδ3 antibody (5.12 mg/ml), corresponding to 128 µg, was added to the 100 µg of APC. The antibody was diluted to a total volume of 100 µl with sterile PBS and the PBS-diluted antibody was then added to the vial containing solid APC and mixed gently. 1 µl of the modifier solution provided in the kit was added per 10 µl of antibody-PBS mixture (i.e. 10 µl) and the mixture was incubated at room temperature, protected from light, for 3 hours. 1 µl of quencher solution provided in the kit was added per 10 µl of antibody mixture (i.e. 10 µl) and mixed gently and incubated for 30 min. The APC-

labelled Vδ3 antibody was diluted with sterile PBS to a total volume of 5 ml and a titration assay was carried out to determine the recommended staining concentration for cell samples. The labelled antibody was aliquoted and stored at 4°C protected from light. Sterile aliquots of antibody were used for cell sorting.

2.5.4 Cell sorting

Flow sorters use a principle of electrostatic deflection of charged droplets to isolate cells of interest. The cell sample is passed through a column of pressurised sheath fluid and as they are ejected from the nozzle, pass through the analysis point consisting of laser beams which provide scattered light and fluorescence signals, which are then compared to pre-set sort criteria for the cells of interest. There is a time delay between the analysis point and where the droplets break off at the end of the stream and it is referred to as the drop delay. Once the drop delay is calculated, an electric charge is applied to the stream at the precise moment the first drop forms, allowing individual drops to be independently charged. Once the charged droplets reach the end of the stream, they are passed through high-voltage charged deflection plates, which cause the charged droplets to be deflected towards the oppositely charged plate and into collection tubes, while uncharged droplets are aspirated to waste (Davies, 2007).

2.6 Isolation and expansion of human cells

2.6.1 Isolation of human PBMC

Human blood for cell isolation was obtained from the Irish Blood Transfusion Service (St. James's Hospital, Dublin 8). The IBTS provides *pro bono* blood components to Irish third level educational facilities or health care facilities for the purposes of research and education. This blood is from voluntary, anonymous, non-remunerated donors donated primarily for therapeutic application to patients. PBMC were prepared from human buffy coat packs by standard density gradient centrifugation over Lymphoprep™ (Nycomed Pharma, Oslo, Norway), a solution of sodium diatrizoate and polysaccharide with a density similar to that of leukocytes (1.077 g/ml). In the presence of blood it aggregates erythrocytes and granulocytes, which then sediment

under centrifugation, while lymphocytes and monocytes remain in solution. Blood was diluted in sterile PBS at a ratio of 1:1 and carefully layered on top of Lymphoprep at a ratio of 2:1. The tubes were centrifuged for 25 min at 400 g with brakes off (acceleration and deceleration set to zero) to prevent sudden changes in speed, which would disturb the layers (Fig. 2.3). The buffy coat layer was then carefully extracted using a Pasteur pipette into a new tube and washed twice with PBS, centrifuging for 5 min at 800 g in between washes.

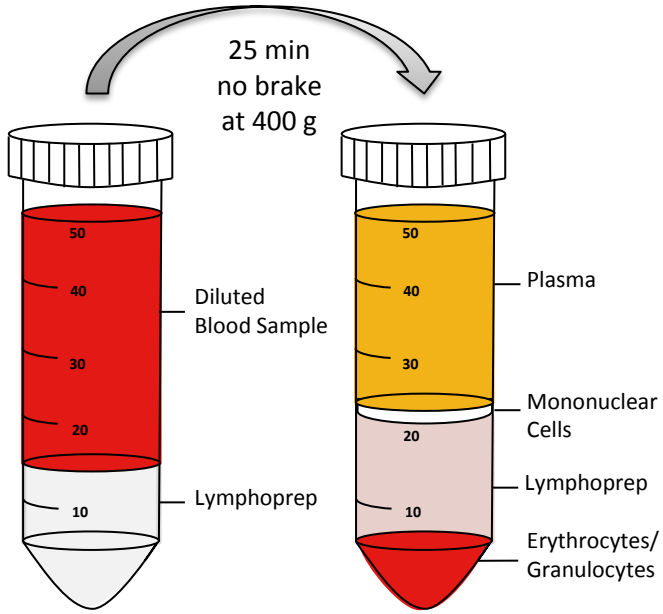

Figure 2.3. PBMC isolation using density gradient centrifugation. PBS-diluted blood is carefully layered on top of Lymphoprep by holding the tube containing lymphoprep at a 45° angle while pipetting the blood on top of the lymphoprep without disturbing the gradient. The tubes were centrifuged at 400 g for 25 min with no brakes. The left hand picture shows a tube containing blood layered over lymphoprep. The right hand picture shows the different layers following centrifugation.

2.6.2 Vδ2 T cell expansion

Cells were enriched by positive selection magnetic bead separation of PBMC (Miltenyi Biotec, Bergisch-Gladbach, Germany). Magnetic-activated cell sorting (MACS) is a method for separating cell populations depending on their surface antigens, and uses superparamagentic nanoparticles (50 nm diameter) and columns. The procedure allows cells to be separated by incubating them with magnetic nanoparticles coated with antibodies directed against desired surface antigens, thus allowing the desired cells expressing the antigen to attach to the magnetic nanoparticles. The cell solution

is then transferred onto a column placed in a strong magnetic field. Thus, the cells attached to the nanoparticles stay on the column, while those not expressing the antigen flow through. PBMC were re-suspended in cold MACS Buffer (sterile PBS containing 0.5% BSA and 2 mM EDTA, 4°C). 100 µl of anti-γδ TCR hapten-antibody and 400 µl MACS buffer was added per 5 x 10^8 cells and incubated on ice for 10 min. 200 µl of anti-hapten FITC-conjugated magnetic bead particles and 300 µl MACS buffer per 5 x 10^8 cells was then added and incubated for 15 min on ice. By adding a 5^{th} of the recommended bead concentration, the % of Vδ2 T cells was increased from <2% to 50-80%. The aim was to purify the population but not completely, so that it still contains $CD14^+$ cells (monocytes) which have been shown to be required to present HMB-PP to the Vδ2 T cells (Miyagawa, et al., 2001). The magnetically-labeled cells were washed in MACS buffer (3 ml per 1 x 10^7 cells) and centrifuged at 300 g for 10 min and the cell pellets were re-suspended in buffer (5 ml per 1 x 10^7 cells). Large MS columns were attached to large MACS magnets. The columns were primed by passing 3 ml of cold buffer through the column. A MACS pre-separation filter was used to prevent cell clumps from clogging the column. After the cell suspension was passed through the column, the filter was removed and the column was washed three times using 3 ml of MACS buffer each time. γδ T cell negative populations were eluted from the column, while the column containing γδ T cell positive cells was removed from the magnet and flushed with 5 ml of buffer and collected in a separate tube and re-suspended in cRPMI. Cell surface phenotyping and purity were determined by staining with mAb against CD3 and Vδ2 and examining by flow cytometry (Fig. 2.4). It was shown that virtually all $Vδ2^+$ T cells express the Vγ9 chain. Therefore, Vγ9Vδ2 T cells were subsequently identified by a Vδ2 mAb only (Dunne, et al., 2010). Vγ9 expression was confirmed by flow cytometry in a proportion of the donors. The magnetically enriched PBMC containing a high purity of Vδ2 T cells were plated in 24-well tissue culture plates at a cell density of 1 x 10^6 cells per ml and expanded by stimulating with (E)-4-hydroxy-3-methyl-but-2enylpyrophosphate (HMB-PP, 10 nM) and culturing with IL-2-supplemented (50 IU/ml, Peprotech or Miltenyi Biotec) cRPMI medium. The medium was changed every 3-4 days by replacing with fresh IL-2-supplemented cRPMI to provide a constant supply of IL-2 to the cells which is required for T cell survival and expansion (Smith, 1988). The cells were harvested after 14-28 of culture (Fig. 2.4).

Alternatively, Vδ2 T cells were expanded directly from PBMC using HMB-PP (10 nM), the aminobisphosphonate zoledronate (5 µM), or extracts from *Clostridium difficile* (see chapter 6.0) or combinations of these for 14 days.

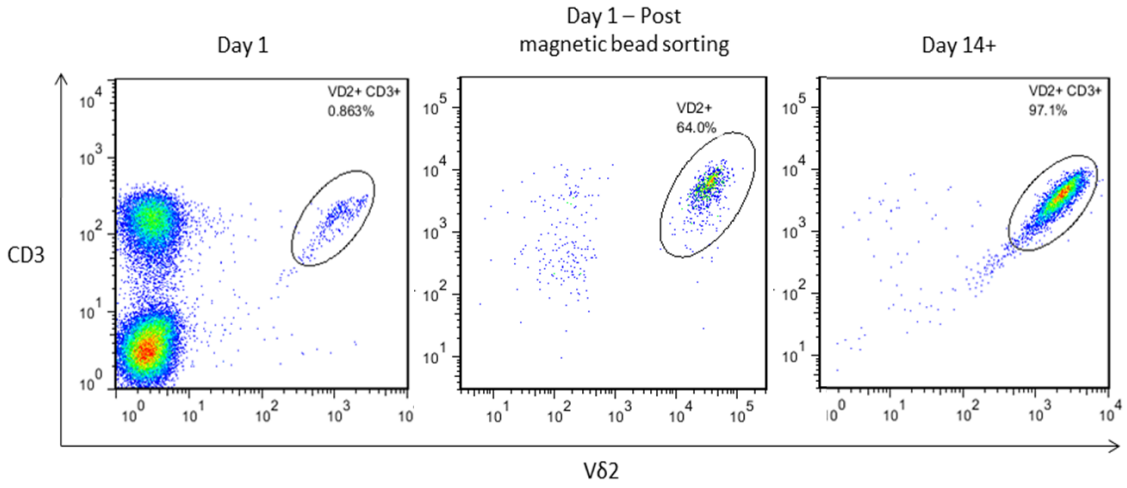

Figure 2.4. Flow cytometry dot plots of Vδ2 purity in peripheral blood and following *in vitro* expansion. Left and centre panels, the % of Vδ2 T cells within the lymphocyte population on day 1 before and after magnetic bead cell-sorting, respectively. Right panel, % of Vδ2 T cells after 14 days expansion with HMB-PP and IL-2.

2.6.3 Vδ3 T cell expansion

PBMCs were enriched by positive selection magnetic bead separation of γδ TCR^+ cells (Miltenyi Biotec) using the same method as described above. The γδ-enriched cells were stained with mAb against CD3 (10 µl per 1 x 10^6 cells) and Vδ3 (20 µl per 1 x 10^6 cells) and 30 µl PBS per 1 x 10^6 cells for 15 min. The cells were washed in PBS and the pellet re-suspended in 0.5 ml PBS per 1 x 10^7 cells and sorted using a Moflo XDP cell sorter (Beckman Coulter) which allowed retrieval of a pure (>97%) population of $CD3^+$ $Vδ3^+$ cells. These cells were then plated in 96-well tissue culture plates at a cell density of 1 x 10^3 cells and 2 x 10^5 irradiated allogeneic PBMC per well and stimulated with PHA-P (phytohaemagglutinin, 1 µg/ml, Sigma-Aldrich) and cultured in 200 µl of IL-2-supplemented (250 IU/ml) cRPMI for 4 weeks. PHA is a plant lectin derived from *Phaseolus vulgaris*, which causes mitogenic stimulation of lymphocytes through the T cell receptor, resulting in their proliferation (Morgan, et al., 1976). Due to the unspecific nature of PHA in activating all T cells, a pure population of Vδ3 is required for expansion. The medium was changed every 3-4 days by replacing with fresh IL-2-supplemented cRPMI. Cell surface phenotyping and purity after expansion were

determined by flow cytometric analysis by staining with mAb against CD3 and Vδ3 (Fig. 2.5).

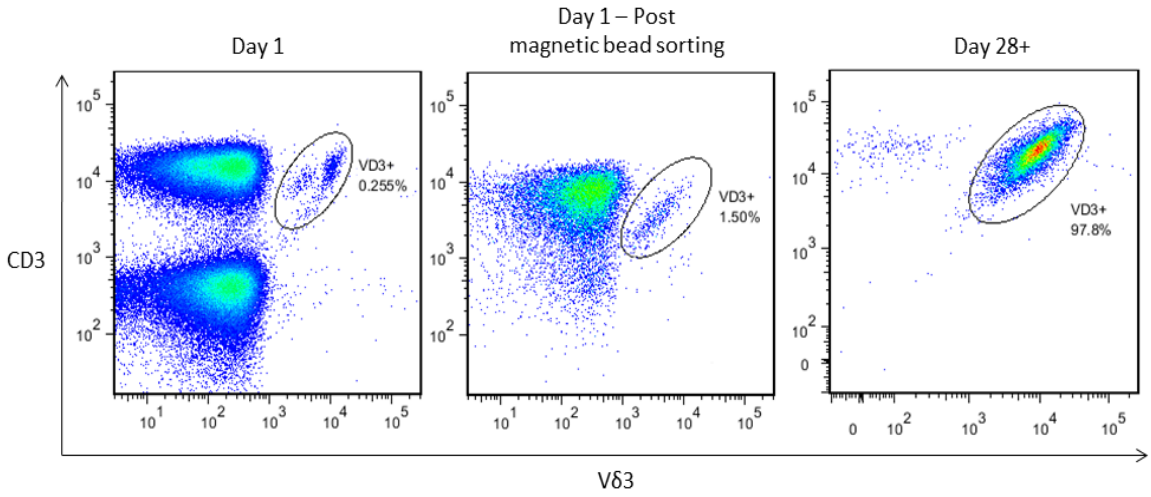

Figure 2.5. Flow cytometry dot plots of Vδ3 T cells in peripheral blood and following *in vitro* expansion. Left and centre panels, the % of Vδ3 T cells within the lymphocyte population on day 1 before and after magnetic cell-sorting, respectively. Right panel, % of Vδ3 T cells after 28 days expansion with PHA-P, irradiated PBMC and IL-2.

2.6.4 B cell isolation

B cells were obtained from human PBMC by positive selection magnetic bead cell sorting (Miltenyi Biotec) or negative selection magnetic bead cell sorting (Easysep kit, Stemcell Technologies) of $CD19^+$ lymphocytes. For positive selection magnetic bead cell sorting, 100 µl CD19 microbeads and 400 µl buffer were added per 5 x 10^8 cells and mixed well and incubated for 15 min on ice. The magnetically-labeled cells were washed in MACS buffer (3 ml per 1 x 10^7 cells) and centrifuged at 300 g for 10 min and the cell pellets were re-suspended in buffer (5 ml per 1 x 10^7 cells). The magnetic separation was carried out as described above, with CD19 positive cells being collected within the column and CD19 negative cells flushed through. The negative selection magnetic bead kit labeled unwanted cells (all cells except $CD19^+$ cells) with dextran-coated bispecific Tetrameric Antibody Complexes (TAC) containing mAb directed against cell surface antigens on human blood cells (CD2, CD3, CD14, CD16, CD36, CD43, CD56, CD66b, glycophorin A). The cells were suspended at a concentration of 5 x 10^7 cells per ml in recommended medium and mixed with 50 µl/ml human B cell enrichment cocktail for 10 min. The magnetic particles were vortexed for 30 sec and

added at 75 µl/ml to the cell suspension for 5 min. The cell suspension was brought to a total volume of 5 ml (for <2 x 10^8 cells) and placed inside the large magnet for 5 min in an uncapped round bottom 15 ml tube. In one continuous motion, the magnet and tube were inverted and the desired fraction was poured off into a new tube. The magnetically labelled unwanted cells remained bound inside the original tube, held by the magnetic field. The B cells were used fresh or otherwise were frozen for long-term storage in Nalgene cryovials. CD19 and CD20 antibodies were used to determine the cell purity (Fig. 2.6).

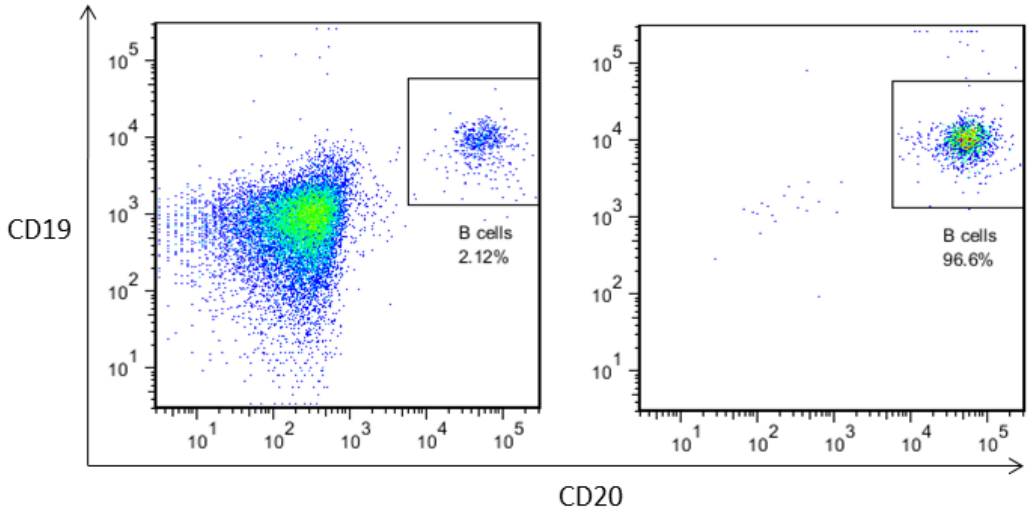

Figure 2.6. Peripheral blood B cells before and after enrichment. Flow cytometry dot plots of B cell frequency in PBMC of a healthy donor (left panel) and following magnetic-bead cell sorting (right panel).

2.6.5 Dendritic cell preparation

Monocyte-derived DC were obtained from human PBMC by positive selection magnetic bead cell sorting (Miltenyi Biotec) of $CD14^+$ cells. For magnetic bead cell sorting, 100 µl CD14 Microbeads and 400 µl cold buffer were added per 5 x 10^8 cells and mixed well and incubated for 15 min on ice. The magnetically-labeled cells were washed in MACS buffer (3 ml per 1 x 10^7 cells) and centrifuged at 300 g for 10 min and the cell pellets were re-suspended in buffer (5 ml per 1 x 10^7 cells). The magnetic separation was carried out as described above, with CD14 positive cells being collected within the column and CD14 negative cells flushed through. The resulting monocytes were induced to differentiate into immature DC by culturing with IL-4 (70 ng/ml) and

GM-CSF (50 ng/ml) (Immunotools, Friesoythe, Germany). The cells were plated in DC media (RPMI 1640 supplemented with 10% heat inactivated, filtered endotoxin-free HyClone foetal calf serum, 1% penicillin-streptomycin, 1% fungizone, 50 µM L-glutamine, 50 µM β-mercaptoethanol, 1% non-essential amino acid mixture, 1% essential amino acid mixture, 1 mM sodium pyruvate and 25 mM HEPES; Gibco-BRL, Paisley, UK and Thermo-Scientific, Logan, UT) in 6-well tissue culture plates at a cell density of 1 x 10^6 cells per ml. Several serum batches were tested and the one that resulted in the highest number of immature DC was subsequently used for deriving DC. After 3 days, media was replaced with fresh DC media containing IL-4 and GM-CSF. On day 6, immature DC were harvested and used immediately. CD14 was used to identify monocytes prior to culture with cytokines, while CD11c was used to identify DC after 6 day culture (Fig. 2.7).

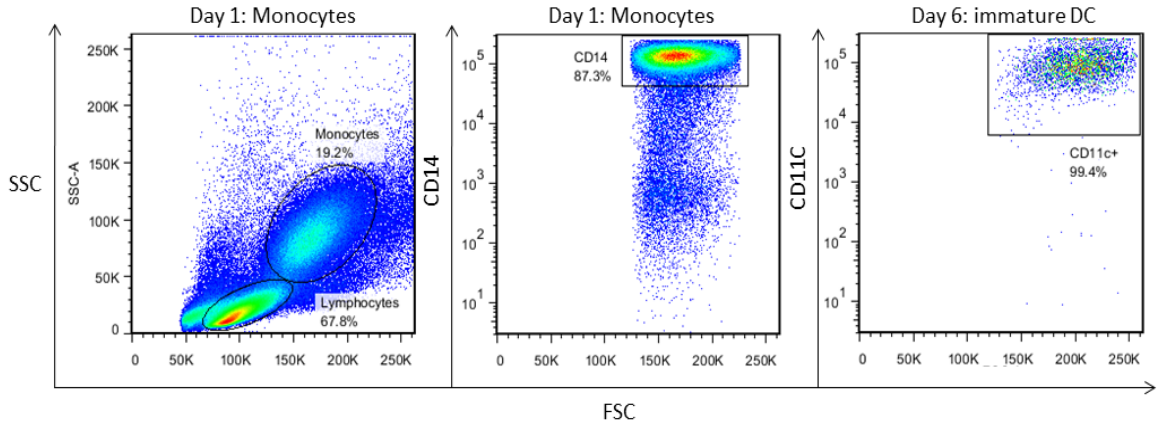

Figure 2.7. Flow cytometric dot plots of monocyte-derived DC. Left panel, monocytes in a PBMC sample on a dot plot of SS vs FS. Centre panel, dot plot showing monocytes after magnetic bead enrichment of $CD14^+$ cells. Right panel, monocyte-derived iDC ($CD11c^+$) after culturing monocytes with IL-4 and GM-CSF for 6 days.

2.6.6 Preparation of non-γδ T cell lines

αβ T cells were obtained by purifying $CD3^+$ cells from human PBMC by positive selection magnetic bead cell sorting (Miltenyi Biotec) and stimulating with 1 µg/ml PHA-P, which stimulates T cell activation and proliferation. For the magnetic bead cell sorting, 70 µl CD3 Microbeads and 280 µl buffer were added per 5 x 10^8 cells and mixed well and incubated for 15 min on ice. The magnetically-labeled cells were

washed in MACS buffer (3 ml per 1 x 10^7 cells) and centrifuged at 300 g for 10 min and the cell pellets were re-suspended in buffer (5 ml per 1 x 10^7 cells). The magnetic separation was carried out as described above, with CD3 positive cells being collected within the column and CD3 negative cells flushed through. Cells were used fresh for T cell proliferation assays. For αβ T cell lines, the cells were plated in 24-well tissue culture plates at a cell density of 1 x 10^6 cells per ml and expanded in cRPMI supplemented with IL-2 (50 IU/ml) for 2 weeks. The medium was replaced twice weekly by removing half of the supernatant and replacing with fresh IL-2-supplemented medium. Cell purity prior to and following expansion was determined using mAb against CD3 and analysed by flow cytometry (Fig. 2.8).

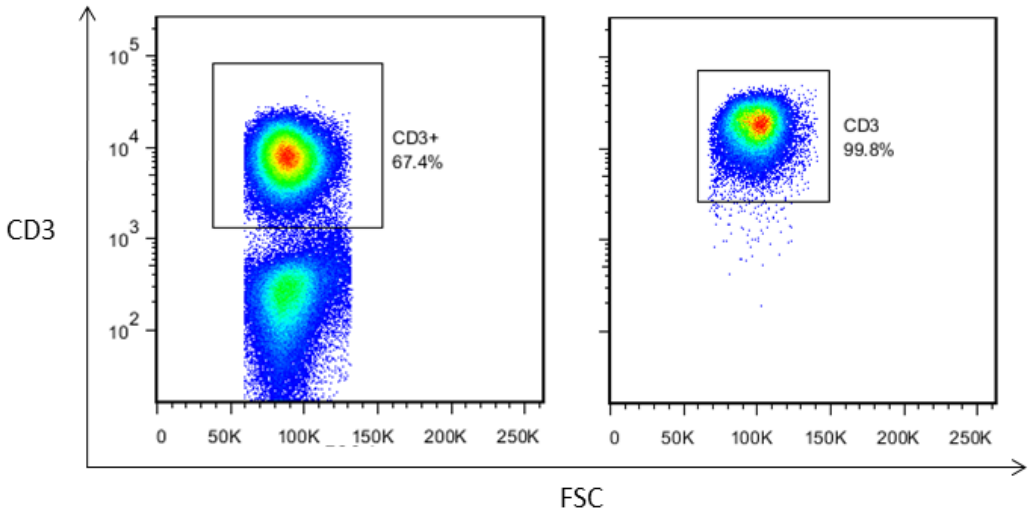

Figure 2.8. Flow cytometric dot plots of Non-γδ CD3 T cells. A PBMC sample was examined for CD3 expression by flow cytometry before and after magnetic bead cell sorting of CD3 T cells. Left Panel, CD3 T cells within PBMC. Right panel, CD3 T cells following magnetic bead cell sorting.

2.6.7 iNKT cell expansion

PBMC were enriched by magnetic bead selection of Vα24$^+$-Jα18$^+$ Vβ11$^+$ iNKT cells (Miltenyi Biotec). 100 µl of beads and 400 µl of MACS buffer were added per 1 x 10^8 cells and incubated for 15 min. The magnetically-labeled cells were then washed with 2 ml MACS buffer per 1x10^8 cells and centrifuged at 300 g for 10 min. The magnetic separation was carried out as described above, with CD3 positive cells being collected

within the column and CD3 negative cells flushed through. $CD3^+$ $V\alpha24^+$ $V\beta11^+$ cells were then sorted by flow cytometry and then cultured in 96 well round bottom plates at 1 x 10^3 iNKT cells and 2 x 10^5 irradiated allogeneic PBMC per well in iNKT medium (RPMI supplemented with 10% Hyclone fetal calf serum, 1% penicillin-streptomycin, 1% fungizone, 50 µM L-glutamine, 50 µM β-metrcaptoethanol, 1% non-essential amino acids, 1% essential amino acids, 1 mM sodium pyruvate and 25 mM HEPES). The cells were stimulated with 1 µg/ml PHA and 250 IU/ml IL-2. The medium was replaced with fresh iNKT medium at 24 h intervals for the first 48 h, and then every 3-4 days for 3-4 weeks.

2.6.8 HeLa cervical cancer cells

HeLa cells (immortalised cervical cancer cells) transfected with CD1a, CD1b, CD1c, CD1 and mock-transfected, which were a kind gift from Dr. Steven Porcelli (Albert Einstein College of Medicine, New York) were maintained in culture flasks in complete DMEM medium (DMEM + GlutaMax TM-1 [+] 4.5 g/L glucose, [-] pyruvate, supplemented with 10% HyClone fetal calf serum, 1% penicillin-streptomycin, 1% fungizone, and 25 mM HEPES; Gibco-BRL, Paisley, UK and Thermo-Scientific, Logan, UT) and split 1 in 5 or as required, twice weekly. The cells were used when they reached 80% confluency. Due to the adherent nature of the cells, they were trypsinised using 0.05% Trypsin-EDTA (Gibco-BRL, Paisley, UK) before use. Cell line purity was determined using mAb specific for CD1a, CD1b, CD1c and CD1d (Fig. 2.9).

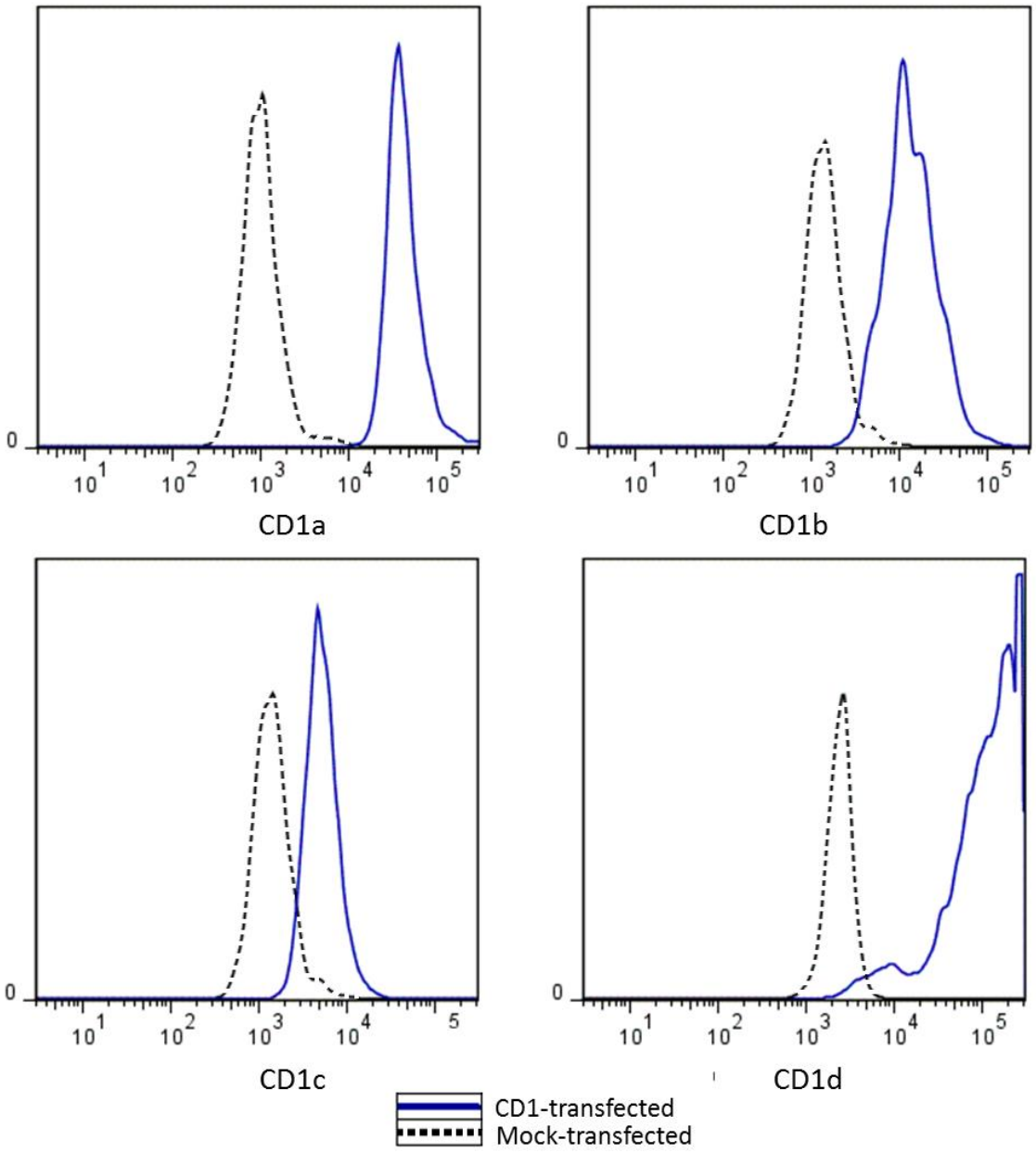

Figure 2.9. Flow cytometric dot plots of CD1-transfected HeLa cells. Cells were trypsinised and stained with mAb for CD1a, CD1b, CD1c and CD1d. Mock-transfected HeLa cells were negative for all four markers.

2.7 Analysis of cell surface markers and secreted factors from cell cultures

2.7.1 Analysis of co-stimulatory marker expression

The expression of antigen presentation markers was measured using flow cytometry. Cells of interest were cultured for 24 h. The plates were centrifuged and supernatants were collected and frozen at -20°C for later analysis of cytokines by ELISA. The cells cultures were stained for surface markers for the cells of interest and markers of

antigen presentation CD40, CD80, CD86 and HLA-DR. Surface expression of these markers was compared by mean fluorescence intensity obtained using flow cytometry.

2.7.2 Analysis of cytokine release from cell cultures

A sandwich ELISA system was used to quantify cytokines present in cell culture supernatants. Cells of interest were cultured for 24 h in round-bottomed 96-well tissue culture plates at a density of 1 x 10^5 cells per well in complete RPMI. The supernatants were harvested after 24 h and assayed for levels of IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17 or TNF-α by ELISA using antibody pairs (Table 2.10) purchased from R&D Systems (Abingdon, UK) or Biolegend. Nunc Immunoplates (Thermo Scientific, Denmark) were coated with mouse anti-human capture antibody, which bound any antigen present. The capture antibody was diluted in PBS to working concentration and plated in triplicate at 50 µl per well overnight to allow the antibody to coat the plate. The following day the plates were washed in wash buffer (PBS with 0.05% Tween). Antibody standards were diluted to create a 7 point curve using 2-fold serial dilutions in PBS. The standards and supernatants were plated in triplicate at 50 µl per well and left to incubate in the dark at room temperature for 2 h. The only cytokine that would bind to the plate is the one that the capture antibody is specific for. The plates were washed to remove unbound antigen and coated with the goat anti-human biotinylated detection antibody for 2 h. The detection antibody would bind only to the cytokine it is specific for. Therefore, any cytokine of interest in the supernatant should have bound to the capture antibody, and the detection antibody bound to a different epitope on the same cytokine. After washing the plates, horseradish-peroxidase-conjugated streptavidin was added (5 µl/ml) for 15 min, followed by washing. Colour substrate tetramethylbenzidine (TMB, Sigma Aldrich) was added at 50 µl per well and left to incubate in the dark for 15 min, as the solution is light sensitive. Addition of TMB caused the colour to change to blue at a level proportionate to the concentration of cytokine in the sample. Stop solution (2N H_2SO_4, Sigma) was added at 25 µl per well to stop the reaction, and the plates were read spectrophotometrically at 450 nm and 620 nm using a Tecan Sunrise (Tecan, Switzerland). Readings from the reference filter were automatically subtracted from readings at 450 nm to correct for optical imperfections in the plate. Optical density values were transferred into an Excel

(Microsoft) file, where a standard curve was computed to determine the cytokine levels (pg/ml). The procedure is summarised in Fig. 2.10.

Table 2.10. ELISA antibodies used and their standard concentrations.

ELISA kit	Top standard concentration	Capture antibody working concentration	Detection antibody working concentration	Company
IFN-y Duoset	1000 pg/ml	4 µg/ml	50 ng/ml	R&D Systems
IFN-y Deluxe	500 pg/ml	1:200	1:200	Biolegend
IL-2 Duoset	1000 pg/ml	4 µg/ml	200 ng/ml	R&D Systems
IL-4 Duoset	2000 pg/ml	4 µg/ml	75 ng/ml	R&D Systems
IL-4 Deluxe	250 pg/ml	1:200	1:200	Biolegend
IL-6 Duoset	600 pg/ml	2 µg/ml	50 ng/ml	R&D Systems
IL-10 Duoset	2000 pg/ml	2 µg/ml	150 ng/ml	R&D Systems
IL-10 Deluxe	250 pg/ml	1:200	1:200	Biolegend
IL-12p40 Deluxe	4000 pg/ml	1:200	1:200	Biolegend
IL-12p70 Duoset	2000 pg/ml	4 µg/ml	100 ng/ml	R&D Systems
IL-12p70 Deluxe	1000 pg/ml	1:200	1:200	Biolegend
IL-13 Duoset	6000 pg/ml	2 µg/ml	150 ng/ml	R&D Systems
IL-17 Duoset	1000 pg/ml	4 µg/ml	150 ng/ml	R&D Systems
TNF-a Deluxe	500 pg/ml	1:200	1:200	Biolegend

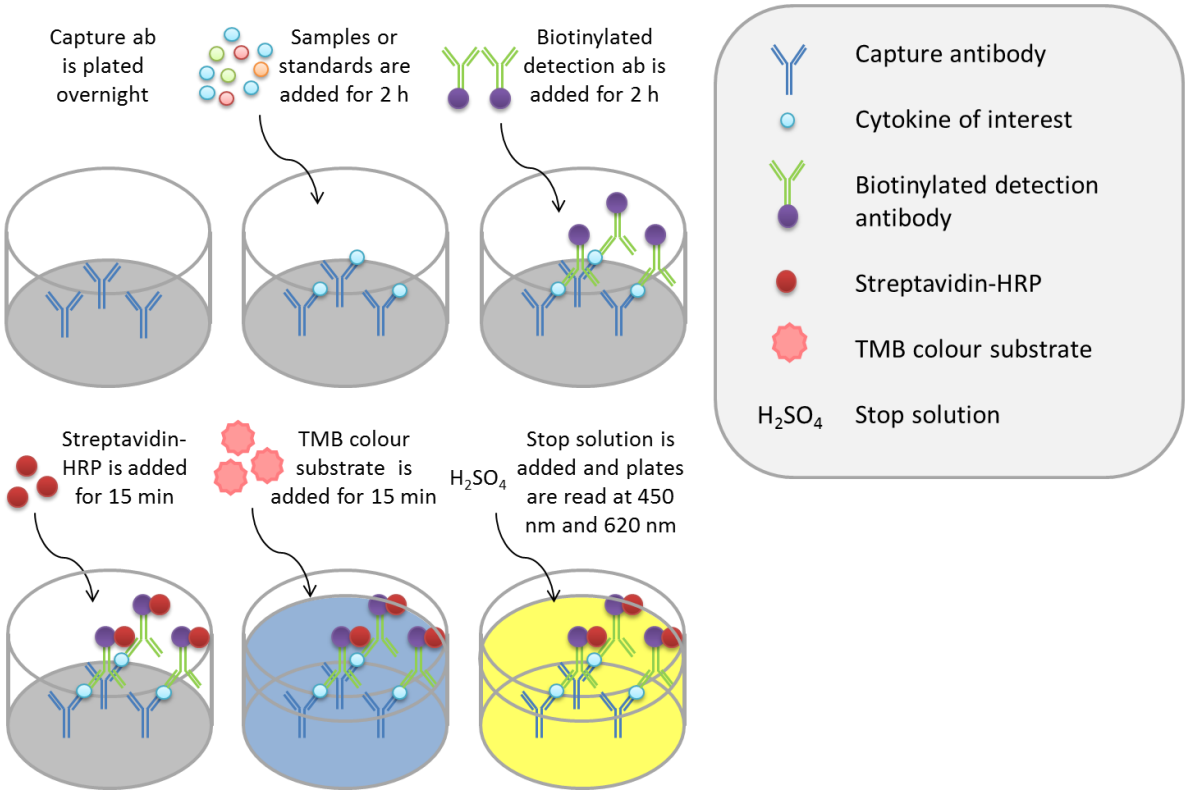

Figure 10. Step-by-step ELISA protocol. Immunoplates were coated with capture antibody overnight (Table 6). Standards of known cytokine concentrations and cell culture supernatants were added for 2 h to allow for antigens to bind to the antibody. A secondary, biotinylated antibody was then added for 2 h followed by Streptavidin-HRP which bound to biotin. TMB substrate bound to Streptavidin-HRP and produced a colour change from clear to blue. Once the colour is fully developed, stop solution was added to stop the reaction, and this turned the colour to yellow. The plates were read immediately.

2.7.3 Analysis of intracellular cytokine production

Whilst ELISA analysis of cell culture supernatants allows quantitative determination of cytokines, it cannot discern the cellular source of the secreted cytokines. Using flow cytometric analysis, the cellular cytokine source may be determined by staining cells with cell surface antibodies for the particular cells and against individual cytokines. In order to accumulate cytokines within cells to obtain a detectable signal, protein secretion was inhibited using monensin. Monensin is derived from *Streptomyces cinnamonensis* and is a Na^+ ionophore that inhibits trans-Golgi transport by collapsing intracellular Na^+ and H^+ gradients that are required for protein transport

(Mollenhauer, et al., 1990). Cells of interest were co-cultured or cultured separately in the presence of monensin (10 µl/ml, Biolegend) for 4-16 h to promote intracellular accumulation of cytokines. The cultures were set up on their own or in the presence of phorbol myristate acetate (PMA, 10 ng/ml) and ionomycin (1 ug/ml). PMA activates cells through protein kinase C, while ionomycin is a calcium ionophore which affects activation potential across cell membranes. Together, these two compounds stimulate all cells to proliferate and produce cytokines, thus serving as a positive control (Chatila, et al., 1989). After the incubation, the cells were washed and stained for cell surface expression of markers for the cells of interest. The cells were washed with PBA and fixed with 4% paraformaldehyde (4 g PFA, 90 ml PBS and 40 µl NaOH heated to 60°C and cooled to 4°C prior to use). The cells were then washed and treated with 0.2% saponin (0.05 g saponin in 25 ml PBA) which permeabilises the cell membrane, allowing the antibodies to enter the cell and bind to the cytokines. The cells were stained for intracellular expression of IFN-γ, IL-4, IL-6, IL-10, IL-12/23, IL-13, IL-17a, IL-23 and TNF-α using fluorochrome-conjugated mAb obtained from Biolegend and eBioscience. The cells were then washed to restore membrane integrity and analysed by flow cytometric analysis.

2.7.4 Measurement of antibody production by B cells

Antibody production was assessed using an immunoglobulin cytometric bead array kit (BD Biosciences, US). B cells were cultured alone or with T cells in an equal ratio for 7 days. The supernatants were harvested and tested for IgA, IgM and total IgG. IgM and IgA were multiplexed, whereas IgG was run as a single plex. The standards were reconstituted in assay diluent for 15 min, and two-fold serial dilutions were performed as follows: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 0. Wells of 96 well plates were coated with capture antibody, followed by either sample or standard and allowed to incubate for 1 h at room temperature. The plate was then washed and coated with PE detection reagent and incubated for 2 h at room temperature, protected from light. The plate was washed, and the samples were fixed in 4% PFA and re-suspended in wash buffer. The flow cytometer was set up using the instrument setup beads which included unstained beads and single stained beads for each APC, APC-Cy7 and PE. IgG, IgA and IgM populations were identified on dot plots of APC-Cy7

vs APC (Fig. 2.11A-C) followed by geometric MFI of PE (Fig. 2.11D). A standard curve was computed using the standards and was used to determine the immunoglobulin concentration in the samples.

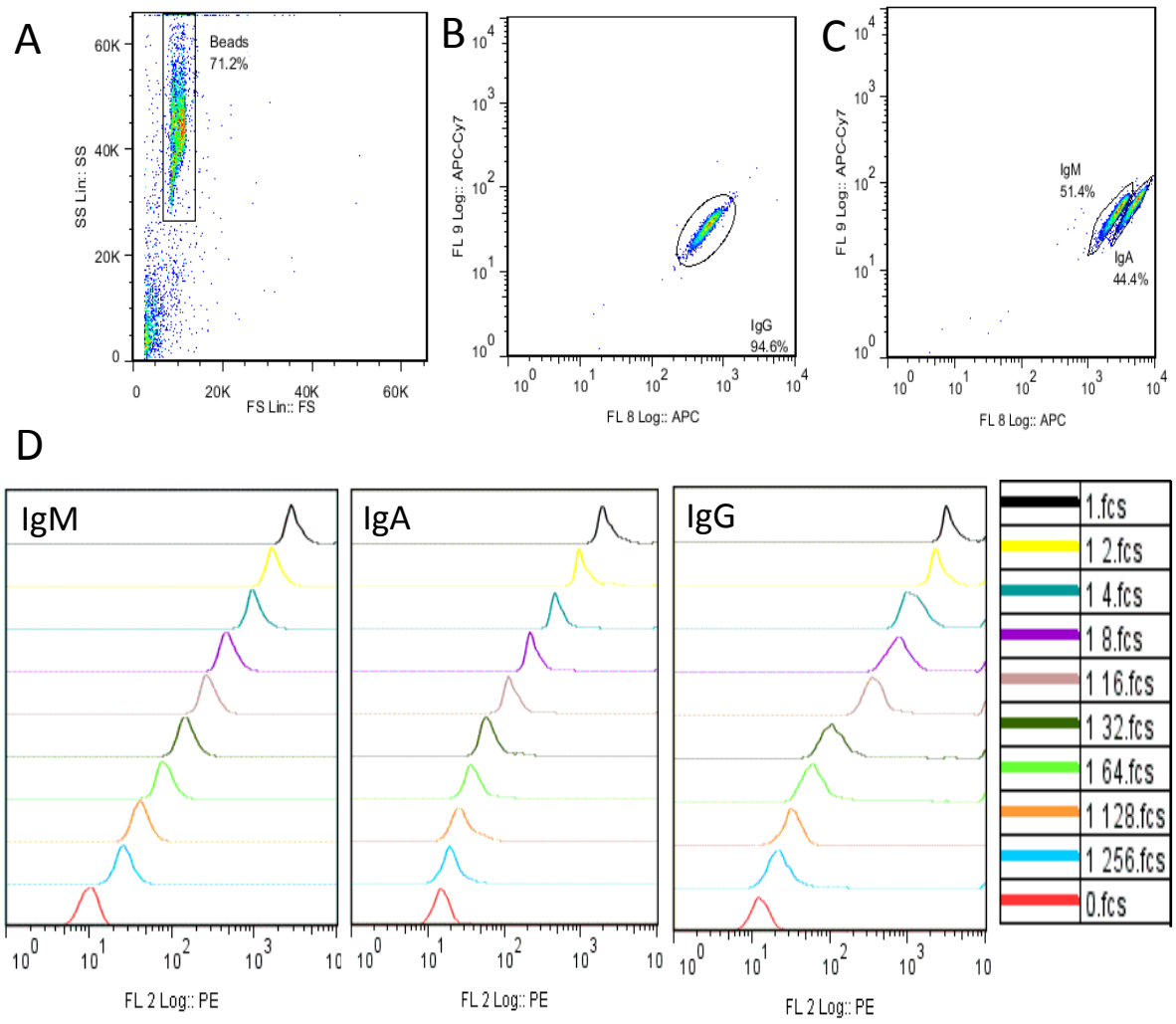

Figure 11. Immunoglobulin production by B cells. A, a FS vs SS dot plot depicting the bead population. **B**, dot plot of IgG-positive beads; **C**, dot plots of IgM- and IgA-positive beads. **D**, histograms of the standards are shown for each immunoglobulin subtype from highest to lowest standard.

2.7.5 Blocking experiments

To examine the molecules involved in activation of DC and B cells, co-cultures were carried out in the presence or absence of low-endotoxin, azide-free functional grade blocking antibodies (10 µg/ml) against CD86, CD40L, IFN-γ and IFN-γ receptor, IL-4 and IL-4 receptor, or TNF-α or isotype control mAb for 24 h. Similar cell cultures (without

the addition of mAb) were set up in transwell plates to prevent cell contact. The effects of blocking on DC and B cell phenotypes, cytokine expression and release and antibody production were determined as described above.

2.7.6 Examination of T cell proliferation and resulting cytokine release

The ability of specific cells to stimulate proliferation of labeled autologous or allogeneic T cells was tested by flow cytometric analysis of CellTrace™ (Invitrogen) dilution. PBMC were enriched for CD3 positive cells using a Miltenyi isolation kit and stained using a freshly prepared CellTrace™ solution. A vial of component A of the CellTrace kit was dissolved in 20 µl DMSO (component B) to obtain a 5 mM stock solution. This solution was diluted 1 in 5. The solution was added at 1 µl per ml of cell sample (cells at $1x10^6$ per ml) for a final working concentration of 5 µM. The cells were stained for 20 min at 37°C in the dark. The unbound dye was quenched by adding 5 times the amount of staining volume and incubating for 5 min. The cells were pelleted and re-suspended in fresh pre-warmed culture medium. The cells were incubated for 10 min before to allow the stain to undergo acetate hydrolysis. The CellTrace™-labelled allogeneic resting $CD3^+$ T cells were added to the stimulating cell culture at a ratio of 10:1 and cultured for 6 days. Supernatants were collected on days 3-5 of the co-culture. PHA-P-stimulated αβ T cells cultured with IL-2 and irradiated PBMC were used as positive controls. On day 6, cells were stained using cell surface markers and analysed by flow cytometry. A characteristic plot of proliferation peaks is shown below (Fig. 2.12). Celltrace violet was detected on FL7 on the violet laser.

In addition to examining T cell proliferation in response to Vδ2-matured DC and B cells, we examined cytokine production by the T cells. Resting $CD3^+$ T cells were added at 10:1 or 1:1 ratios to the stimulating cell culture for 3 days. The supernatants were harvested and examined for cytokine secretion by ELISA.

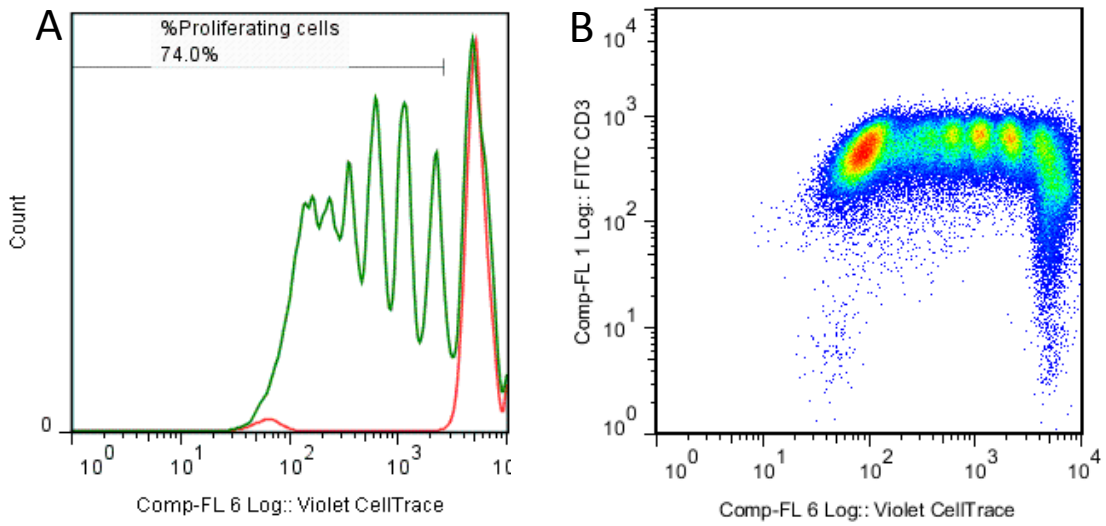

Figure 2.12. T cell proliferation peaks shown by dilution of Cell Trace dye. A, negative control sample of unproliferating cells is shown in red, while a positive control sample of proliferating cells is shown in green. **B**, dot plot representatitve of the proliferating peaks is shown.

2.7.7 Cytotoxicity assay

Cytolytic degranulation against tumour cell lines was examined by flow cytometric analysis of CD107a expression. CD107a, also known as lysosomal membrane protein-1 (LAMP-1) is mainly found on the membrane of late endosomes-lysosomes, it has also been detected in early endosomal membranes, plasma membranes and the circulation. Lysosomes are membrane bound organelles rich in hydrolytic enzymes, responsible for degrading molecules through endocytosis or phagocytosis or through autophagy. Cytotoxic T lymphocyte and NK cell activation results in degranulation and secretion of cytotoxic granules which leads to CD107a to be detectable on the surface of effector cells. CD107a expression can be measured and has been shown to directly correlate with target cell death (Penack, et al., 2005).

2.8 Vδ3 and iNKT cell stimulation using glycolipids

Glycolipid stocks were stored at -70°C. Immediately prior to use in cell assays, the stocks were thawed at room temperature, mixed by vortexing for 1 min, heated to 80°C for 2 min and sonicated for 10 min. Working solutions were made by further diluting in cRPMI. The lipids were applied to HeLa cells immediately and pulsed overnight.

2.9 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism v 5.0 (San Diego, CA, USA). Paired and unpaired *t*-tests were used to compare the means between different treatment. Group comparisons were analysed using two-way ANOVA and Bonferroni post hoc tests. *P* values of <0.05 were considered statistically significant.

Chapter 3

Characterisation of γδ T cell subsets in fresh blood and expanded cell lines

3.1 Introduction

Human γδ T cells constitute a minor population of T cells in peripheral blood and a larger one in the epithelial tissues. They play important roles in sensing pathogens and other dangers and mediating rapid immune responses. Humans have three main subsets of γδ T cells - Vδ1, Vγ9Vδ2 and Vδ3 T cells - and these TCR can respond to a variety of stress-induced ligands on host cells, tumours and infected cells or phosphoantigens produced by various microbes or transformed host cells in an MHC-unrestricted way (Hayday, 2000). γδ T cells also express TLR and NK receptors, allowing them to also respond to many microbial components (Morita, et al., 2007). Once activated they can directly exert cytotoxicity against tumour cells (Dieli, et al., 2001, Wrobel, et al., 2007, Fisch, et al., 1990, Mattarollo, et al., 2007, Viey, et al., 2005), rapidly secrete cytokines, chemokines, antiviral and antimicrobial factors (Caccamo, et al., 2006, Agrati, et al., 2009, Cipriani, et al., 2000, Dudal, et al., 2006, Lehner, et al., 2000) and can stimulate other immune cells including monocytes, neutrophils, DC, B cells and other T cells, which in turn activate immune responses (Vantourout and Hayday, 2013, Moser and Eberl, 2007, Bonneville, et al., 2010, Chien, et al., 2014, Hayday, 2009, Holtmeier and Kabelitz, 2005).

Vδ1 T cells, which do not pair with a specific Vγ chain, are the second most common γδ T cell subset in human blood and are predominantly found in tissues such as skin and small intestine where they play important roles in homeostasis, tissue integrity and lipid surveillance (Siegers and Lamb, 2014). They do not recognise pAg or aminobisphosphonates, but instead recognise stress-induced self-ligands such as MICA, MICB and ULBP and lipids presented by CD1 molecules (Ismaili, et al., 2002, Leslie, et al., 2002, Spada, et al., 2000). These molecules are also upregulated on malignant cells (Diefenbach and Raulet, 2002, Gleimer and Parham, 2003, Raulet, 2003) thus allowing them to recognise these. An increased prevalence of Vδ1 T cells has been found in tumours expressing MICA and MICB (Groh, et al., 1999). Vδ1 T cells are also found to be expanded in some viral and bacterial infections and B cell chronic lymphocytic leukaemia. Expression of NKG2D by Vδ1 and Vγ9Vδ2 T cells is crucial for cytotoxicity against various cancers (Diefenbach and Raulet, 2002). Interestingly, unlike Vγ9Vδ2 T cells, Vδ1 T cells reactive to tumours can survive in the circulation for

years, thus reinforcing their role in tumour surveillance (Godder, et al., 2007, Lamb, et al., 1996).

Vδ3 T cells are the third most common subset in peripheral blood, but are found in higher numbers in the gut and liver and their frequency is also increased in chronic viral infections and leukaemia. The ligands that stimulate this subset are yet to be identified, but a subset of these have been reported to recognise CD1d (Mangan, et al., 2013).

Vγ9Vδ2 T cells are the predominant subset in blood. Their ligand specificities are well defined, and these include pAg, which are metabolites of the pathway of isoprenoid biosynthesis, a pathway essential for cell survival employed by most eukaryotic cells and many pathogens. There are two distinct pathways, the mevalonate pathway and the MEP (Fig. 1.3) pathway. The MEP pathway, which is used by many Gram-positive bacteria, mycobacteria, protozoa and parasites (Morita, et al., 2007), results in production of HMB-PP, the most potent ligand known to uniquely activate Vγ9Vδ2 T cells and which is not produced by human cells. Thus, since it is not found endogenously, HMB-PP specifically plays a role in recognition of foreign pathogens by Vγ9Vδ2 T cells. HMB-PP can be converted to IPP, another Vγ9Vδ2 T cell antigen of much lower potency than its precursor. IPP can also be produced via the mevalonate pathway used by most eukaryotes, and thus can serve as a self-ligand in human cells. However, the levels normally produced in human cells are not sufficient to activate Vγ9Vδ2 T cells. Cell transformation or dysregulation can result in overproduction of rate-limiting enzymes in this pathway, such as HMG-CoA reductase, or inhibition of the enzyme responsible for breaking down IPP, both of which result in increased IPP levels, and ultimately recognition by Vγ9Vδ2 T cells (Thedrez, et al., 2007, Gober, et al., 2003, Morita, et al., 2007, Idrees, et al., 2013, Riganti, et al., 2012). Some bacteria such as *Listeria monocytogens* and *Streptomyces* use both pathways (Heuston, et al., 2012). Therefore Vγ9Vδ2 T cells can sense foreign pathogens that employ the MEP pathway (Table 1.2), but can also sense cellular dysregulation and thus play an important role in immunosurveillance (Girardi, et al., 2001, Kabelitz, et al., 2007, Liu, et al., 2008, Zocchi and Poggi, 2004).

Aminobisphosphonates, which are synthetic compounds used for their antiosteoclastic activity, can also promote IPP accumulation by blocking the enzyme downstream of IPP. As a result, aminobisphosphonates such as zoledronate and pamidronate, which can induce Vγ9Vδ2 T cell proliferation *in vivo* and *in vitro* have gained attention in immunotherapies aimed at increasing circulating Vγ9Vδ2 T cell numbers in order to exploit their anti-infection and anti-tumour activities (Caccamo, et al., 2008, Chiplunkar, et al., 2009, Fisher, et al., 2014).

Unlike αβ T cells, Vγ9Vδ2 T cells are also efficient APC, referred to as γδ-T-APC, which upon activation are capable of expressing co-stimulatory molecules, processing peptide antigens and cross-presenting these to CD8 T cells and they can induce proliferation of conventional T cells. All these are features of professional APC such as DC, but in contrast to DC, the effects in γδ-T-APC are not transient (Moser and Brandes, 2006, Moser and Eberl, 2007) and thus are superior to DC in this regard.

High numbers of pure populations of Vγ9Vδ2 T cells can be obtained *in vitro* owing to the discovery that pAg and aminobisphosphonates exclusively expand the Vγ9Vδ2 T cell subset. This can be done directly from PBMC using HMB-PP (Dunne, et al., 2010, Petrasca and Doherty, 2014). BrHPP has been shown to expand Vγ9Vδ2 T cells *in vivo* and *ex vivo* in cancer patients (Bennouna, et al., 2010, Chargui, et al., 2010). Metabolite dimethylallyl diphosphate (DMAPP), which is an isomer of IPP, can also stimulate Vγ9Vδ2 T cells, but its bioactivity, like that of IPP, is 10,000 fold lower than that of HMB-PP (Morita, et al., 2007, Riganti, et al., 2012). As already mentioned above, zoledronate and other aminobisphosphonates are currently used in clinical trials for cancer treatment for their ability to stimulate Vγ9Vδ2 T cell expansion *in vivo* (Caccamo, et al., 2008, Sicard, et al., 2005). Other strategies involve *ex vivo* expansion of γδ T cells in the presence of IL-2 and adoptively transferring these back into the patients (Izumi, et al., 2013). Although several expansion methods have been described and are currently used in clinical trials, there is a need to characterise the functions of differentially-activated γδ T cells in order to determine the best mode of activation to yield the desired immune responses *in vivo*.

3.2 Objectives

Since γδ T cells show promise as immunotherapeutic agents, the aim of this chapter was to investigate and compare the functional capacities of pAg- or zoledronate-expanded Vδ2 T cells and PHA-expanded Vδ3 T cells by examining their phenotypes and cytokine profile. In addition we wished to compare human Vδ1, Vδ2 and Vδ3 T cell subset frequencies, phenotypes and cell surface markers in fresh blood and compare these to those of expanded γδ T cell lines.

The specific objectives were:

- To phenotypically characterise γδ T cell subsets in peripheral blood by examining their differentiation status, CD4 and CD8 expression and expression of stimulatory surface markers
- To phenotypically and functionally characterise the human Vδ2 T cell lines generated using two different methods of activation: using pAg versus aminobisphosphonate
- To phenotypically and functionally characterise Vδ3 T cell lines generated from human blood using PHA activation

3.3 Methods

3.3.1 Phenotypes of peripheral blood γδ T cell subsets

Fresh human PBMC (section 2.6.1) were phenotyped for expression of CD3, Vγ9, Vδ1, Vδ2 and Vδ3, CD4 and CD8 subsets, memory phenotype, and expression of cell activation markers, NK markers and TLR. Since γδ T cell subsets are found at very low frequencies in blood, γδ TCR^+ PBMC were isolated and phenotyped for a clearer distinction of sub-populations (see section 2.6.2). Some markers were included for analysis at a later stage and therefore are depicted using lower n numbers.

3.3.2 Expansion and phenotyping of Vδ2 T cell lines

Vδ2 T cells were obtained by stimulating magnetically-purified γδ T cells with HMB-PP and culturing for 14-28 days in IL-2-supplemented media (see section 2.6.2). The expanded cell lines were then examined for CD3, Vγ9 and Vδ2 expression, CD4 and CD8 subsets, memory phenotype and expression of cell activation markers, NK markers and TLR. Vδ2 T cells were also expanded using the aminobisphosphonate, zoledronate from PBMC, without magnetic bead enrichment (see section 2.6.2). The resulting Vδ2 percentages and cytokine profiles were examined by flow cytometry and compared to those of the phosphoantigen-stimulated Vδ2 T cells. Cytokines for which statistical significance was not reached were examined using additional n numbers than those that reached significance at lower n numbers.

3.3.3 Expansion and phenotyping of Vδ3 T cell lines

Vδ3 T cells were derived by magnetically-enriching PBMC for γδ T cells and sorting $CD3^+V\delta3^+$ T cells by flow cytometry. The pure sample of Vδ3 T cells was stimulated with PHA-P and cultured with irradiated PBMC in IL-2-supplemented media for 28 days (see section 2.6.3). The expanded cell lines were then examined for CD3 and Vδ3 expression and CD4 and CD8 subsets. Cytokine profiles of Vδ3 T cell lines were examined by flow cytometry and ELISA.

3.4 Results

3.4.1 Phenotypes of peripheral blood γδ T cell subsets

The frequency of γδ T cell subsets in peripheral blood of healthy donors was characterised by flow cytometry. It was confirmed that Vδ2 T cells are the predominant subset, as commonly reported in the literature, followed by Vδ1 T cells and lastly Vδ3 T cells, which are the least common subset (Fig. 3.1A). Various Vγ chains can pair up with human Vδ chains. We found that Vγ9 expression, as expected, reflected the expression of Vδ2 in the same donors (Fig. 3.1B,C). Since the Vδ2 chain is known to specifically pair with the Vγ9 chain, we looked for its presence in several donors. We investigated whether all Vδ2 T cells were positive for the Vγ9 chain and found that while there were some $Vγ9^+$ cells lacking Vδ2 expression (Fig. 3.2), there were virtually no Vδ2 T cells that lacked Vγ9 expression, thus confirming that by staining for the Vδ2 chain is sufficient for identifying all Vγ9Vδ2 T cells. Next, we examined whether Vδ3 T cells paired with the Vγ9 chain and found that only a small percentage of Vδ3 T cells were also positive for Vγ9 (Fig. 3.3). The $Vγ9^+Vδ3^-$ cells observed were $Vδ2^+$.

We also examined the percentage of the Vδ1, Vδ2 and Vδ3 T cell subsets as a percentage of γδ TCR^+ cells. Since γδ T cell subsets are found at low frequency in PBMC (usually <3% of lymphocytes), we carried this out in magnetically sorted PBMC which contained 40-70% γδ TCR^+ lymphocytes. It was confirmed, as in PMBC, that Vδ2 T cells were the main subset, followed by Vδ1 T cells and Vδ3 T cells (Fig. 3.4).

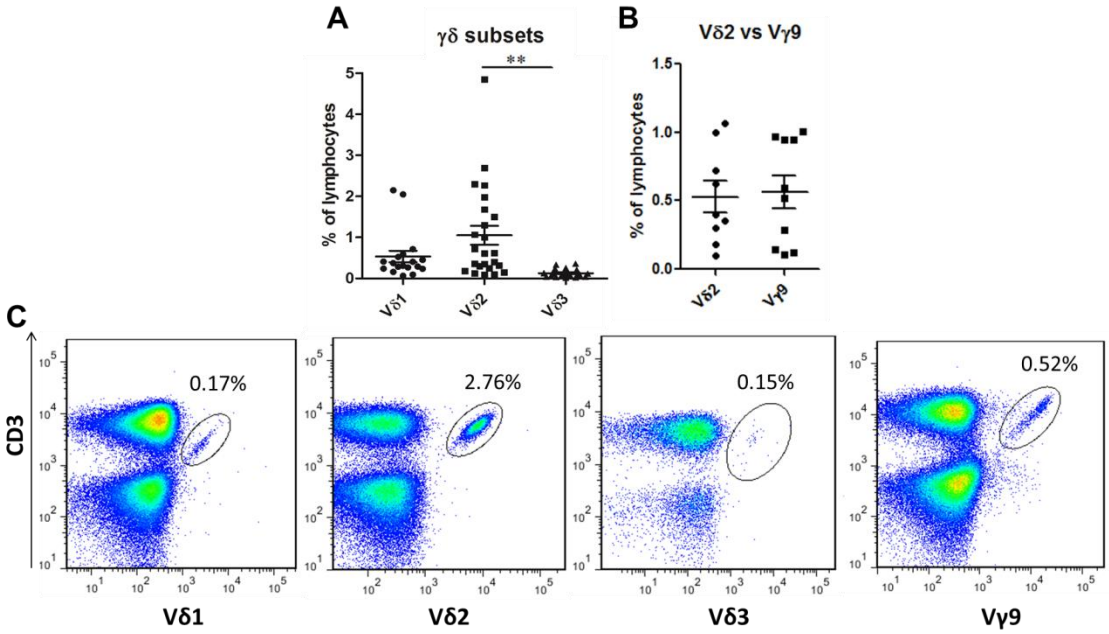

Figure 3.1. Frequency of γδ T cell subsets in peripheral blood. PBMC were isolated from human buffycoat packs and stained for surface expression of CD3, Vδ1, Vδ2, Vδ3 and Vγ9 (n=17-24). The difference in n numbers between the subsets is due to the inclusion of additional donors phenotyped for Vδ2 expression. **A**, frequency of Vδ1, Vδ2 and Vδ3 T cells in fresh PBMC samples. **B,** expression of Vδ2 versus Vγ9 in a proportion of the donors phenotyped in part A (n=10). **C,** representative flow cytometric dot plots showing Vδ1, Vδ2, Vδ3 and Vγ9 T cell subsets, respectively. ** $p<0.01$ using a paired *t* test.

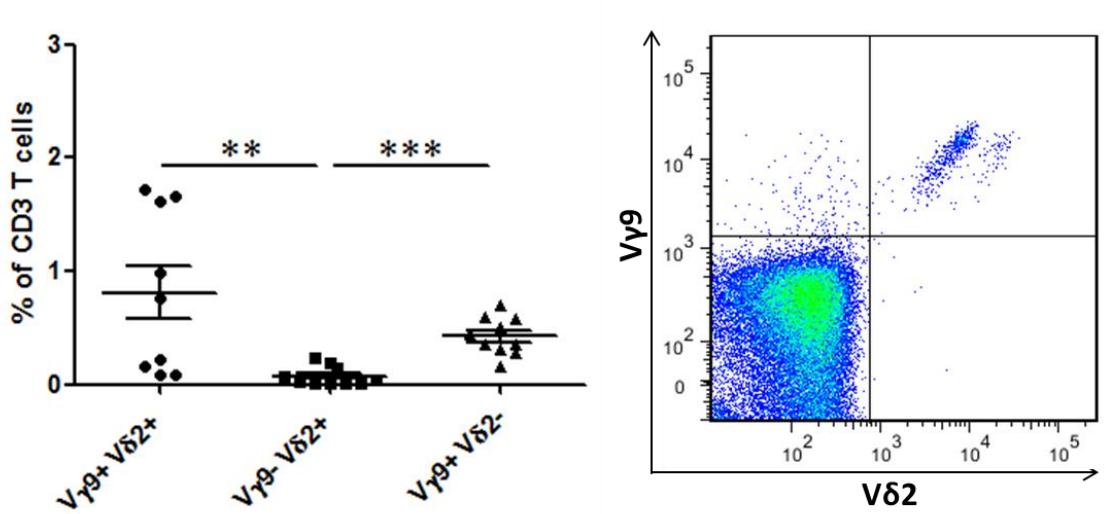

Figure 3.2. Flow cytometric analysis of Vγ9 and Vδ2 expression in fresh PBMC. PBMC were isolated from human buffycoat packs and stained for cell surface co-expression of Vγ9 and Vδ2 by flow cytometry. Left panel, percentage of Vγ9 and Vδ2 expression by $CD3^+$ T cells (n=10). Right panel, representative flow cytometric dot plot showing Vγ9 and Vδ2 expression by $CD3^+$ T cells. These donors are the same as those depicted in Fig. 3.1B. ** $p<0.01$, *** $p<0.001$ using a paired *t* test.

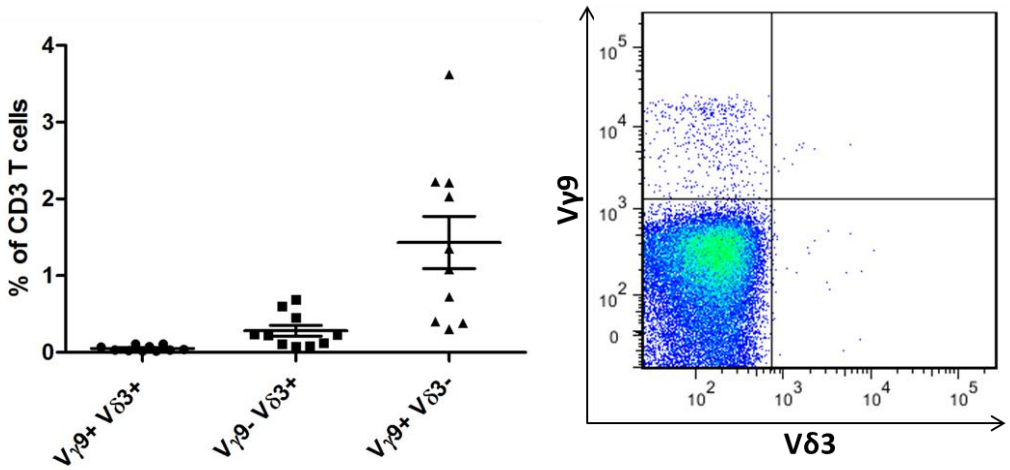

Figure 3.3. Flow cytometric analysis of Vγ9 and Vδ3 expression in fresh PBMC. PBMC were isolated from human buffycoat packs and stained for cell surface co-expression of Vγ9 and Vδ2 by flow cytometry. Left panel, percentage of Vγ9 and Vδ3 expression in $CD3^+$ T cells (n=10). Right panel, representative flow cytometric dot plot showing Vγ9 and Vδ3 expression in $CD3^+$ T cells.

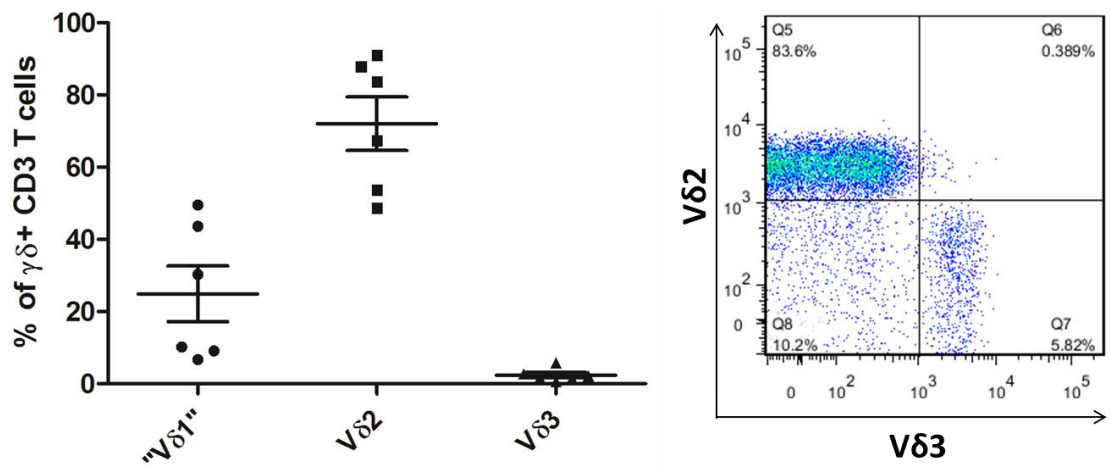

Figure 3.4. Frequency of γδ T cell subsets in γδ T cell-enriched PBMC. PBMC were isolated from human buffycoat packs and magnetically sorted for γδ TCR^+ cells. γδ T cell-enriched PBMC containing a high percentage of γδ T cells were stained for surface expression of CD3, γδ TCR, "Vδ1", Vδ2 and Vδ3 (n=6). Left panel, frequency of "Vδ1", Vδ2 and Vδ3 T cells as a percentage of γδ TCR^+ T cells. Right panel, representative flow cytometric dot plot showing Vδ2 and Vδ3 T cell subsets. $Vδ2^-$ $Vδ3^-$ γδ TCR^+ cells were termed Vδ1 T cells. This was because the magnetic particles used to enrich for γδ TCR^+ cells are FITC-labeled, and therefore we could not directly detect Vδ1 T cells using anti-Vδ1 antibody, as this was also FITC-labeled.

3.4.2 Frequency of CD4 and CD8 T cells in peripheral blood γδ T cell subsets

Next, we defined the CD4 and CD8 subsets in Vδ1 (Fig. 3.5A), Vδ2 (Fig. 3.5B) and Vδ3 (Fig. 3.5C) T cells and found that the majority were $CD4^{-}CD8^{-}$ in each of the subsets, with the next most common being CD8 T cells, and $CD4^{+}CD8^{+}$ T cells being least common. Similar results were also observed in enriched γδ TCR^{+} PBMC (Fig. 3.6), although there was a reduction in $CD4^{+}$ cell frequency following magnetic bead cell sorting of γδ TCR^{+} cells. There, $CD4^{-}CD8^{-}$ comprised >90% of cells, while in fresh PBMC it ranged from 50% to 80%.

3.4.3 Memory phenotypes of peripheral blood γδ T cell subsets

We also investigated the memory phenotype of γδ T cell subsets and found that they mainly exhibited a naïve phenotype (Fig. 3.7), while very few exhibited an effector memory phenotype, which was to be expected in resting cells of a healthy individual.

3.4.4. Expression of stimulatory surface markers in peripheral blood γδ T cell subsets

Since it is known that Vδ2 T cells express NK cell markers and TLR receptors, we looked for expression of these and other markeres by Vδ1, Vδ2 and Vδ3 T cells. It was found that while a small proportion (~5%) of Vδ1 and Vδ2 T cells expressed CD69 (Fig. 3.8), a marker of T cell activation, a larger proportion (~15%) of Vδ3 T cells expressed CD69. When examining for TLR2 expression by the γδ T cells subsets, it was found that 5-10% of each subset expressed TLR2 (Fig. 3.9), thus conferring the ability to recognise various pathogens. We also looked for expression of NKG2D, an NK cell marker which mediates recognition of tumours cells expressing NKG2D ligands, by the γδ T cell subsets and found that 5-10% expressed NKG2D (Fig.3.10). We also found expression of CD161, another NK marker, on ~10% of Vδ1 T cells and on ~15-20% of Vδ2 and Vδ3 T cells (Fig. 3.11).

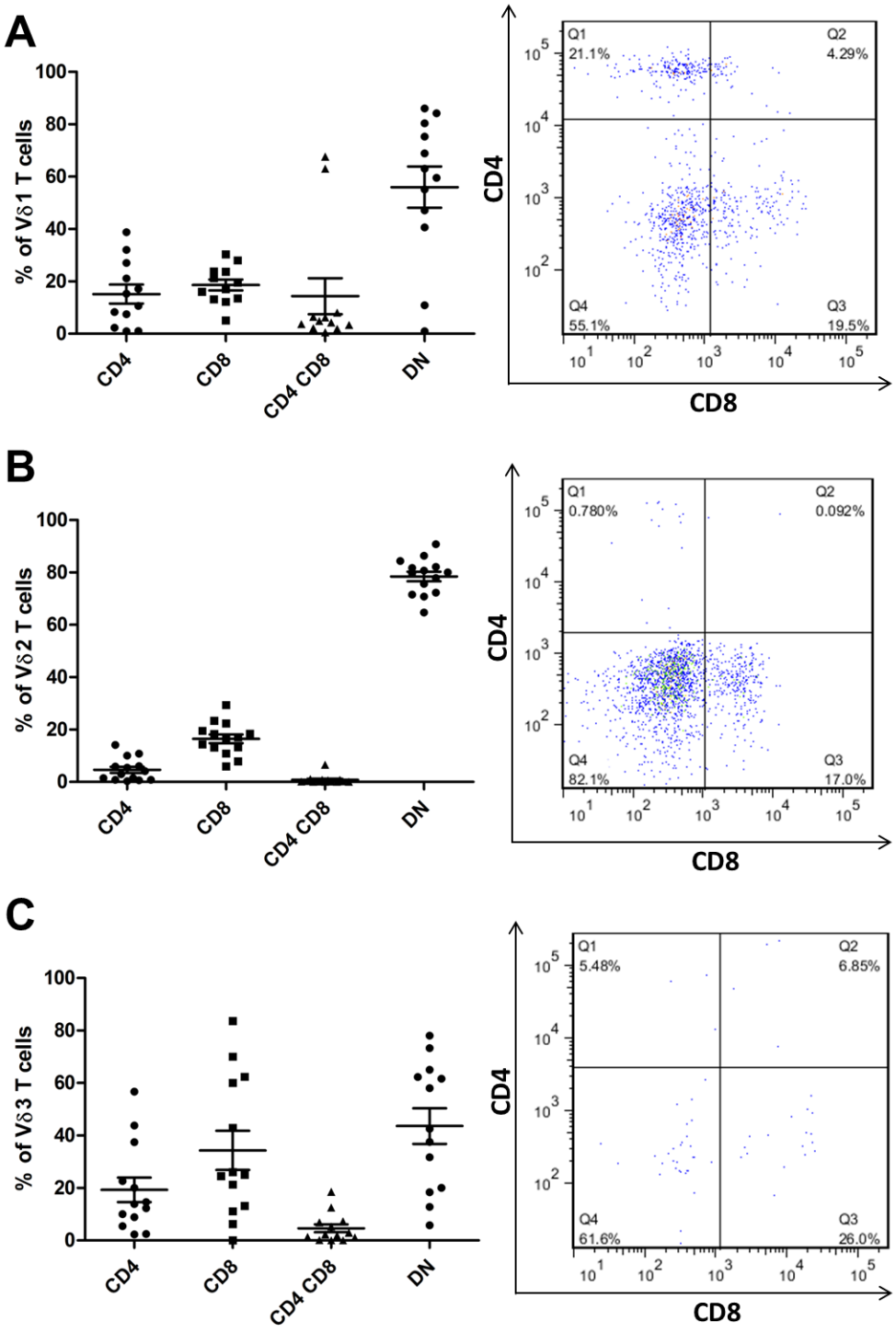

Figure 3.5. Flow cytometric analysis of CD4 and CD8 subsets in fresh PBMC. PBMC were isolated from human buffycoat packs and stained for cell surface expression of CD3, CD4, CD8, Vδ1, Vδ2 and Vδ3. A-C left panels, CD4 and CD8 subsets of Vδ1 (**A**; n=12), Vδ2 (**B**; n=13) and Vδ3 (**C**; n=13) T cells. Right panels, representative flow cytometric dot plots showing CD4 and CD8 subsets of Vδ1, Vδ2 and Vδ3 T cells.

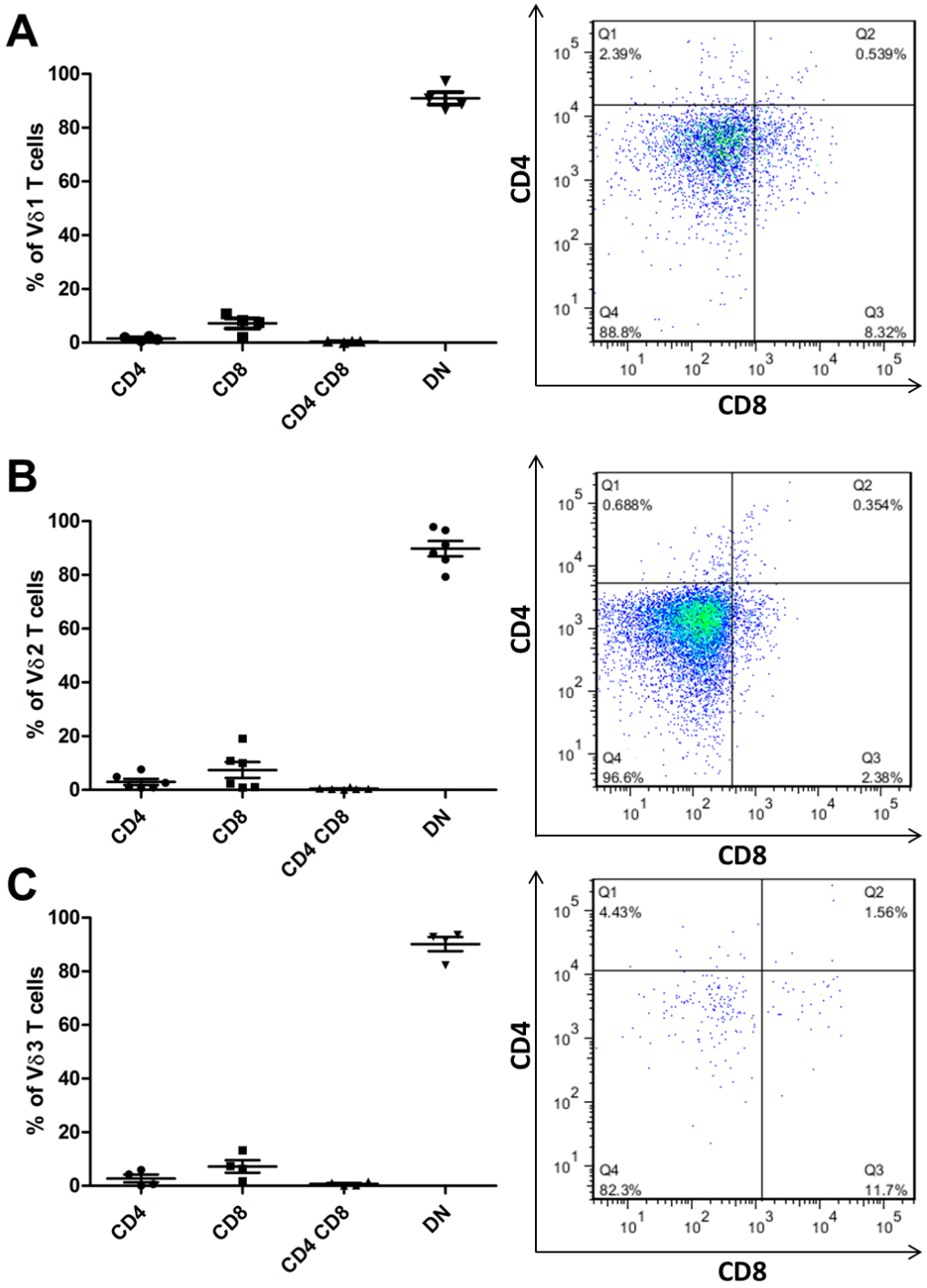

Figure 3.6. Flow cytometric analysis of CD4 and CD8 subsets in γδ-enriched PBMC. PBMC were enriched for γδ TCR$^+$ cells and stained for cell surface expression of CD3, CD4, CD8, Vδ1, Vδ2 and Vδ3. A-C left panels, CD4 and CD8 subsets of Vδ1 (**A**; n=4), Vδ2 (**B**; n=6) and Vδ3 (**C**; n=4) T cells. Right panels, representative flow cytometric dot plots showing CD4 and CD8 subsets of Vδ1, Vδ2 and Vδ3 T cells.

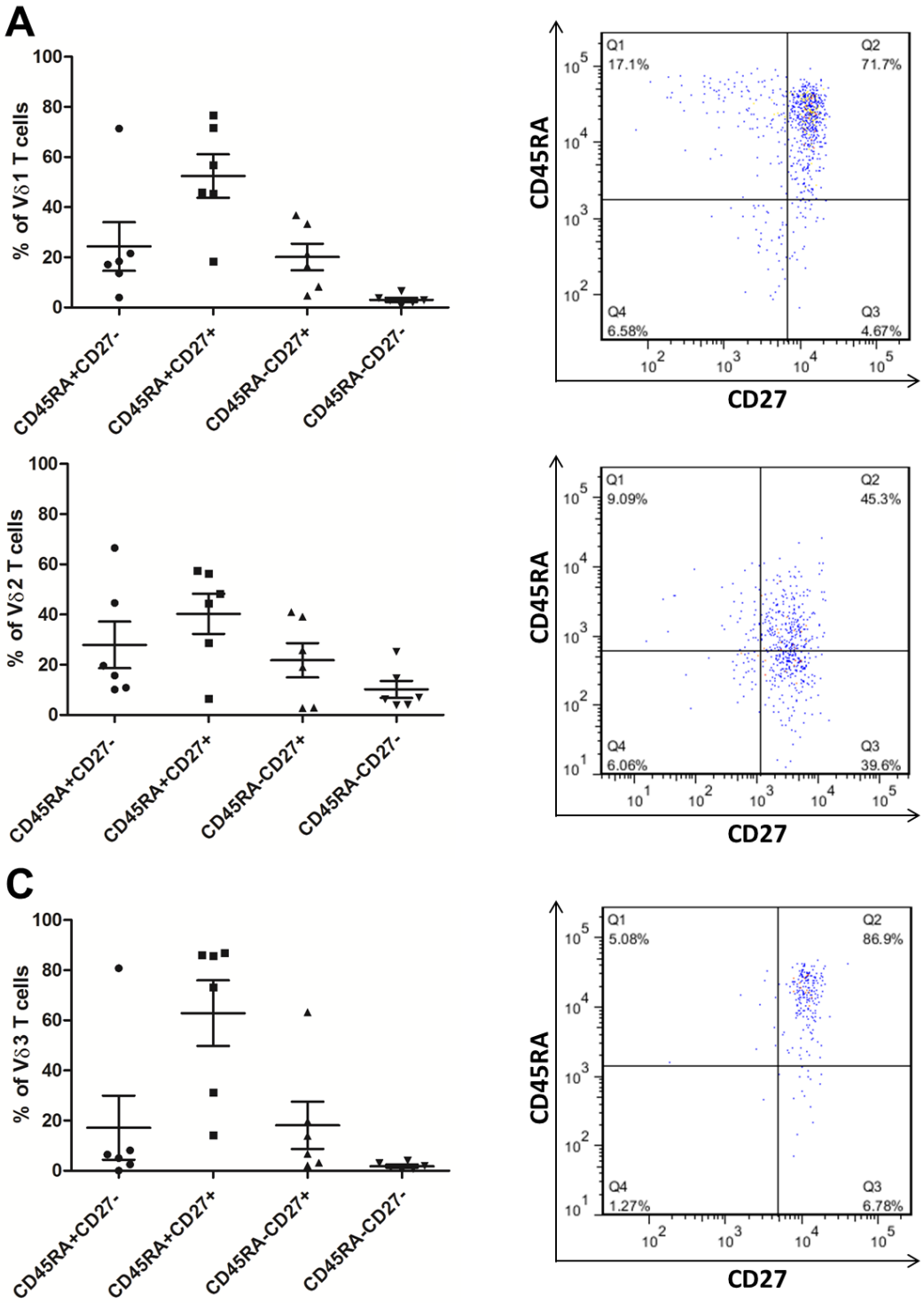

Figure 3.7. Flow cytometric analysis of memory phenotype in fresh PBMC. PBMC were isolated from human buffycoat packs and stained for cell surface expression of CD3, Vδ1, Vδ2, Vδ3, CD45RA and CD27. $CD45RA^{+}CD27^{-}$ cells exhibit a terminally differentiated phenotype; $CD45RA^{+}CD27^{+}$ cells represent naïve cells; $CD45RA^{-}CD27^{+}$ cells exhibit central memory and $CD45RA^{-}CD27^{-}$ cells exhibit an effector memory phenotype. A-C left panels, CD45RA and CD27 expression by Vδ1 (**A**; n=6), Vδ2 (**B**; n=6) and Vδ3 (**C**; n=6) T cells. Right panels, representative flow cytometric dot plots showing CD45RA and CD27 expression by Vδ1, Vδ2 and Vδ3 T cells.

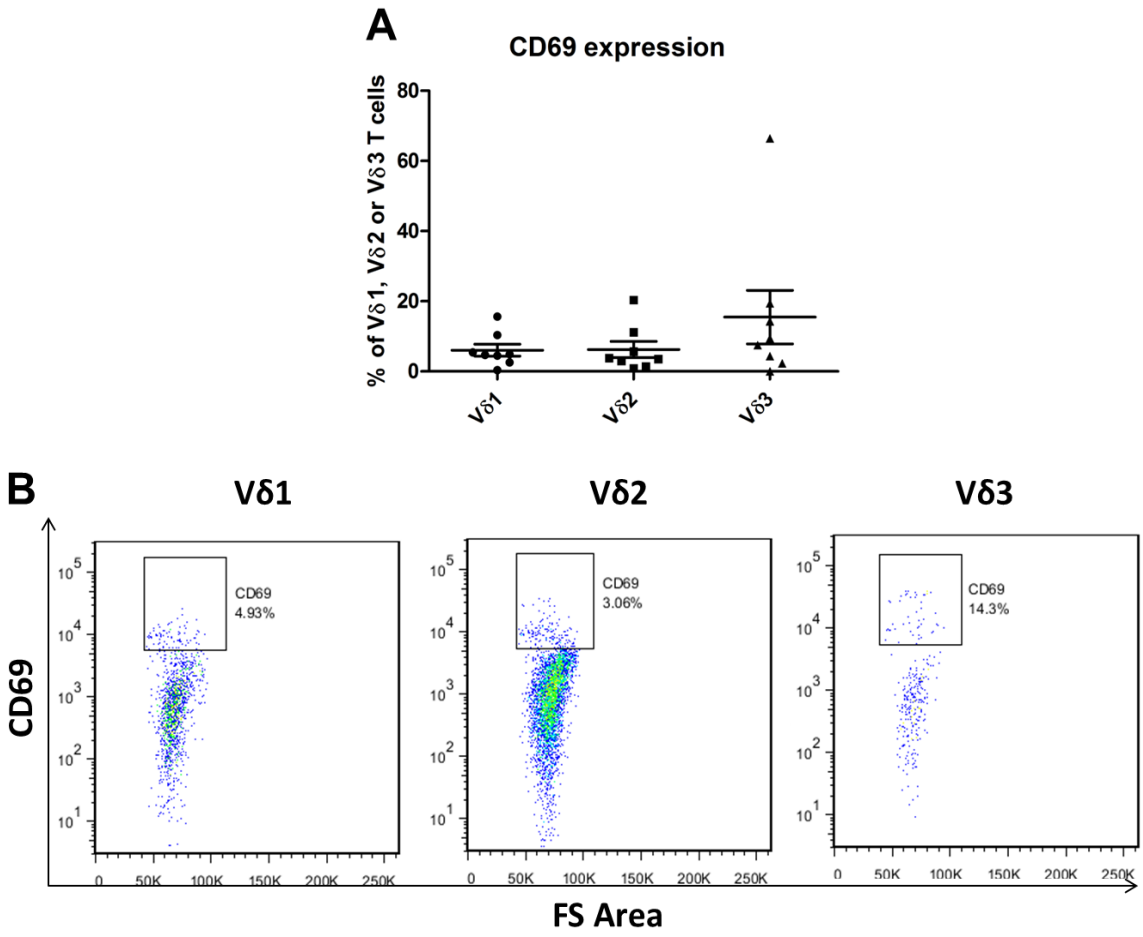

Figure 3.8. Flow cytometric analysis of CD69 expression by γδ subsets. PBMC were isolated from human buffycoat packs and stained for cell surface co-expression of CD3, CD69, Vδ1, Vδ2 and Vδ3 and analysed by flow cytometry. **A**, CD69 expression as a percentage of γδ T cell subsets (n=8). **B**, representative flow cytometric dot plots showing CD69 expression by the three γδ T cell subsets.

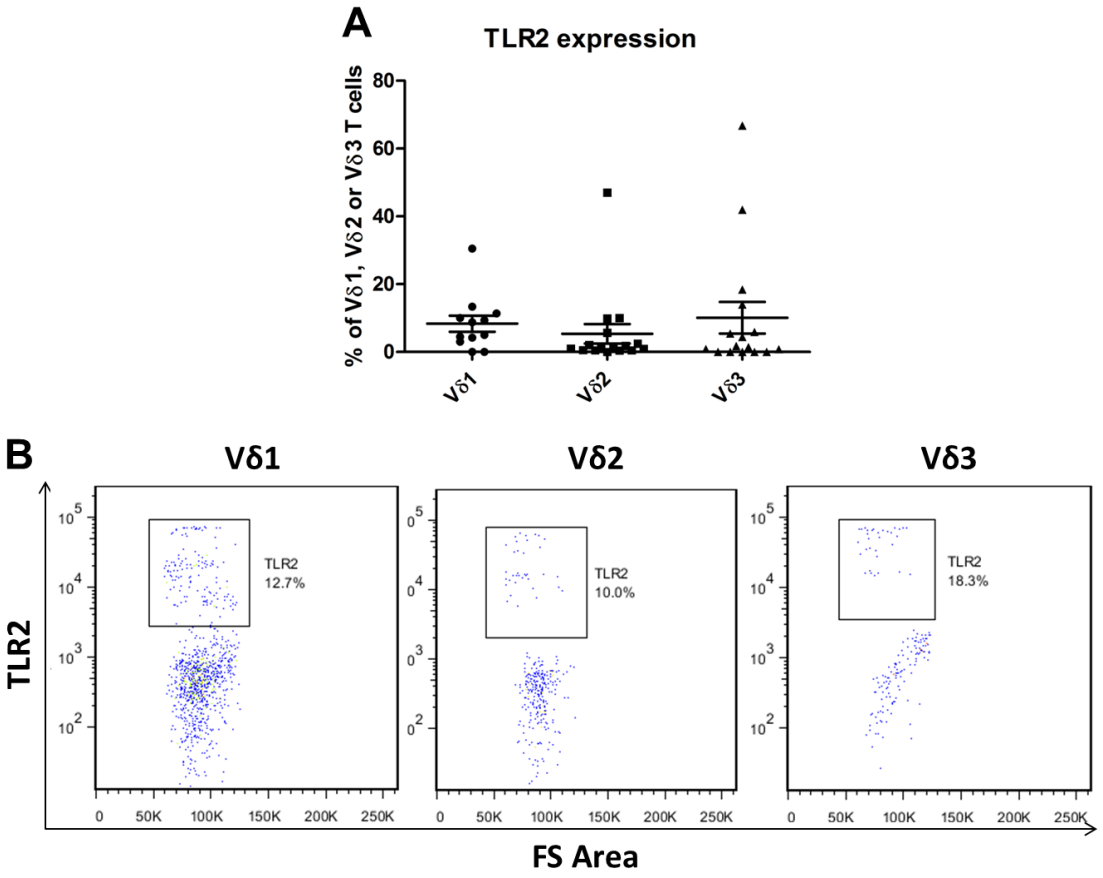

Figure 3.9. Flow cytometric analysis of TLR2 expression by γδ subsets. PBMC were isolated from human buffycoat packs and stained for cell surface co-expression of CD3, TLR2, Vδ1, Vδ2 and Vδ3 and analysed by flow cytometry. **A**, TLR2 expression as a percentage of γδ T cell subsets (n=16). **B**, representative flow cytometric dot plots showing TLR2 expression by the three γδ T cell subsets.

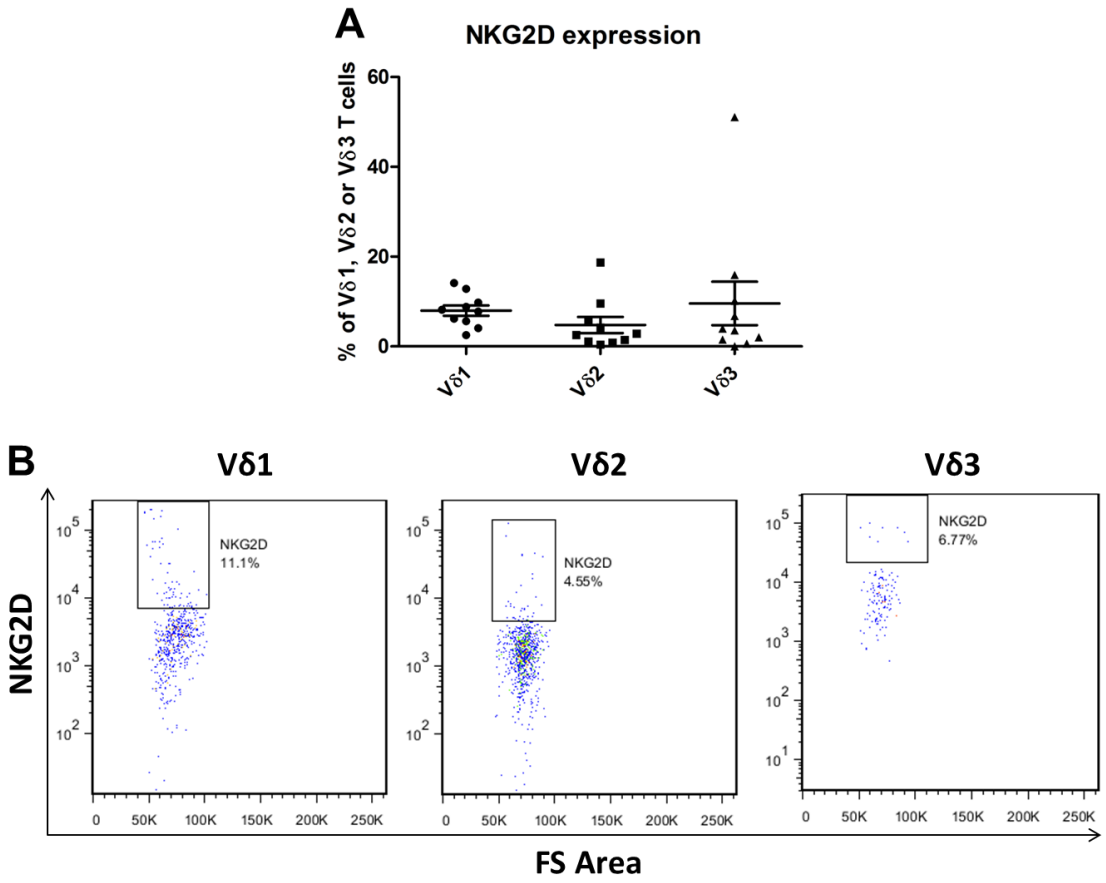

Figure 3.10. Flow cytometric analysis of NKG2D expression by γδ subsets. PBMC were isolated from human buffycoat packs and stained for cell surface co-expression of CD3, NKG2D, Vδ1, Vδ2 and Vδ3 and analysed by flow cytometry. **A**, NKG2D expression as a percentage of γδ T cell subsets (n=10). **B**, representative flow cytometric dot plots showing NKG2D expression by the three γδ T cell subsets.

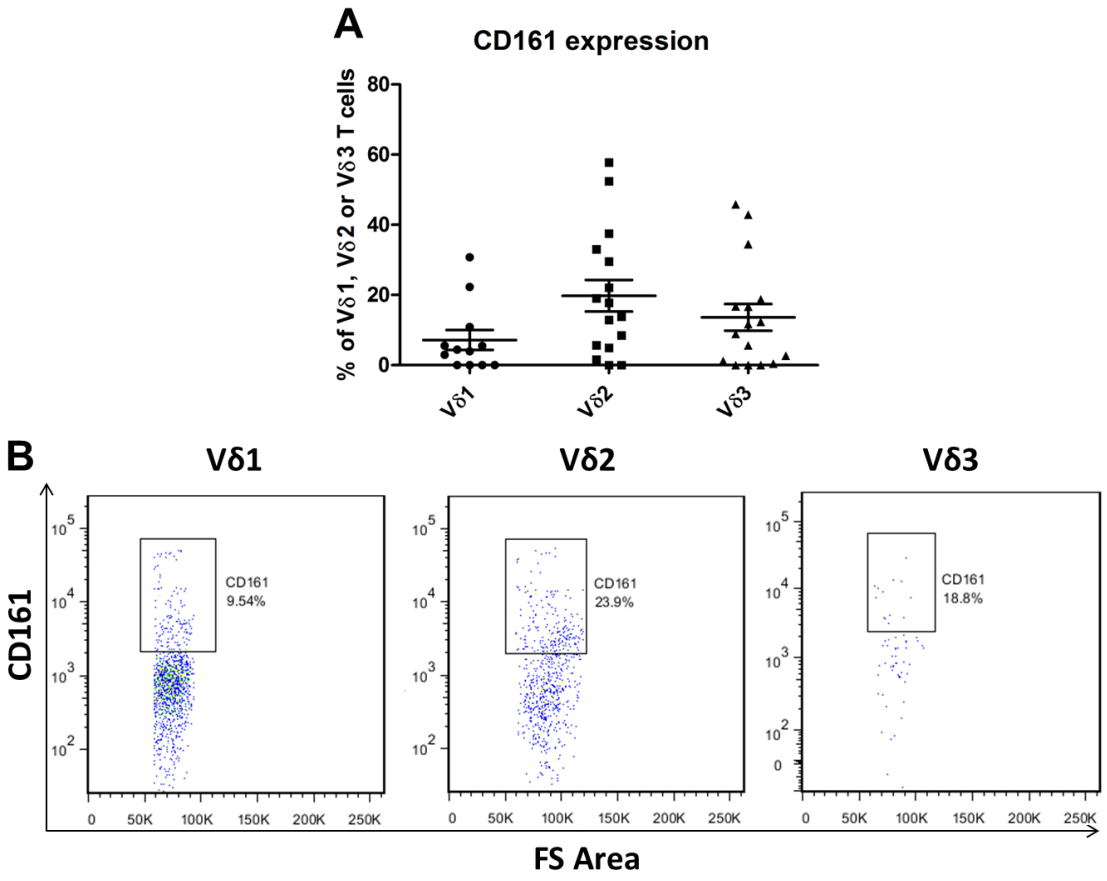

Figure 3.11. Flow cytometric analysis of CD161 expression by γδ subsets. PBMC were isolated from human buffycoat packs and stained for cell surface co-expression of CD3, CD161, Vδ1, Vδ2 and Vδ3 and analysed by flow cytometry. **A**, CD161 expression as a percentage of γδ T cell subsets (n=16). **B**, representative flow cytometric dot plots showing CD161 expression by the three γδ T cell subsets.

3.4.5. Assessing purity of HMB-PP expanded Vγ9Vδ2 T cell lines

Magnetically sorted γδ TCR^+ cells were stimulated with HMB-PP and cultured in the presence of IL-2 for 2-4 weeks. The cells were then phenotyped for CD3, Vγ9 and Vδ2 expression and only cell lines containing >85% $Vδ2^+$ lymphocytes were used for subsequent experimental analysis (chapter 4.0). Most cell lines expanded well, with the majority reaching >94% purity (Fig. 3.12). In addition, it was found that virtually all (>99%) of Vδ2 T cells co-expressed Vγ9 (Fig. 3.13) and there were virtually no $Vγ9^-Vδ2^+$ cells.

3.4.6. Expanded Vδ2 T cell lines are CD8 positive or double negative and exhibit an effector memory phenotype.

Analysis of CD4 and CD8 expression by Vδ2 T cell lines revealed that there were virtually no CD4 or $CD4^+CD8^+$ Vδ2 T cells, with the majority being $CD4^-CD8^-$ and a small percentage (~15%) being CD8 T cells (Fig. 3.14), which may be due to the selective expansion of CD8 and DN cells. The expanded cell lines mainly exhibited an effector memory phenotype (Fig. 3.15), as they were mainly $CD45RA^-CD27^-$, which was expected of cells that have been exposed to an antigen and cultured for several weeks. There were also some terminally differentiated cells which were $CD45RA^+CD27^-$.

3.4.7. Expanded Vδ2 T cells express markers of T cell activation and TLR

We examined whether Vδ2 T cell lines expressed T cell activation markers CD69 and CD56 and found that the majority (>90%) were $CD69^+$ (Fig. 3.16), with a small proportion of those (~7%) co-expressing $CD56^+$. We also tested for the presence of TLR2 and found that in contrast to fresh Vδ2 T cells, the majority of expanded Vδ2 T cells did not express TLR2 (<0.5%) (Fig. 3.17) after several weeks of culture.

3.4.8 Expanded Vδ2 T cells are capable of producing IFN-γ, IL-4 and TNF-α

We wanted to characterise the cytokine profiles of our expanded Vδ2 T cell lines. Cells were rested by culturing them overnight for 24 h in the absence of IL-2. They were then re-stimulated with HMB-PP or left unstimulated for 4 h in the presence of monensin. The expression and secretion of IFN-γ, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17, IL-

23 and TNF-α by Vδ2 T cells was examined. It was found that a small percentage of resting Vδ2 T cells expressed IFN-γ (Fig. 3.18A,E), and a high percentage expressed IL-4 (Fig. 3.18B,E), while there was virtually no TNF-α (Fig 3.18C,F) or IL-13 (Fig. 3.18D,F) detected in Vδ2 T cells that were not restimulated. HMB-PP induced a ~3-fold increase in IFN-γ-producing cells and a 20-fold increase in TNF-α producing cells while there was no increase in IL-4 or IL-13 expression. There was also no expression of IL-10, IL-12 (Fig. 3.18G), IL-6, or IL-23 (data not shown) in resting or HMB-PP-activated Vδ2 T cells.

As the data presented above revealed the percentage of cells expressing cytokines, we next quantified the levels of cytokine secretion in the cultures. ELISA revealed that in addition to an increase in IFN-γ- and TNF-α-producing Vδ2 T cells, there was also an increase in IFN-γ (Fig. 3.19A) and TNF-α (Fig. 3.19C) secretion after 24 h. While there was a high percentage of IL-4-expressing Vδ2 T cells, these did not secrete IL-4, while the HMB-PP-activated Vδ2 T cells produced significantly increased levels of IL-4 (Fig 3.19B) after 24 h. It was also shown that there was no increase in levels of IL-6 (Fig. 3.19D) or IL-10 (Fig. 3.19E) by HMB-PP-activation. Furthermore, it was found that after a further 2 days there was a reduction in IFN-γ, IL-4 and TNF-α secretion compared to two days prior. There was no IL-12 or IL-17 secretion from Vδ2 T cells (data not shown).

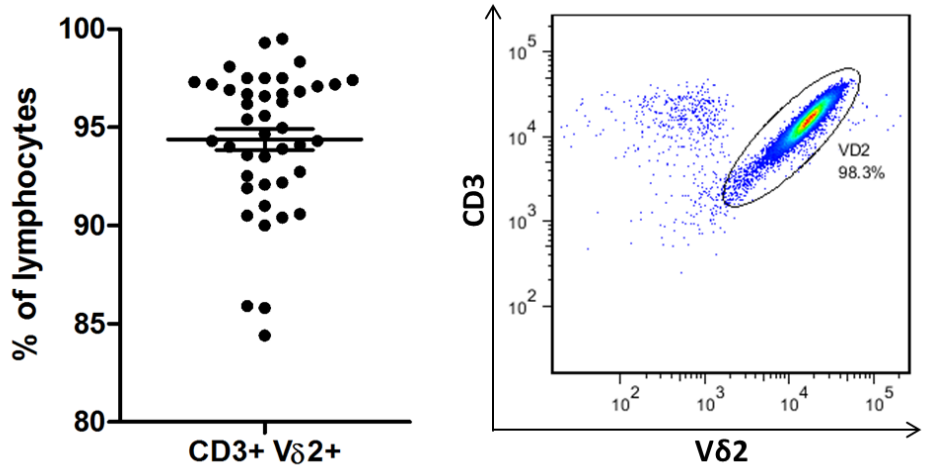

Figure 3.12. Flow cytometric analysis of purity of expanded Vδ2 T cell lines. Vδ2 T cell lines were generated by stimulating magnetically sorted γδ T cells with HMB-PP and culturing for 14-28 days in IL-2 supplemented RPMI. Expanded Vδ2 T cells were analysed for expression of CD3 and Vδ2 by flow cytometry. Left panel, mean (±SEM) percentage of $CD3^+$ $V\delta2^+$ T cells in expanded cell lines (n=43). Right panel, representative flow cytometric dot plot showing percentage of $CD3^+$ $V\delta2^+$ lymphocytes.

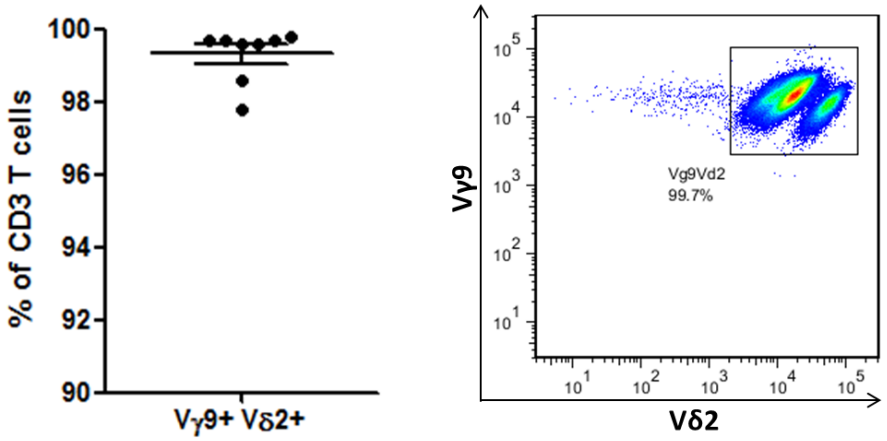

Figure 3.13. Flow cytometric analysis of Vγ9 and Vδ2 expression in expanded Vδ2 T cell lines. Vδ2 T cell lines were generated by stimulating magnetically sorted γδ T cells with HMB-PP and culturing for 14-28 days in IL-2 supplemented RPMI. Expanded Vδ2 T cells were analysed for co-expression of Vγ9 and Vδ2 by flow cytometry. Left panel, mean (±SEM) percentage of Vγ9 and Vδ2 co-expressing CD3 T cells (n=8). Right panel, representative flow cytometric dot plot showing Vγ9 and Vδ2 expression by $CD3^+$ T cells.

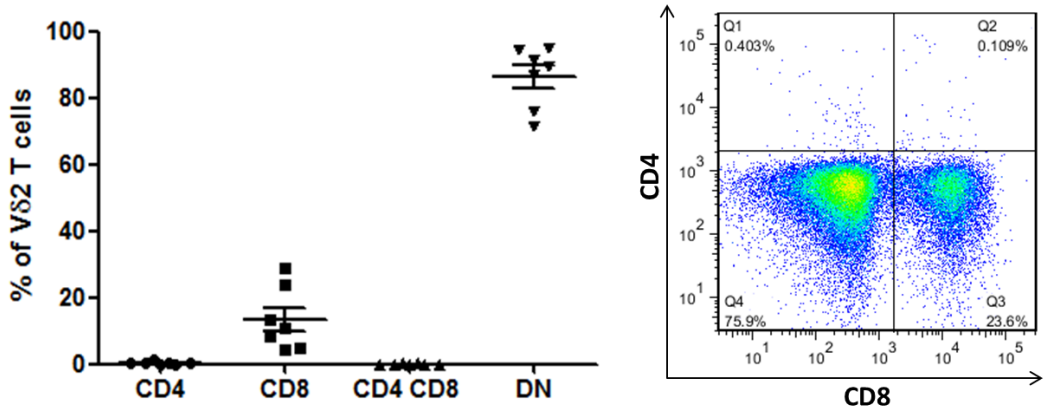

Figure 3.14. Flow cytometric analysis of CD4 and CD8 subsets in expanded Vδ2 T cell lines. Vδ2 T cell lines were generated by stimulating magnetically sorted γδ T cells with HMB-PP and culturing them for 14-28 days in IL-2 supplemented RPMI. Expanded Vδ2 T cells were examined for expression of CD4 and CD8. Left panel, mean (±SEM) percentage of CD4 and CD8 subsets in $CD3^+$ $V\delta2^+$ T cells (n=7). Right panel, representative flow cytometric dot plot showing CD4 and CD8 subsets.

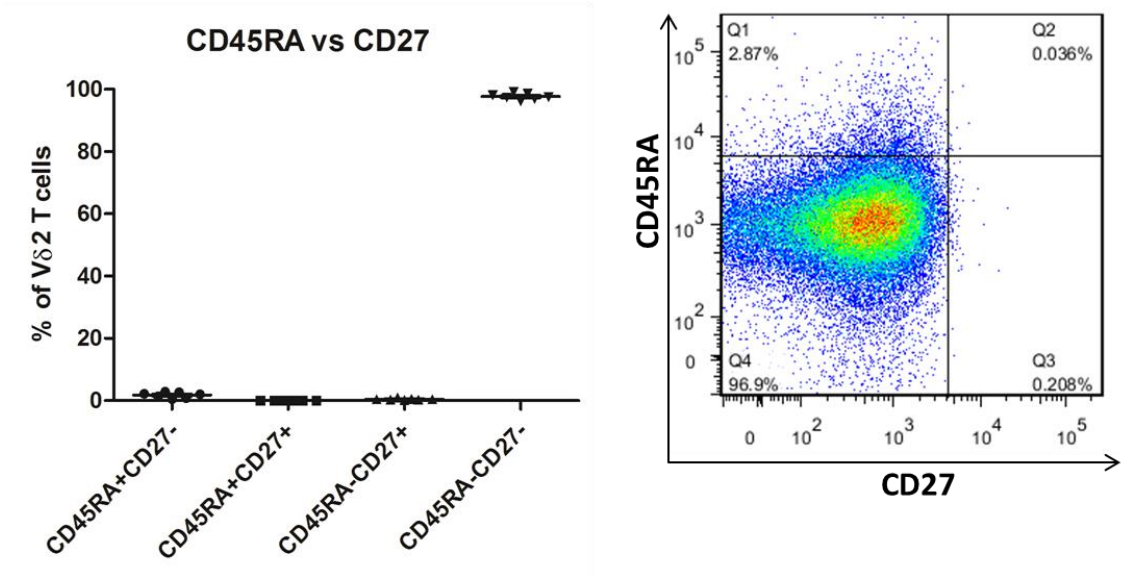

Figure 3.15. Flow cytometric analysis of memory phenotype in expanded Vδ2 T cell lines. Vδ2 T cell lines were generated by stimulating magnetically sorted γδ T cells with HMB-PP and culturing for 14-28 days in IL-2 supplemented RPMI. Expanded Vδ2 T cells were examined for expression of CD45RA and CD27. Left panel, mean (±SEM) expression of CD45RA and CD27 in $CD3^+$ $V\delta2^+$ T cells (n=7). Right panel, representative flow cytometric dot plot showing the memory phenotype. $CD45RA^+CD27^-$ cells represent terminally differentiated cells (T_{EMRA}), while $CD45RA^+CD27^+$ cells exhibit a naïve phenotype. $CD45RA^-CD27^+$ cells represent central memory T cells (T_{CM}) and $CD45RA^-CD27^-$ cells are effector memory T cells (T_{EM}).

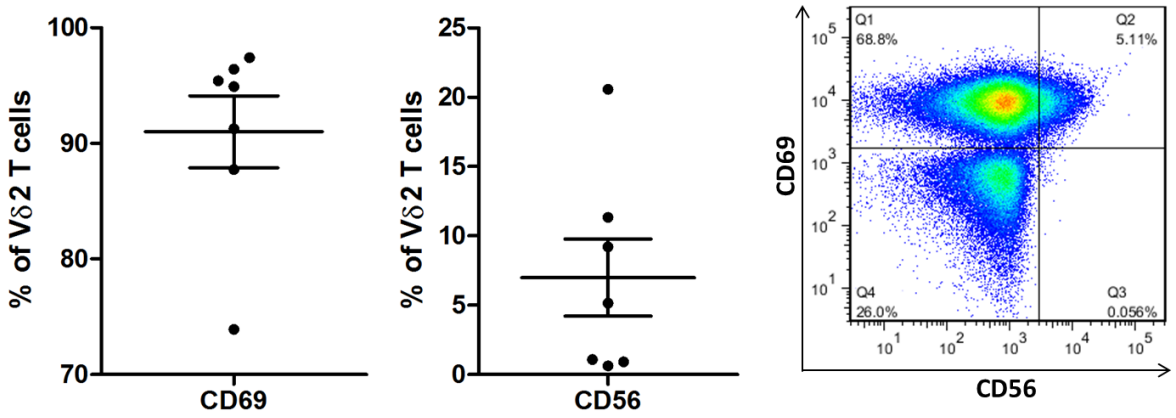

Figure 3.16. Flow cytometric analysis of markers of T cell activation in expanded Vδ2 T cell lines. Vδ2 T cell lines were generated by stimulating magnetically sorted γδ T cells with HMB-PP and culturing for 14-28 days in IL-2 supplemented RPMI. Expanded Vδ2 T cells were examined for expression of CD56 and CD69. Left panel, mean (±SEM) CD69 expression in expanded Vδ2 T cell lines (n=7). Centre panel, mean (±SEM) CD56 expression in expanded Vδ2 T cell lines (n=7). Right panel, representative flow cytometric dot plot showing CD69 and CD56 expression.

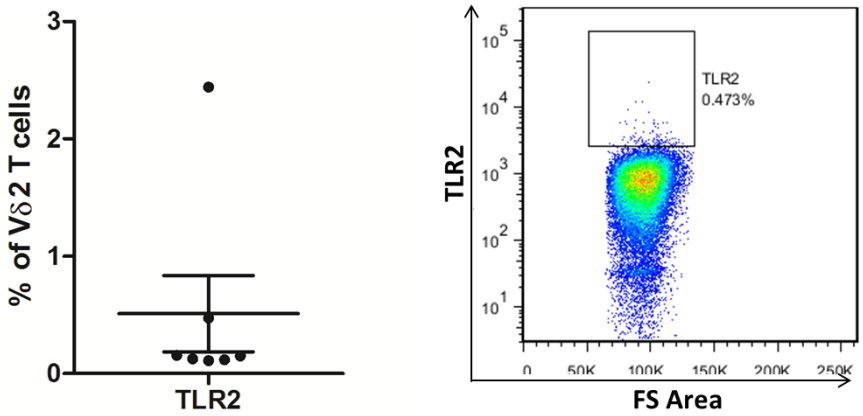

Figure 3.17. Flow cytometric analysis of TLR2 expression in expanded Vδ2 T cell lines. Vδ2 T cell lines were generated by stimulating magnetically sorted γδ T cells with HMB-PP and culturing for 14-28 days in IL-2 supplemented RPMI. Expanded Vδ2 T cells were examined for expression of TLR2. Left panel, mean (±SEM) TLR2 expression in expanded Vδ2 T cell lines (n=7). Right panel, representative flow cytometric dot plot showing TLR2 expression.

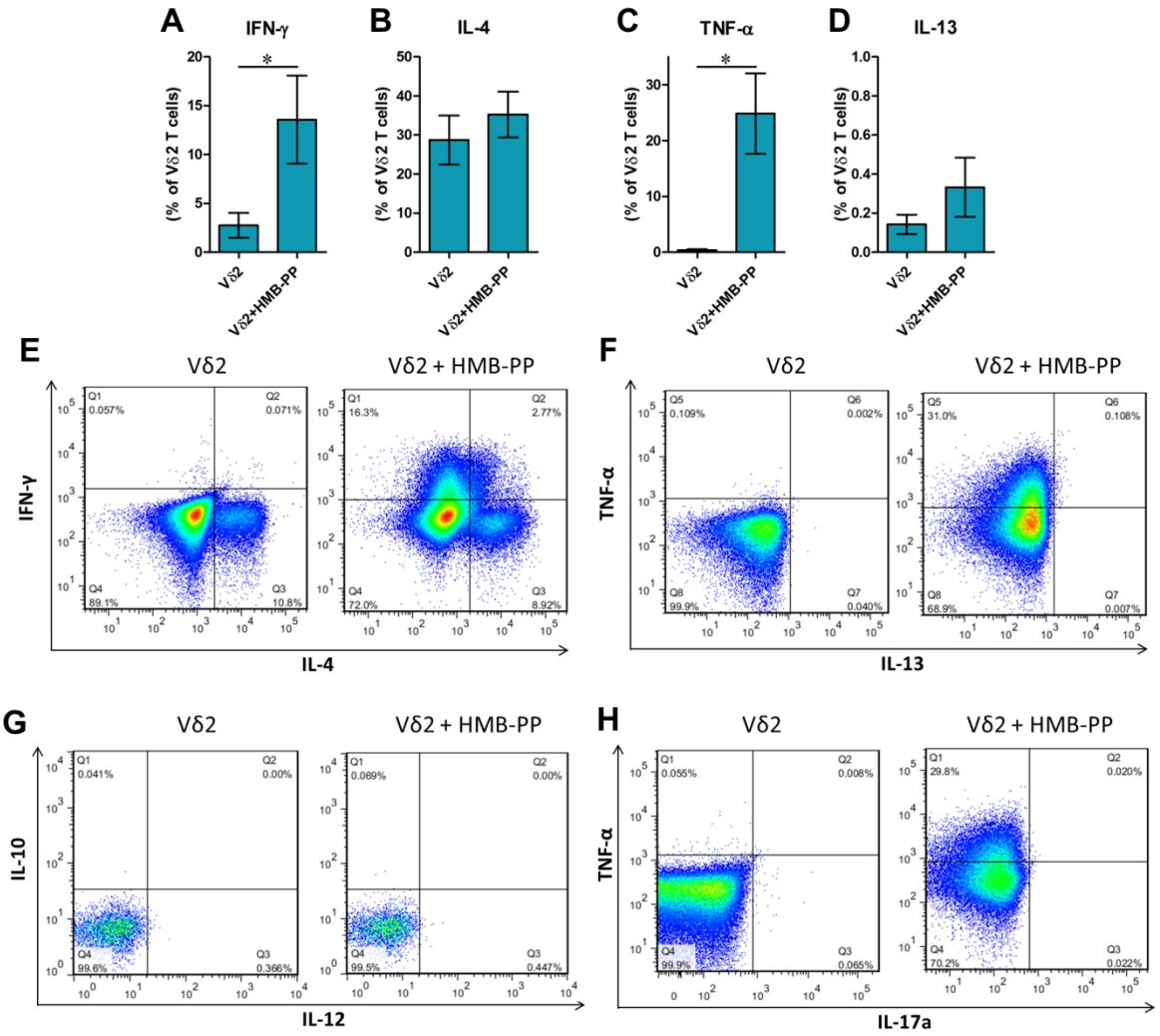

Figure 3.18. Intracellular cytokine production by Vδ2 T cells. Vδ2 T cell lines were generated by stimulating magnetically sorted γδ T cells with HMB-PP and culturing for 14-28 days in IL-2 supplemented RPMI. Expanded Vδ2 T cells were transferred to IL-2-free media overnight. The cells were then cultured with monensin in the presence or absence of HMB-PP for 4 h and examined for intracellular IFN-γ, IL-4, TNF-α and IL-13 expression by flow cytometry. A-D, mean (±SEM) percentage of Vδ2 T cells that express IFN-γ (**A**; n=9), IL-4 (**B**; n=14), TNF-α (**C**; n=4) and IL-13 (**D**; n=4). E-H, representative flow cytometric dot plots showing IFN-γ and IL-4 (**E**), TNF-α and IL-13 (**F**), IL-10 and IL-12 (**G**) and TNF-α and IL-17a (**H**) expression by resting (left panels) or HMB-PP-activated (right panels) Vδ2 T cells. *p<0.05 using a paired *t*-test.

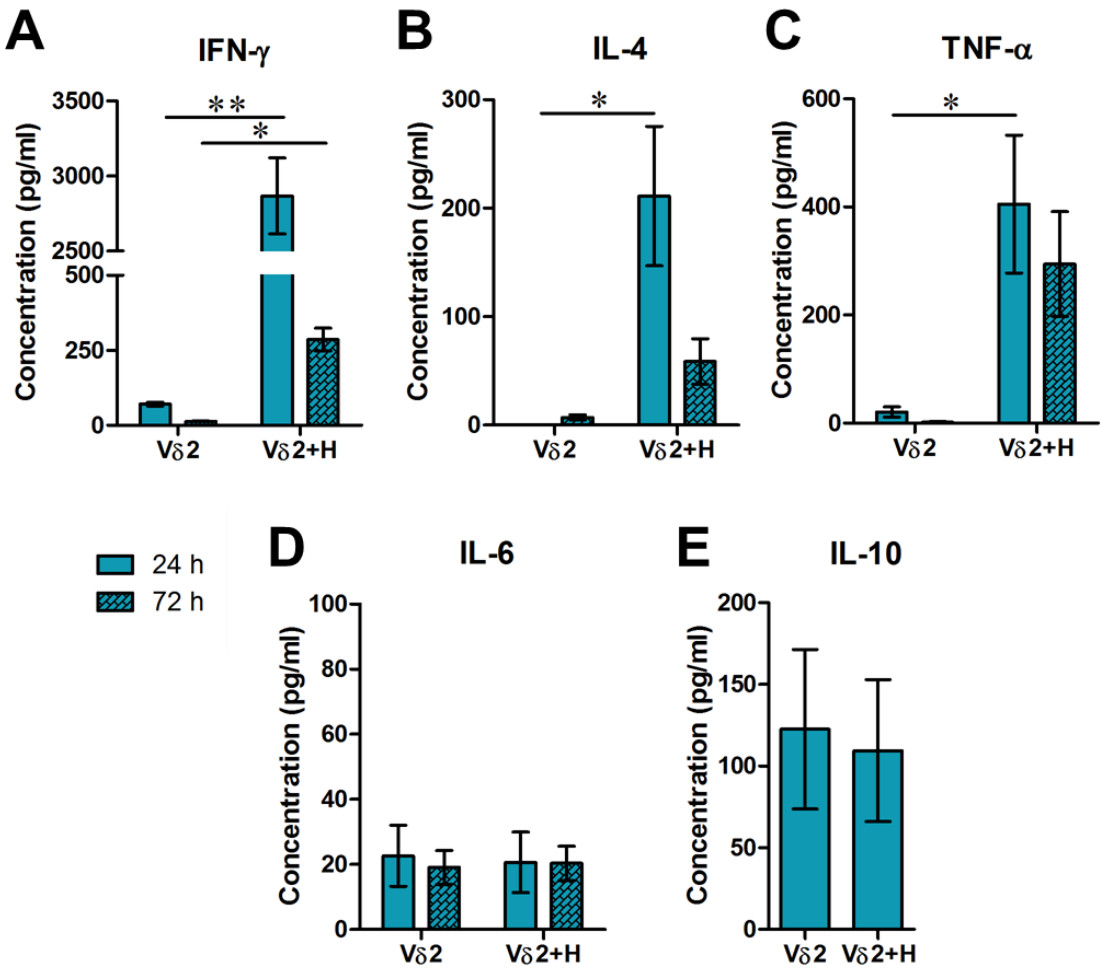

Figure 3.19. Cytokine secretion from Vδ2 T cell supernatants. Vδ2 T cell lines were generated by stimulating magnetically sorted γδ T cells with HMB-PP and culturing for 14-28 days in IL-2 supplemented RPMI. Expanded Vδ2 T cells were transferred to IL-2-free media and cultured for 24 or 72 h in the presence or absence of HMB-PP. The supernatants were then assayed for levels of IFN-γ, IL-4, TNF-α, IL-6 and IL-10. **A-E**, IFN-γ (**A**; n=3), IL-4 (**B**; n=4-6), TNF-α (**C**; n=-3-6), IL-6 (**D**; n=3-8) and IL-10 (**E**; n=9) secretion by Vδ2 T cells in supernatants. *p<0.05, **p<0.01 using a paired *t*-test.

3.4.9 Aminobisphosphonate stimulation is more efficient than phosphoantigen stimulation in expanding Vδ2 T cells and they induce different cytokine profiles

PBMC were stimulated with HMB-PP or zoledronate and expanded for 2 weeks. Two distinct batches of HMB-PP were assessed. The purity was then assessed by flow cytometry. It was found that zoledronate appeared to be more efficient than the new batch of HMB-PP in inducing Vδ2 T cell expansion (Fig. 3.20A,B). However, this was due to the lower activity of the new batch of HMB-PP. The new batch increased Vδ2 T cell purity to ~30%, while the old batch increased purity to ~50%. There was no difference in stimulating capacity between the old HMB-PP batch and the optimum zoledronate dose used. Furthermore, it was found that both HMB-PP and zoledronate induced the expansion of the same CD4 and CD8 subsets (Fig. 3.21).

Unstimulated HMB-PP- or zoledronate-expanded Vδ2 T cells produced very low levels of IFN-γ (Fig. 3.22A,G) and TNF-α (Fig. 3.22C,G) but high background levels of IL-4 (Fig. 3.22B,G) and virtually no IL-10 (Fig. 3.22D,G), IL-13 (Fig. 3.22E,G) or IL-17 (Fig. 3.22F,G).

Interestingly, restimulated zoledronate- and HMB-PP-expanded Vδ2 T cells had distinct cytokine profiles, with zoledronate-restimulated zoledronate-expanded Vδ2 T cells unable to produce IFN-γ or TNF-α, while HMB-PP-restimulated HMB-PP-expanded Vδ2 T cells produced IFN-γ and TNF-α, while neither increased IL-4, IL-10, IL-13 or IL-17 levels. In addition, when expanded with zoledronate and HMB-PP simultaneously, HMB-PP was unable to restore the zoledronate-induced inhibition of IFN-γ and TNF-α production by Vδ2 T cells.

3.4.10 Assessing purity of expanded Vδ3 T cell lines

Expanded Vδ3 T cell lines were more difficult to expand into a pure population of Vδ3 T cells, owing to the fact that the antigen, PHA-P, is non-specific and drives proliferation of all T cells. Therefore, if the flow cytometry cell sort did not yield a very pure population of Vδ3 T cells, PHA-P would have also expanded any other T cells present. Only cell lines containing >85% $V\delta3^+$ lymphocytes were used for subsequent experiments (chapter 5.0). With the exception of a few donors, most Vδ3 T cells

reached the desired purity (Fig. 3.23). Like Vδ2 T cell lines, Vδ3 T cell lines were mostly $CD4^-CD8^-$, and some were $CD8^+$ (Fig. 3.24).

3.4.11 Resting Vδ3 T cells produce very low cytokine levels

The cytokine profile of the expanded Vδ3 T cell lines was characterised by testing for production of IFN-γ, IL-4, TNF-α and IL-17. It was found that Vδ3 T cells expressed these at very low levels (Fig. 3.25), and these were not increased by PHA-P stimulation (data not shown). PMA and ionomycin stimulation was able to induce an increase in IFN-γ (Fig. 3.25A,E), IL-4 (Fig 3.25B,E) and TNF-α (Fig. 3.25C,F) but not IL-17a (Fig. 3.25D,F) expression by Vδ3 T cells. In contrast, PHA induced a significant increase in IFN-γ secretion (Fig. 3.26A) by Vδ3 T cells and a moderate increase in IL-4 secretion (Fig. 3.26B). No IL-17 secretion by Vδ3 T cells was detected by ELISA (data not shown).

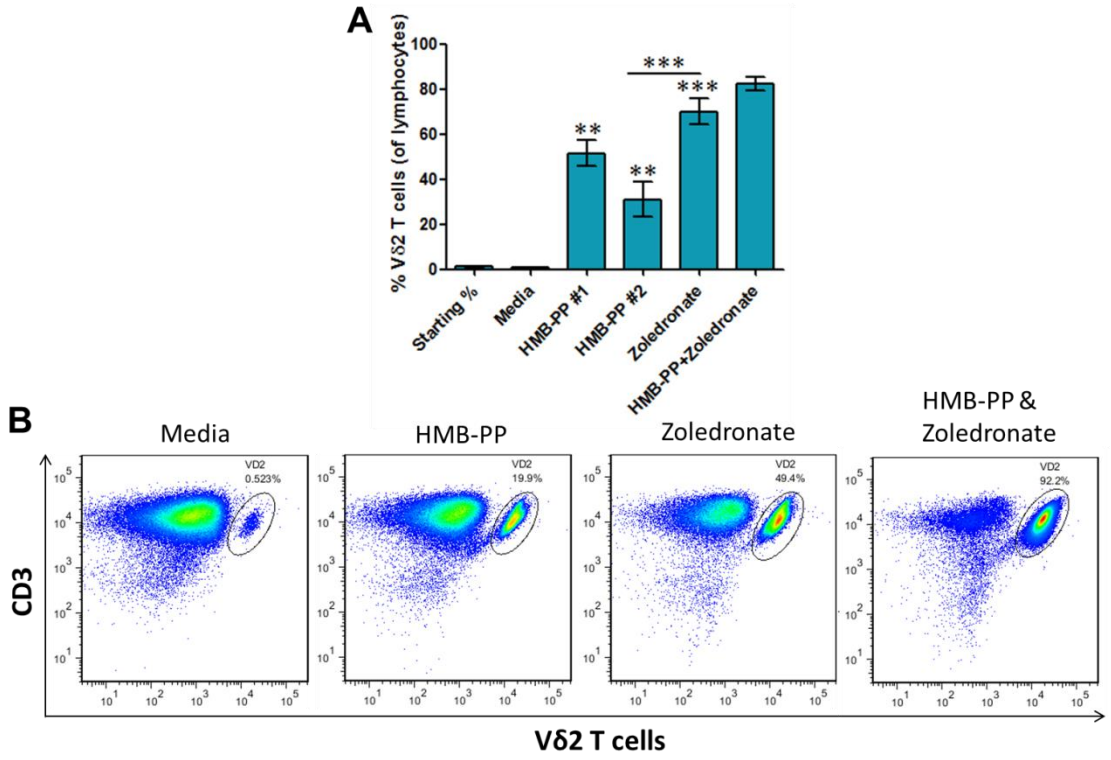

Figure 3.20. Flow cytometric analysis of purity of expanded Vδ2 T cell lines. PBMC were stimulated with Zoledronate, HMB-PP or a combination of the two and cultured for 14 days in IL-2-supplemented RPMI. Two different HMB-PP batches: the old one (#1) and new one (#2) were compared for their stimulating capability. Expanded Vδ2 T cells were analysed for expression of CD3 and Vδ2 by flow cytometry. **A**, mean (±SEM) percentage of $CD3^+$ $V\delta2^+$ T cells in expanded cell lines (n=10). **B**, representative flow cytometric dot plots showing percentage of $CD3^+$ $V\delta2^+$ lymphocytes with the 3 different stimulations.

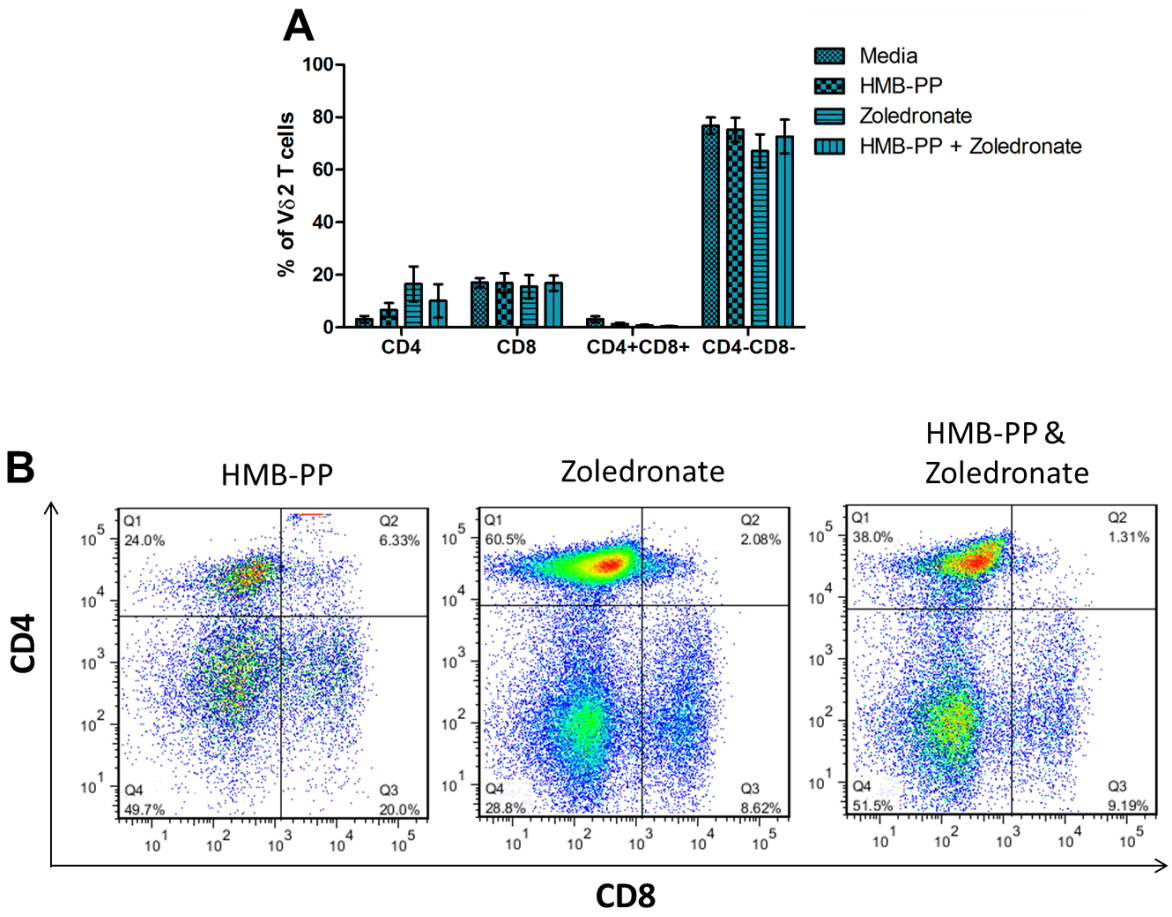

Figure 3.21. Flow cytometric analysis of CD4 and CD8 subsets in expanded Vδ2 T cell lines. PBMC were stimulated with zoledronate, HMB-PP or a combination of the two and cultured for 14 days in IL-2-supplemented RPMI. Expanded Vδ2 T cells were analysed for expression of CD4 and CD8 subsets of $CD3^{+}V\delta2^{+}$ T cells by flow cytometry. **A**, mean (±SEM) percentage of $CD3^{+}$ $V\delta2^{+}$ T cells in expanded cell lines (n=10). **B**, representative flow cytometric dot plots showing CD4 and CD8 subsets of $CD3^{+}V\delta2^{+}$ T cells with the 3 different stimulations.

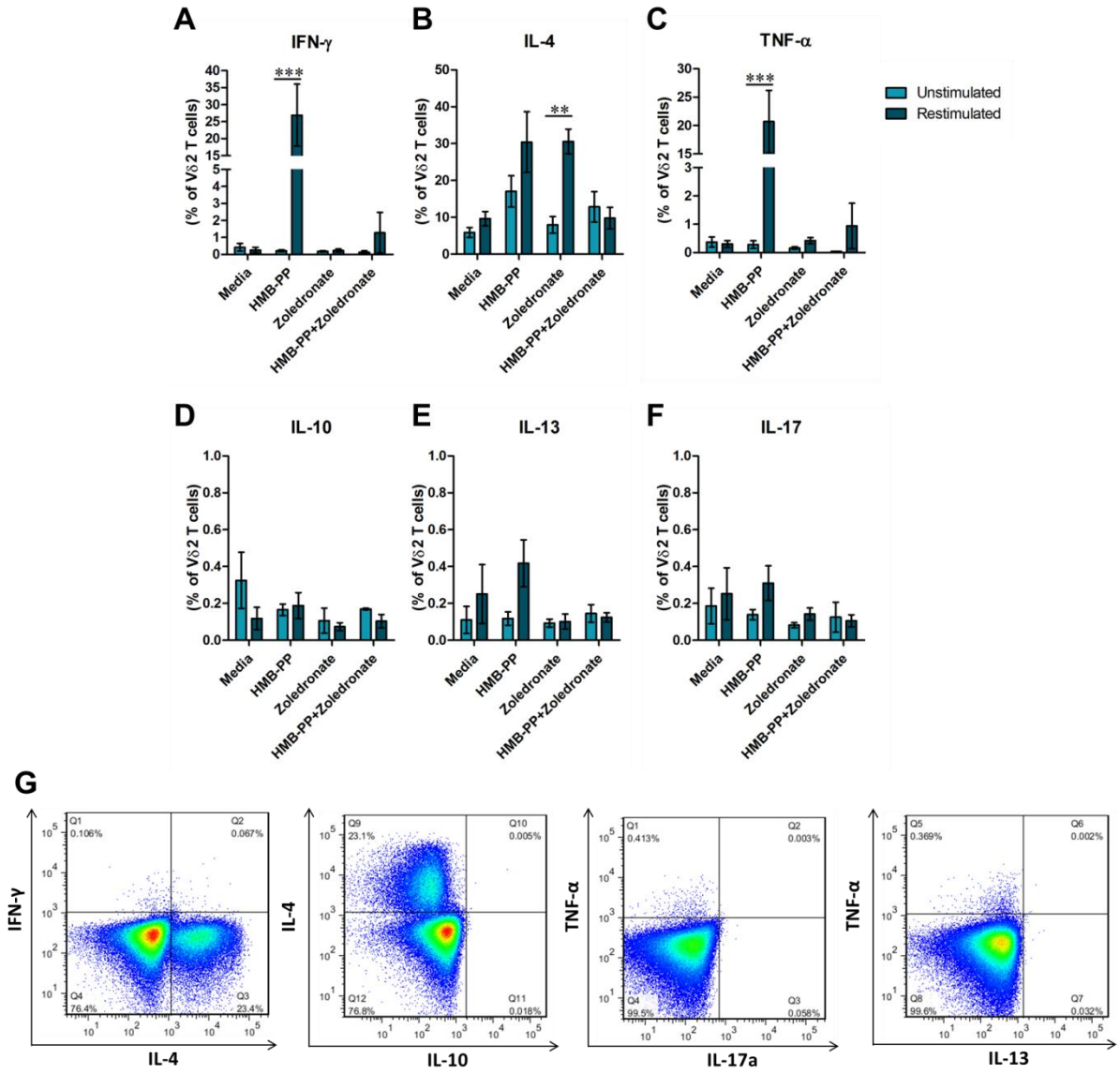

Figure 3.22 Intracellular cytokine expression by phosphoantigen and aminobisphosphonate-expanded Vδ2 T cell lines. PBMC were stimulated with zoledronate, HMB-PP or a combination of the two and cultured for 14 days in IL-2-supplemented RPMI. The expanded Vδ2 T cell lines were transferred to IL-2-free media overnight and then cultured with monensin for 4 h. Half of the cells were left unstimulated, and the other half were restimulated with the original stimulus used for the expansion. The cell cultures were then examined for intracellular expression of IFN-γ, IL-4, TNF-α, IL-10, IL-13 and IL-17a. **A-F**, mean (±SEM) percentage (n=3) of IFN-γ (**A**), IL-4 (**B**), TNF-α (**C**), IL-10 (**D**), IL-13 (**E**) and IL-17a (**F**) in expanded Vδ2 T cell lines. **G**, representative flow cytometric dot plots showing cytokine production in Zoledronate-expanded Vδ2 T cell lines. ***p*<0.01, ****p*<0.001 using a two-way ANOVA (Bonferroni post-hoc test).

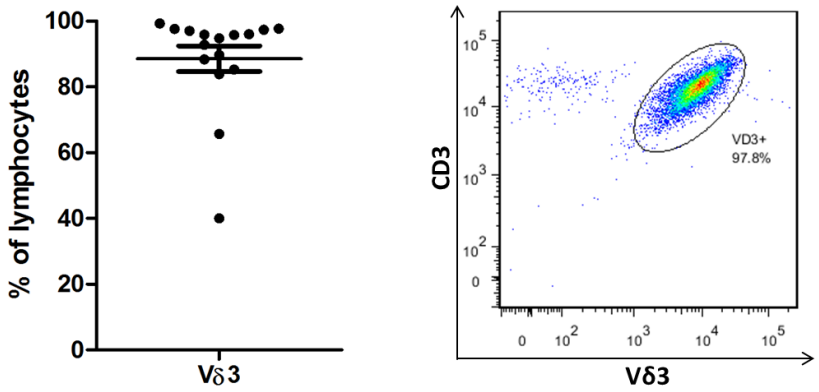

Figure 3.23. Flow cytometric analysis of purity of expanded Vδ3 T cell lines. PBMC were magnetically sorted for γδ TCR^+ cells and then sorted for $CD3^+V\delta3^+$ by flow cytometry. The pure Vδ3 T cell lines were stimulated with PHA-P and irradiated PBMC and cultured for 28 days in IL-2-supplemented RPMI. Expanded Vδ3 T cells were examined for CD3 and Vδ3 expression by flow cytometry. Left panel, mean (±SEM) percentage of $CD3^+$ $V\delta3^+$ T cells in expanded lines (n=16). Right panel, representative flow cytometric dot plot showing percentage of $CD3^+$ $V\delta3^+$ lymphocytes.

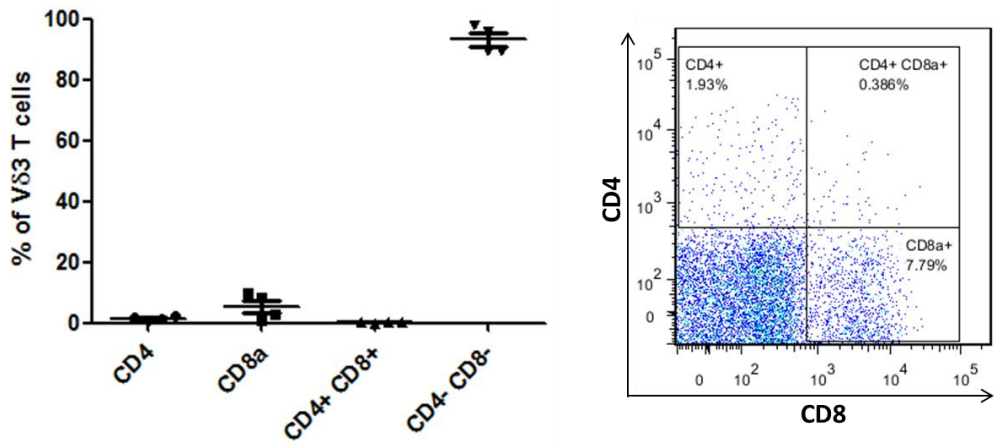

Figure 3.24. Flow cytometric analysis of CD4 and CD8 subsets in expanded Vδ3 T cell lines. PBMC were magnetically sorted for γδ TCR$^+$ cells and then sorted for CD3$^+$Vδ3$^+$ by flow cytometry. The pure Vδ3 T cell lines were stimulated with PHA-P and irradiated PBMC and cultured for 28 days in IL-2-supplemented RPMI. Expanded Vδ3 T cell lines were examined for surface expression of CD4 and CD8. Left panel, mean (±SEM) percentage of CD4 and CD8 subsets in CD3$^+$ Vδ3$^+$ T cells (n=4). Right panel, representative flow cytometric dot plot showing CD4 and CD8 expression by Vδ3 T cells.

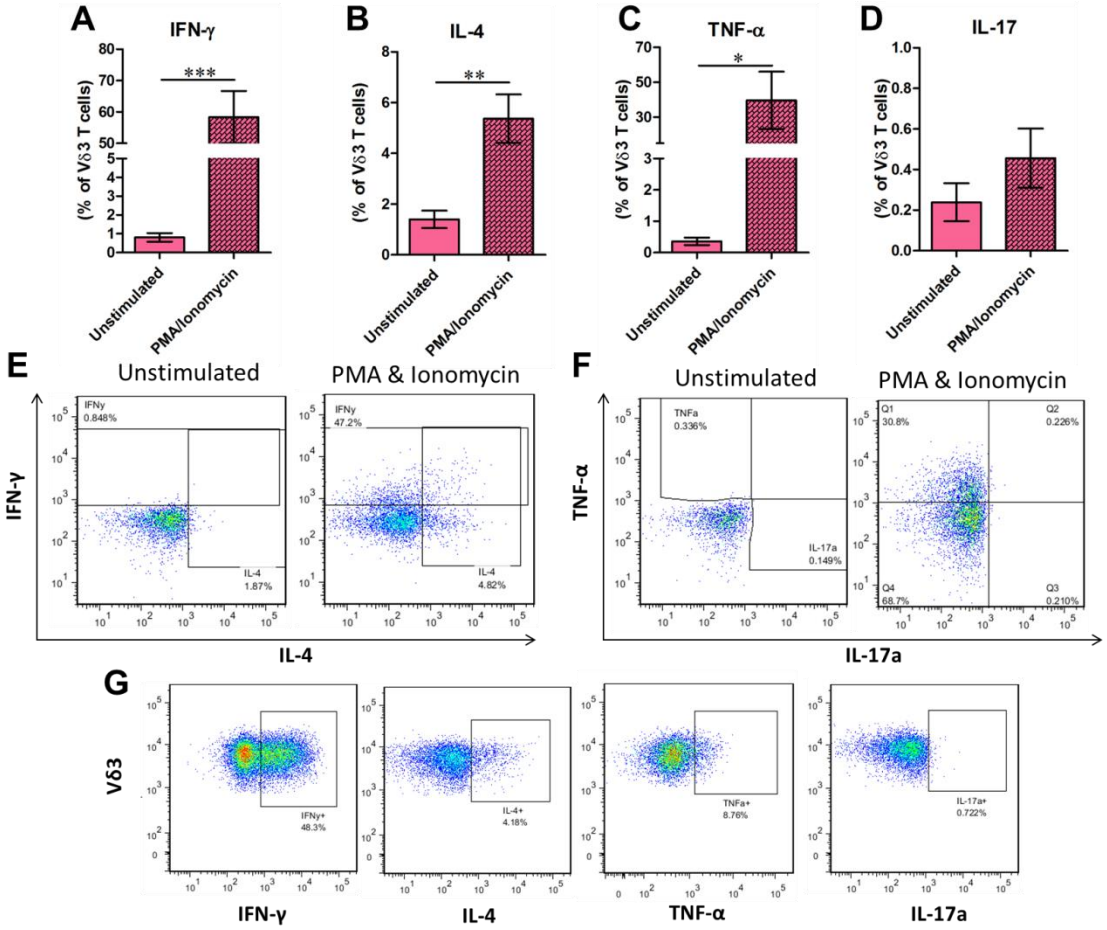

Figure 3.25. Cytokine production by expanded Vδ3 T cell lines. PBMC were magnetically sorted for γδ TCR$^+$ cells and then sorted for CD3$^+$Vδ3$^+$ by flow cytometry. The pure Vδ3 T cell lines were stimulated with PHA-P and irradiated PBMC and cultured for 28 days in IL-2-supplemented RPMI. Expanded Vδ3 T cell lines were transferred to IL-2-free media overnight. The cells were then cultured for 4 h with monensin in the presence or absence of PMA and ionomycin, which acted as a positive control. The cells were then stained for intracellular expression of IFN-γ, IL-4, TNF-α and IL-17a. **A-D**, intracellular expression of IFN-γ (**A**), IL-4 (**B**), TNF-α (**C**) and IL-17a (**D**) by untreated (n=7) or PMA and ionomycin-stimulated (n=3) Vδ3 T cells. **E**, representative flow cytometric dot plots showing IFN-γ and IL-4 production by Vδ3 T cells. **F**, representative flow cytometric dot plots showing TNF-α and IL-17a production by Vδ3 T cells. **G**, representative flow cytometric dot plots showing cytokine production by Vδ3 T cells following PMA & ionomycin stimulation for 4 h. *p<0.05, **p<0.01, ***<0.001 using a paired t test.

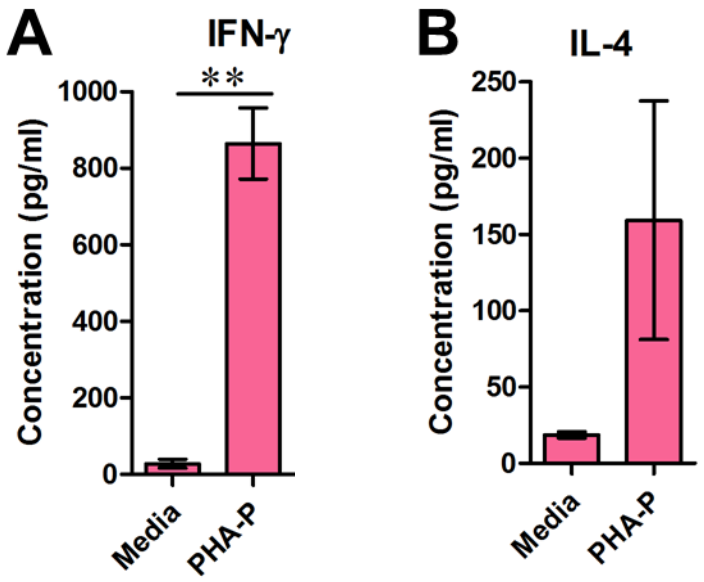

Figure 3.26. Cytokine secretion by Vδ3 T cells. PBMC were magnetically sorted for γδ TCR^+ cells and then sorted for $CD3^+V\delta3^+$ by flow cytometry. The pure Vδ3 T cell lines were stimulated with PHA-P and irradiated PBMC and cultured for 28 days in IL-2-supplemented RPMI. Expanded Vδ3 T cells were cultured in IL-2-free media overnight and cultured for 4 h in the presence or absence of PHA-P. The supernatants were harvested and assayed for levels of IFN-γ and IL-4 by ELISA. **A**, IFN-γ secretion by unstimulated, PHA-P-treated (n=6) Vδ3 T cells. **B**, IL-4 secretion by unstimulated or PHA-P-treated Vδ3 T cells (n=4). $^{**}p<0.01$ using a paired *t*-test.

3.5 Discussion

γδ T cells have attracted interest as immunotherapeutic agents owing to their ability to respond rapidly when faced with a wide array of pathogens or transformed cells, without the requirement for MHC presentation. They recognise danger through expression of the TCR, TLR and stimulatory NK receptors and respond by killing target cells and activating various other immune cells, thus acting as a bridge between the innate and adaptive immune system (Hayday, 2000, Vantourout and Hayday, 2013). Their frequencies are often reduced in bacterial and viral infections and cancers. A major goal has been to manipulate the functional properties of γδ T cells optimise their antitumour activities. This could be done directly through *in vivo* stimulation, or adoptive transfer of *ex vivo*-expanded cell lines. Thus, the aim of this chapter was to assess expansion methods for two γδ T cell subsets and compare the resulting phenotypes to those of γδ T cells in freshly-isolated PBMC. We compared the phenotypes of the three γδ T cell subsets in peripheral blood of healthy donors and examined the phenotypes and cytokine profiles of expanded Vδ2 and Vδ3 T cell lines.

We confirmed that Vδ2 T cells were the predominant subset in these donors, followed by Vδ1 T cells, and lastly, Vδ3 T cells (Halary, et al., 2005, Kalyan and Kabelitz, 2013). We also showed that, as previously described, the majority of Vδ2 T cells paired with the Vγ9 chain (Dunne, et al., 2010), and thus subsequently defined Vγ9Vδ2 T cells using solely an antibody specific for the Vδ2 TCR chain. In contrast, Vδ3 T cells were mainly $Vγ9^-$, thus indicating that they pair with different Vγ chains. Vδ1 T cells have been previously found not to pair with specific Vγ chains (Siegers and Lamb, 2014).

It has been shown that most peripheral γδ T cells lack CD4 and CD8 expression (Hayday, 2000, Kabelitz, et al., 2000) which is in line with lack of MHC restriction. We saw that Vδ1, Vδ2 and Vδ3 subsets in PBMC were predominantly DN for CD4 and CD8, although there were small proportions of CD4 and CD8 γδ T cells. Interestingly, there were virtually no CD4 T cells following expansion, as was also seen by Dunne and colleagues (Dunne, et al., 2010). Thus the expansion method for γδ T cells appeared to exclude CD4 T cells, and hence they were found in low numbers in expanded lines.

Furthermore, we found that there was no difference in CD4 and CD8 frequencies between HMB-PP- and zoledronate-activated Vδ2 T cells.

Analysis of differentiation status revealed that fresh Vδ1, Vδ2 and Vδ3 T cells exhibited predominantly naïve phenotypes, as expected of resting cells that have not been in contact with antigens. Naïve cells accounted for the majority of Vδ1, Vδ2 and Vδ3 T cells. In all subsets a fifth were of T_{EMRA} phenotype and another fifth were of T_{CM} phenotype, while T_{EM} were the least common in all 3 subsets. These results, which used only a small number of donors were not in full agreement with other studies (Dieli, et al., 2003, Dunne, et al., 2010, Caccamo, et al., 2005) that have shown that naïve cells constitute for just a fifth of Vδ2 T cells, while T_{EMRA} account for just a small percentage, while T_{CM} make up the majority and T_{EM} account for a high proportion of Vδ2 T cells. However, there was substantial inter-donor variability in the cited studies, which was also observed in the donors examined in this study. Furthermore, expanded Vδ2 and Vδ3 T cells exhibited a mainly T_{EM} status, as expected following antigen exposure and culture for several weeks, which was also shown by others (Dunne, et al., 2010).

We also found that a minority of Vδ1 and Vδ2 T cells expressed CD69, a marker of T cell activation, which was also seen by Dunne et al (2010) and it was found on a proportion of Vδ3 T cells. Upon pAg activation, the majority of Vδ2 T cells expressed CD69, which was also previously reported (Dunne, et al., 2010, Pechhold, et al., 1994). Although CD56 expression was reported to be upregulated upon pAg stimulation (Zheng, et al., 2013), we found CD56 expression on only a small percentage of pAg-stimulated Vδ2 T cells, and these co-expressed CD69. However, this was assessed 2 months following pAg stimulation and the cells may have expressed higher levels of CD56 earlier post-activation. Dunne found that a third of Vδ2 T cells expressed CD56 following pAg stimulation. TLR2 expression was observed in each of the subsets, which agrees with other studies which showed that Vδ2 T cells express various TLR including TLR1, TLR2 and TLR3 (Pietschmann, et al., 2009, Wesch, et al., 2006, Wesch, et al., 2011, Beetz, et al., 2008). However, TLR2 was virtually undetectable on our pAg-expanded Vδ2 T cells. Vδ2 T cells also frequently express NKG2D (Biassoni, et al., 2001), which allows them to directly detect and kill tumour cells. We found that a

proportion of Vδ1, Vδ2 and Vδ3 T cells expressed the NK cell marker NKG2D. We also observed CD161 expression by all three subsets. Recently, CD161 has been linked with T_H17-expressing cells, and its presence on γδ T cells is consistent with their ability to produce T_H17 cytokines under certain conditions. Overall, we found great inter-individual variability among donors, and thus higher numbers would be required to better define these phenotypes.

A single stimulation with HMB-PP of enriched γδ T cells was sufficient to yield cell lines of high purity after two weeks of culture. We found that most donors reached high purity using this method. However, expansion from PBMC was not successful in all donors, as some donors did not expand well upon HMB-PP stimulation, and on average, purity of ~30% was obtained. It is important to note that the old batch of HMB-PP was more potent than the new one. In contrast, most donors responded to stimulation using the aminobisphosphonate zoledronate, resulting in purities of ~70% in the same donors. Using both zoledronate and HMB-PP simultaneously did not significantly increase Vδ2 T cell purities.

Vδ2 T cells are known to rapidly produce T_H1 cytokines IFN-γ and TNF-α upon activation, but can also be primed to produce T_H17 cytokines under certain conditions (Eberl, et al., 2009). We investigated the expression of T_H1, T_H2 and T_H17 cytokines, and confirm this by showing that HMB-PP-activated Vδ2 T cells produced IFN-γ and TNF-α, but no T_H2 or T_H17 cytokines. Interestingly, we found that while a high percentage of resting Vδ2 T cells expressed IL-4 within 4 h of stimulation, these did not secrete IL-4 within 24-72 h. However, following HMB-PP-activation there was a significant increase in IL-4 secretion by Vδ2 T cells, even though the numbers expressing IL-4 remained the same. IFN-γ and TNF-α levels by HMB-PP-activated Vδ2 T cells were also significantly increased within 24 h. At the 72 h time point, cytokine levels were lower than 2 days earlier, suggesting that the cytokines were used up or recycled by the cells. Another interesting finding was that stimulation with zoledronate seemed to induce T_H2 but not T_H17 cytokines and also prevented T_H1 cytokine production by Vδ2 T cells, highlighting the difference in activation mode between pAg and aminobisphosphonate stimulation of Vδ2 T cells.

Vδ3 T cells are found at very low frequencies in human blood, and they must be activated and expanded for *in vitro* studies. Thus far, only one method to expand Vδ3 T cells has been described (Mangan, et al., 2013). As no ligand for this cell subset has yet been identified, these cells can only be stimulated by the non-specific mitogen PHA, which stimulates all T cells. Thus, to yield highly pure populations of this subset *in vitro*, the cells must first be sorted from PBMC by flow cytometry to recover a highly pure population. An excess number of irradiated PBMC were used to provide cell contact and secreted factors required to stimulate T cell expansion. Expansion was carried out in conjunction with IL-2, which is required for cell survival and proliferation (Smith, 1988). Within 4 weeks, we found an increase in cell numbers of up to 1,000 fold, and these cells were of high purity (>90%). However, it is important to note that this was not the case when cell sorting did not yield pure populations of Vδ3 T cells prior to *in vitro* expansion.

We found that, like Vδ2 T cells, Vδ3 T cells were mainly DN for CD4 and CD8, and there was a small percentage of CD8 T cells. These findings reflect those found by Mangan et al (2013), which also found that Vδ3 T cells expressed high levels of NKG2D, CD56, CD28, HLA-DR, CD161 and low levels of CD69, CD25 and NKG2A but no NKG2C, thus indicating that Vδ3 T cells, like Vδ2 T cells express phenotypic markers that are also found on NK cells which confer the ability to recognise tumour cells (Mangan, et al., 2013).

Analysis of cytokine production revealed that resting Vδ3 T cells expressed very low levels of IFN-γ, TNF-α and IL-4 and no IL-17. Upon stimulation with PMA and ionomycin, IFN-γ, TNF-α and IL-4 levels were significantly increased. While PHA did not affect cytokine expression by Vδ3 T cells, PHA induced a significant increase in IFN-γ secretion, and a small increase in IL-4 secretion. Moreover, we found that IL-17 levels remained the same following PMA and ionomycin stimulation. This is in contrast to the findings by Mangan and colleagues which found that they produced high levels of IL-17 upon PMA and ionomycin stimulation. In addition, they also assessed IL-10 levels and found that Vδ3 T cells did not produce IL-10. Thus, like Vδ2 T cells, activated Vδ3 T cells can produce T_H1 cytokines, but not T_H2, T_H17 or T_{reg} cytokines (Mangan, et al.,

2013). The cytokine profiles of expanded Vδ2 and Vδ3 T cells are summarised in Fig. 3.27.

A summary comparing the differences between surface receptors and cytokine production by fresh and expanded Vδ2 or Vδ3 T cells is depicted in Fig. 3.28.

γδ T cells are found in very small numbers, and thus to study their effects or to harness their therapeutic potential, they need to be expanded. There are various methods described for expanding Vδ2 T cells *in vitro*. HMB-PP is the most potent activator, but others include the synthetic BrHPP (Bennouna, et al., 2010, Chargui, et al., 2010), IPP (Tanaka, et al., 1995) and aminobisphosphonates such as zoledronate (Dieli, et al., 2007). Cancer immunotherapy strategies often involve adoptive transfer of *ex vivo* Vδ2 T cells into patients (Izumi, et al., 2013, Bennouna, et al., 2008, Kobayashi, et al., 2007, Nakajima, et al., 2010, Bonneville and Scotet, 2006) where circulating Vδ2 T cell numbers are decreased due to the disease. An increased number of circulating Vδ2 T cells has been found to increase the susceptibility of tumour cells to Vδ2-mediated cell lysis (Wrobel, et al., 2007, Benzaid, et al., 2011, Todaro, et al., 2009) and has been associated with increased tumour clearance (D'Asaro, et al., 2010, Capietto, et al., 2011, Hannani, et al., 2012). However, the same potent effector activities could also result in undesirable immune responses such as inflammation and autoimmunity. Thus, a better understanding of the outcomes of manipulating the interactions between γδ T cells with other immune cells is required to direct desired immune responses, and will be examined in the subsequent chapters.

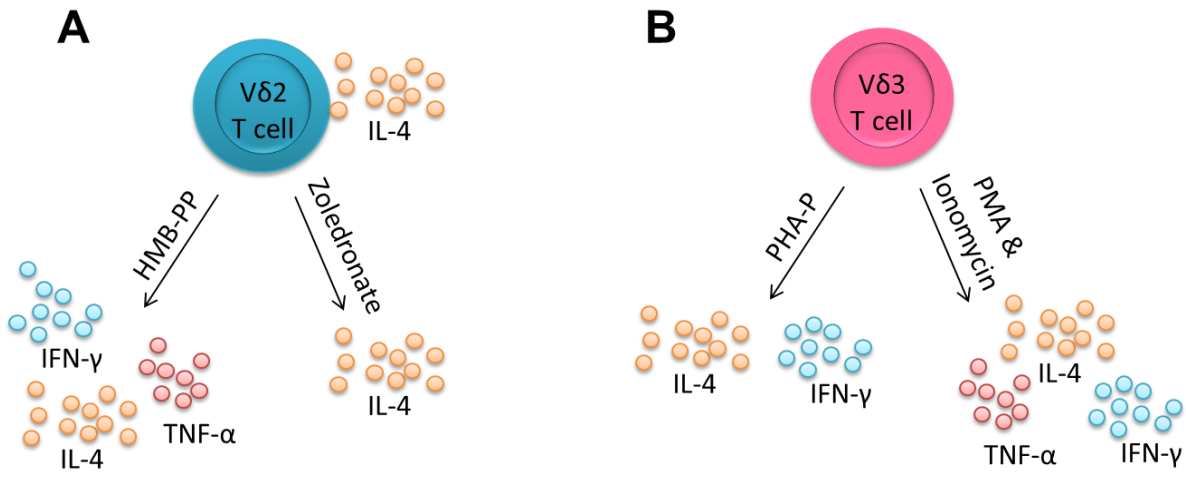

Figure 3.27. Summary of cytokine production by Vδ2 and Vδ3 T cells. A, Resting Vδ2 T cells produce IL-4 and very low levels of other T_H1 and T_H2 cytokines, and upon HMB-PP activation they also produce IFN-γ and TNF-α, while Zoledronate only induced IL-4 production. **B**, resting Vδ3 produce very low levels of T_H1 and T_H2 cytokines and upon PHA-P stimulation they produce IL-4 and IFN-γ, while PMA and ionomycin stimulation also induced IL-4 production.

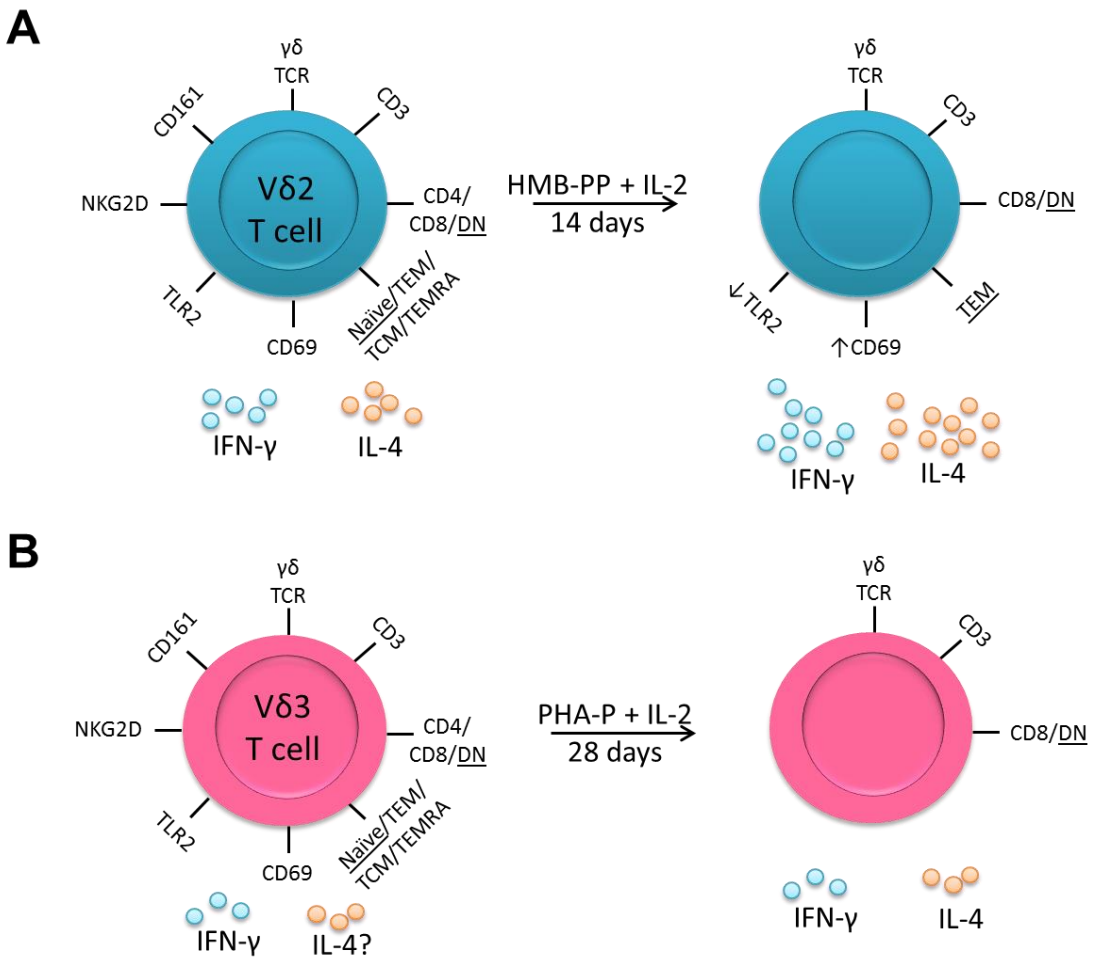

Figure 3.28. Summary of cell surface markers and cytokine production by fresh versus expanded Vδ2 and Vδ3 T cells. A, Resting, fresh Vδ2 T cells express low levels of activation markers, TLR and NK markers, and are predominantly CD4 and CD8 negative, but also express CD4 or CD8. Furthermore, they exhibit a predominantly naïve phenotype, although there is a high proportion of central memory cells (T_{CM}) and terminally differentiated cells (T_{EMRA}). Following expansion using HMB-PP and IL-2 for 14 days, resting Vδ2 T cells express a reduced level of TLR2 but an increased expression of activation markers, and although a small percentage express CD8, there are no $CD4^+$ cells. In addition, most of the cells express an effector memory phenotype (T_{EM}). Lastly, expanded Vδ2 T cells produce higher levels of IFN-γ and IL-4 than fresh Vδ2 T cells. **B**, Resting, fresh Vδ3 T cells also express low levels of TLR, NK receptors and activation markers, and they are predominantly CD4 and CD8 negative, but can also express CD4 or CD8. Similarly, they exhibit a predominantly naïve phenotype but also T_{CM} and T_{EMRA} differentiation status. Following expansion with PHA-P and IL-2 for 28 days, these cells are predominantly double negative for CD4 and CD8 and there are no $CD4^+$ cells. Unlike Vδ2 T cells, expanded Vδ3 T cells do not express increased levels of IFN-γ and IL-4 compared to fresh Vδ3 cells.

Chapter 4

Reciprocal activating interactions between human Vδ2^{+} γδ T cells, dendritic cells and B cells

4.1 Introduction

T cells expressing the Vγ9Vδ2 TCR comprise the most abundant γδ T cell subset in human blood, where they typically account for 1-5% of T cells in healthy adults (Kabelitz and He, 2012, Vantourout and Hayday, 2013, Morita, et al., 2007, Chien, et al., 2014). In many microbial infections, Vγ9Vδ2 T cells dramatically expand, reaching >50% of all T cells at infected sites (Hara, et al., 1992), thus indicating their importance in antimicrobial immunity and their potential for diagnostic and therapeutic use. The Vγ9Vδ2 TCR recognises a variety of pAg, which are low molecular weight pyrophosphate intermediates of isoprenoid biosynthesis. The most potent pAg is HMB-PP, an intermediate of the non-mevalonate pathway that is found in the majority of Gram-negative bacteria, some Gram-positive species and some parasites (Morita, et al., 2007, Eberl, et al., 2003) but is not found endogenously. Eukaryotic cells use the alternative, mevalonate pathway which results in IPP production, and can thus serve as a self-ligand for Vγ9Vδ2 T cells. Aminobisphosphonates, which are clinically approved for use in bone disorders, can also activate Vγ9Vδ2 T cells through IPP accumulation. They do this by inhibiting the enzyme responsible for IPP breakdown. Recently, BTN3A/CD277 was shown to bind to pAg within cells, resulting in activation of Vγ9Vδ2 T cells (Harly, et al., 2012, Sandstrom, et al., 2014) and although the mechanism is not fully understood, it provides insight into Vγ9Vδ2 T cell activation. Activated Vγ9Vδ2 T cells exhibit a range of effector functions including direct cytotoxicity of infected and tumour cells, the induction of inflammatory and immunoregulatory processes and promotion of the survival, differentiation and activation of monocytes, neutrophils, dendritic cells, αβ T cells and B cells (Kabelitz and He, 2012, Vantourout and Hayday, 2013, Morita, et al., 2007, Chien, et al., 2014).

Vγ9Vδ2 T cells can bridge innate and adaptive immune responses by promoting the differentiation of a number of cell types into APC. DC are the most potent professional APC. They exist in peripheral tissues as specialised cells for pathogen recognition and uptake by phagocytosis, endocytosis and pinocytosis, which results in their upregulated expression of antigen presenting and co-stimulatory molecules, secretion of cytokines and migration to lymphoid organs where they present antigen to naïve T

cells (Banchereau, et al., 2000, Mellman and Steinman, 2001). Vγ9Vδ2 T cells, alone and in synergy with pathogen products, can induce differentiation of DC into immunogenic APC that express co-stimulatory markers, produce cytokines and stimulate T cells (Dunne, et al., 2010, Conti, et al., 2005, Devilder, et al., 2006, Ismaili, et al., 2002, Martino, et al., 2005, Shrestha, et al., 2005). Furthermore, HMB-PP-stimulated Vγ9Vδ2 T cells are also capable of promoting survival and differentiation of monocytes into inflammatory DC (Eberl, et al., 2009, Kalyan and Chow, 2009). Vγ9Vδ2 T cells are also capable of inducing recruitment, activation and survival of neutrophils (Agrati, et al., 2009, Davey, et al., 2011) and a recent study has shown that neutrophils exposed to Vγ9Vδ2 T cells acquire the ability to present microbial antigens to $CD4^+$ T cells and to cross-present endogenous antigens to $CD8^+$ T cells (Davey, et al., 2014).

B cells are also capable of presenting antigens to T cells (Rodriguez-Pinto, 2005) and secreting cytokines that activate and regulate adaptive immune responses (Harris, et al., 2000). A number of studies have demonstrated that Vγ9Vδ2 T cells can induce differentiation of B cells into antibody-producing plasma cells (Brandes, et al., 2003, Caccamo, et al., 2006, Bansal, et al., 2012, Caccamo, et al., 2012). They can be found in germinal centers, can acquire features of follicular helper T cells and can induce the production and affinity maturation of class-switched antibodies. However, it is not known if Vγ9Vδ2 T cells contribute to antigen-presentation and cytokine secretion by B cells.

4.2 Objectives

While some studies have shown that Vδ2 T cells can promote maturation of DC into APC capable of cytokine secretion, the relationship between Vδ2 T cells and B cells is not well characterised. Thus, we compared the ability of Vδ2 T cells to induce DC and B cell maturation by examining expression of co-stimulatory markers on DC and B cells, and the resulting cytokine production and antibody release by B cells. In addition, we examined the role of several factors in the activation of DC and B cells by Vδ2 T cells, and investigated the ability of these Vδ2-activated DC and B cells to stimulate resting conventional T cells. In addition, we explored the role of LPS in these interactions, and examined the reciprocal effects of DC and B cells on Vδ2 T cells.

The specific aims were:

- To compare the co-stimulatory marker expression and cytokine production by DC and B cells following culture with resting or HMB-PP-activated Vδ2 T cells or with LPS
- To investigate the role of various molecules and cell contact in the activation of DC by Vδ2 T cells
- To examine the antibody production by B cells cultured with resting or HMB-PP-activated Vδ2 T cells or LPS and to examine the molecular mechanisms involved
- To analyse whether Vδ2-matured DC and B cells can result in activation of resting allogeneic or autologous conventional T cells
- To examine the effect of altering the DC to Vδ2 ratio on cytokine production by these cells
- To investigate the effect on DC, B cells and Vδ2 T cells when the three cell types are cultured together
- To examine whether DC and B cells can in turn activate Vδ2 T cells

4.3 Methods

4.3.1 Cell line generation and co-cultures

All cells were isolated from human buffycoat packs (see section 2.6.1). Vδ2 T cell lines were generated by stimulating magnetically-sorted γδ T cells with HMB-PP and culturing with IL-2 for 14-28 days (see section 2.6.2). Immature DC were generated by isolating monocytes from PBMC and culturing with IL-4 and GM-CSF for 6 days (see section 2.6.5). B cells were derived by isolating $CD19^+$ cells from PBMC (see section 2.6.4). Non-γδ T cells were obtained by magnetically sorting $γδ^-$ $CD3^+$ T cells from PBMC.

Allogeneic co-cultures were set up where the different cell types were derived from distinct PBMC donors. In some cases, we used an autologous system where all cell types used in a co-cultured came from the same donor. The cells (1×10^5 of each cell type) were cultured separately or together in 96-well round bottom plates.

4.3.2 Analysis of co-stimulatory marker expression by DC and B cells

Vδ2 T cells were cultured with either B cells or DC in equal numbers in the presence or absence of HMB-PP for 72 h or 24 h in cRPMI, respectively. The cells were stained for expression of CD11c (DC), CD19 (B cells), CD3 and Vδ2 and markers of antigen presentation CD40, CD80, CD86 and HLA-DR. Surface expression of these markers was compared by MFI readings obtained using flow cytometry (see section 2.7.1). Autologous co-cultures were also carried out.

4.3.3 Analysis of cytokine production

Vδ2 T cells were cultured with either B cells or DC in equal numbers in the presence or absence of HMB-PP for 72 h or 24 h, respectively. The supernatants were then harvested and assayed for levels of IFN-γ, IL-4, IL-6, IL-10, IL-12p70 and TNF-α by ELISA (see section 2.7.2). The cells from the same cultures were cultured with monensin overnight and then stained for cell surface expression of CD3 and Vδ2 (Vδ2 T cells), CD19 (B cells) or CD11c (DC). The cells were then fixed and permeabilised and stained for intracellular expression of IFN-γ, IL-4, IL-6, IL-10, IL-12p40, IL-13 and TNF-α for analysis by flow cytometry (see section 2.7.3). Autologous co-cultures were also carried out.

4.3.4 Measurement of antibody production by B cells

Vδ2 T cells were cultured with B cells at 1:1 ratios in the presence or absence of HMB-PP for 7 days. The supernatants were harvested and analysed using immunoglobulin Cytometric Bead Array kits for IgA, IgM, IgE and total IgG levels (see section 2.7.4).

4.3.5 Blocking experiments

Vδ2 T cells were cultured with either DC or B cells in equal numbers in the presence or absence of HMB-PP and low-endotoxin, azide-free functional grade blocking antibodies against CD86, CD40L, IFN-γ plus IFN-γR, IL-4 plus IL-4R or TNF-α or isotype control mAb for 24 h. Similar cultures were set up in transwell plates to prevent cell contact. The effects of blocking on DC and B cell phenotypes, cytokine expression and

release and antibody production were determined as described above (see section 2.7.5).

4.3.6 Analysis of T cell stimulatory capacities of DC and B cells

DC and B cells are both professional antigen presenting cells capable of internalising, processing and presenting antigenic peptides on MHC molecules to naïve T cells, resulting in their activation. We tested the ability of Vδ2 T cells to influence the ability of B cells and DC to present two antigens, tetanus toxoid and tuberculin, to autologous T cells resulting in their proliferation and maturation into cytokine producing effector T cells. Since T cells reactive to tetanus toxoid or tuberculin are likely to be found in low frequencies, we also tested allogeneic T cells as responders. T cell activation was measured by proliferation, cytokine expression and the ability to release cytokines on subsequent activation with PMA and ionomycin. The ability of Vδ2 T cell-matured DC or B cells to stimulate proliferation of labeled T cells was tested by flow cytometric analysis of CellTrace dilution. Vδ2 T cells were cultured with either B cells or DC in equal numbers in the presence or absence of HMB-PP for 24 h. Tetanus toxoid (40 limes of flocculation, lf, per ml), purified protein derivative of tuberculin (10 ng/ml) or media was added to the culture for 3 h prior to addition of CellTrace labeled αβ T cells. CellTrace-labeled autologous naïve αβ T cells were added at a ratio of 10:1 to the 24 h cultures of B cells or DC with Vδ2 T cells and cultured for 6 days. PHA-P-stimulated αβ T cells cultured with IL-2 and irradiated PBMC were used as positive controls. Percentages of proliferating cells were observed by flow cytometric analysis of CellTrace dilution compared to an unstimulated control, as described in section 2.7.6. These results were compared to an allogeneic stimulation.

Similar co-cultures, except using unlabeled alloreactive αβ T cells, were incubated for 3 days to look for expression of intracellular cytokines by alloreactive T cells (see section 2.6.6). The supernatants harvested on day 3 were assayed for IL-2, IL-4, IL-10 and IFN-γ secretion by ELISA (see section 2.7.2).

4.3.7 The effect of altering the ratio of DC to Vδ2 T cells on cytokine production

The cultures described above were carried out in equal ratios of DC and Vδ2 T cells. Since *in vivo*, these cells are unlikely to be found at equal ratios, the effect of altering the ratios of DC to Vδ2 T cells was investigated by examining cytokine production by flow cytometry. DC and Vδ2 T cells were cultured for 24 h at various ratios. The cells were examined for IFN-γ, IL-4 and TNF-α expression by both DC and Vδ2 T cells by flow cytometry (see section 2.7.3).

4.3.8 The effect of culturing DC, B cells and Vδ2 T cells simultaneously

The effect of culturing DC, B cells and Vδ2 T cells together was examined. DC, B cells and Vδ2 T cells were cultured separately and together for 24 h. The supernatants were harvested and analysed for levels of IFN-γ, IL-4 and TNF-α by ELISA (see section 2.7.2). The cells from the culture were examined for IFN-γ, IL-4 and TNF-α expression by DC, B cells and Vδ2 T cells (see section 2.7.3).

4.3.9 The effect of LPS on DC and B cell activation

Vδ2 T cells were cultured with DC or B cells in the presence of LPS for 24 h and examined for expression of co-stimulatory markers, cytokine production and antibody release, as described in sections 4.3.2, 4.3.3 and 4.3.5, respectively.

The methods used in this chapter are summarised below (Fig. 4.1).

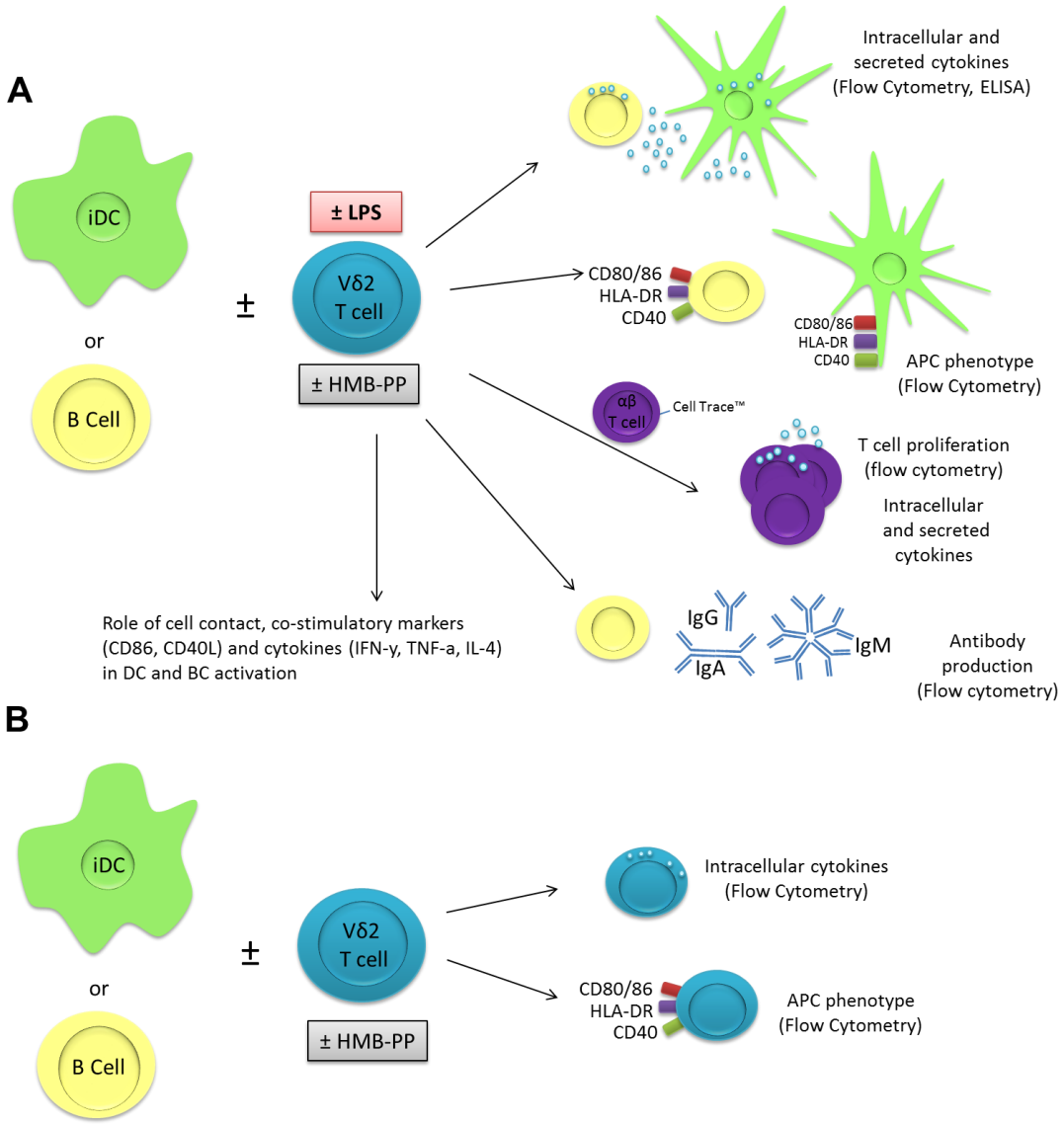

Figure 4.1. Analysis of reciprocal interactions between Vδ2 T cells and B cells or DC. A, monocyte-derived DC or enriched peripheral blood B cells were cultured in the absence or presence of HMB-PP-expanded Vδ2 T cells and left unstimulated or stimulated with LPS or HMB-PP. The DC or B cells were then examined for cytokine expression and secretion, APC phenotypes, ability to stimulate proliferation and cytokine expression by alloreactive and autologous T cells and antibody isotype production. The role of cell contact, co-stimulatory markers and cytokines in DC and B cell activation was examined. **B**, monocyte-derived DC or enriched peripheral blood B cells were cultured in the absence or presence of HMB-PP-expanded Vδ2 T cells and left unstimulated or stimulated with HMB-PP. The Vδ2 T cells were then examined for cytokine production and expression of co-stimulatory molecules.

4.4 Results

4.4.1 Vδ2 T cells induce APC marker expression by DC and B cells

We initially investigated if Vδ2 T cells can induce differentiation of B cells into cells with phenotypes of APC and compared the adjuvant effect with those induced by Vδ2 T cells on monocyte-derived DC. Therefore we examined the expression of CD40, CD86 and HLA-DR by B cells or DC after co-culture with non-stimulated or HMB-PP activated Vδ2 T cells. Vδ2 T cells induced an increase in CD86 (Fig. 4.2A) and HLA-DR (Fig. 4.2C), but not CD40 (Fig. 4.2E) or CD80 (Fig. 4.2G) expression by DC after 24 h and CD86 (Fig. 4.2B), HLA-DR (Fig. 4.2D) and CD40 (Fig. 4.2F) but not CD80 (Fig. 4.2H) expression by B cells after 72 h. CD86 expression was also upregulated on B cells after 24 h (data not shown). To investigate which molecules were involved in DC and B cell activation by Vδ2 T cells or whether it is cell contact dependent, the same co-cultures were set up in the presence of HMB-PP-activated Vδ2 T cells and one of several blocking antibodies or transwell inserts which prevent cell contact between the different cell types in the co-cultures. The results showed that cell contact was important for CD86 expression by DC (Fig. 4.2A), while CD86, TNF-α and IFN-γ were important for HLA-DR (Fig. 4.2C) and CD40 (Fig. 4.3E) expression by DC. In contrast, CD40L and cell contact were important for HLA-DR expression (Fig. 4.2D) but not CD86 (Fig. 4.2B) or CD40 (Fig. 4.2F) expression by Vδ2-stimulated B cells.

4.4.2 Vδ2 T cells induce production of distinct cytokines by DC and B cells

To further characterise the influence of Vδ2 T cells on DC and B cell activation, the same co-cultures were examined for intracellular cytokine expression. The co-cultures, as described above, were treated with monensin for 16 h and the DC or B cells were analysed for intracellular IFN-γ, IL-4 (Fig. 4.3A,B) expression. Vδ2 T cells induced IFN-γ expression by DC (Fig. 4.3C) but not B cells and IL-4 expression by B cells (Fig. 4.3D) but not DC. In addition, we examined TNF-α expression by DC (Fig. 4.4A,B) and B cells (Fig. 4.4BC,D) and found that Vδ2 T cells induced TNF-α expression by both DC and B cells. The blocking studies revealed that CD86 and IFN-γ were important for IFN-γ expression by DC (Fig. 4.3C), but not for IL-4 production by B cells (Fig. 4.3D) or for TNF-α expression by DC or B cells (Fig. 4.4A,C).

4.4.3 Vδ2 T cells induce pro- and anti-inflammatory cytokine secretion from DC and B cell co-cultures

While the flow cytometric cytokine assay revealed the percentage of cells expressing cytokines, the levels of cytokine production from the co-cultures were also quantified using ELISA. After 24 h co-culture of Vδ2 T cells and DC or B cells, the supernatants were analysed for levels of IFN-γ, TNF-α, IL-4, IL-6, IL-10 and IL-12 by ELISA. Since the cellular source of the cytokines produced could not be identified, cytokine production by Vδ2 T cells alone was also examined (see Fig. 3.18, Fig. 3.19). It was found that Vδ2-DC co-cultures produced significantly greater amounts of IFN-γ (Fig. 4.5A), TNF-α (Fig. 4.6A) and IL-6 (Fig. 4.6B) but not IL-4 (Fig. 4.5C), IL-10 (Fig. 4.6C) or IL-12 (Fig. 4.6D) after 24 h compared to Vδ2 T cells or DC cultured alone. In contrast, Vδ2-B cell co-cultures produced TNF-α (Fig. 4.6E) and IL-6 (Fig. 4.6F) but did not augment IFN-γ (Fig. 4.5B), IL-4 (Fig. 4.5D), IL-10 (Fig. 4.6G) or IL-12 (Fig. 4.6H) production compared with Vδ2 T cells cultured alone. In most cases, the induction of cytokine release by DC on B cells by Vδ2 T cells required activation with HMB-PP. None of the molecules tested in the blocking studies, nor cell contact were found to be important for cytokine secretion by these co-cultures. However, surprisingly, blocking of CD86 resulted in augmented IFN-γ secretion after co-culture with Vδ2 T cells (Fig. 4.5A).

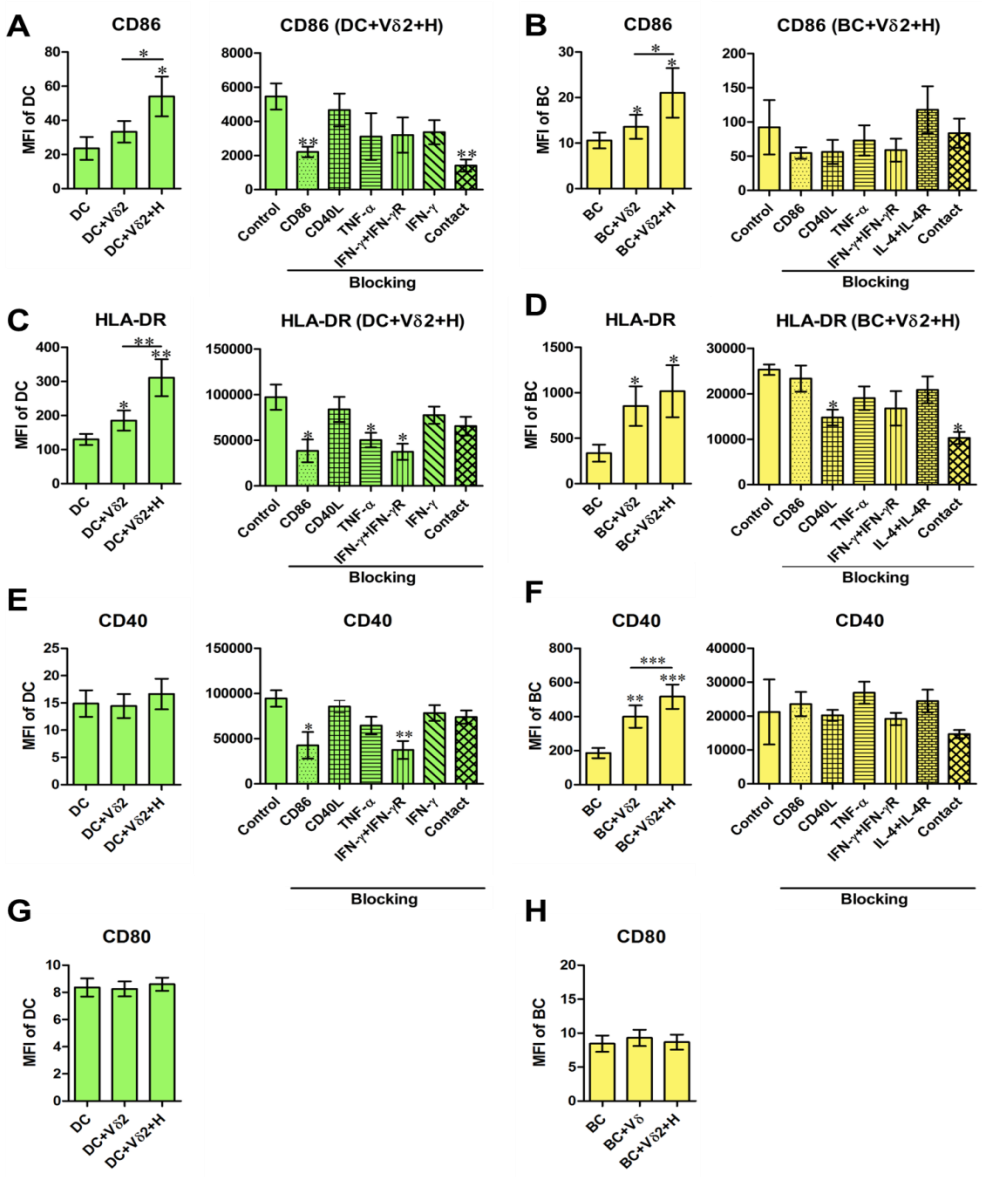

Figure 4.2. Expression of APC markers by dendritic cells (DC) and B cells (BC). Monocyte-derived DC or enriched peripheral blood B cells were cultured for 24 or 72 h, respectively, with HMB-PP-expanded Vδ2 T cells in the absence or presence of HMB-PP (denoted H). Cells were then stained using mAb specific for CD11c or CD19 and CD86, CD80, CD40 and HLA-DR and analysed by flow cytometry. Left panels show average (±SEM) mean fluorescence intensities (MFI) of staining for CD86 expression by DC (**A**; n=11) and BC (**B**; n=12), HLA-DR expression by DC (**C**; n=9) and BC (**D**; n=7), CD40 expression by DC (**E**; n=7) and BC (**F**; n=7) and CD80 expression by DC (**G**; n=8) and BC (**H**; n=14). Right panels show average (±SEM) MFI of staining for CD86 or HLA-DR by DC or B cells after co-culturing them with Vδ2 T cells in the presence of HMB-PP in the absence (control) or presence of blocking mAb specific for CD86, CD40L, TNF-α, IFN-γ + IFN-γR, IL-4 + IL-4R or with the DC or B cells separated from Vδ2 T cells using transwell inserts (contact; n=5 for DC treatments and n=3 for BC treatments). **p*<0.05, ***p*<0.01 using a paired *t* test, compared to DC or BC alone (left panels) or compared to BC control (right panels) and unpaired *t* test compared to DC control (right panels) except where indicated by horizontal lines.

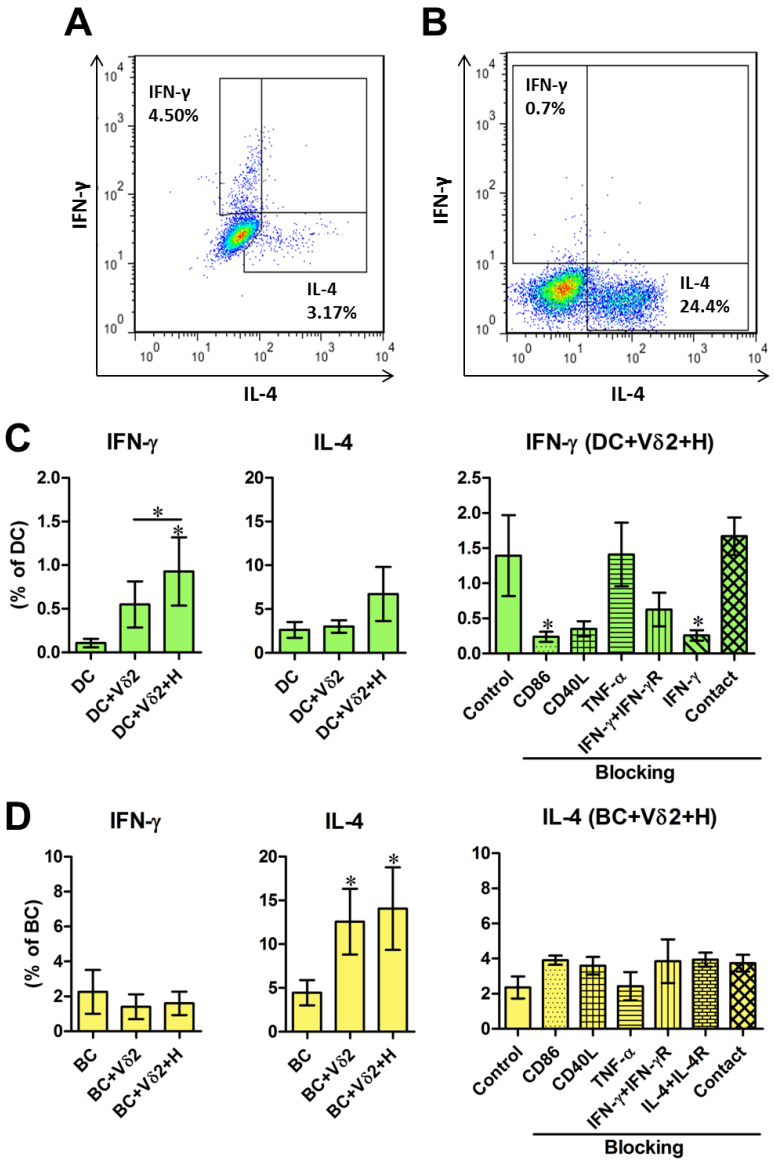

Figure 4.3. Cytokine production by DC and B cells cultured with Vδ2 T cells. DC or B cells were co-cultured with HMB-PP-expanded Vδ2 T cells in the absence or presence of HMB-PP (denoted H) for 24 h. The cultures were then treated with monensin for a further 16 h and stained for cell surface expression of CD11c or CD19 and CD3 and Vδ2 and intracellular expression of IFN-γ and IL-4 and analysed by flow cytometry. **A-B**, Representative flow cytometric dot plots showing IFN-γ and IL-4 expression by gated $CD11c^+$ cells (DC) and $CD19^+$ cells (BC), respectively. **C-D**, Left and center panels show mean (± SEM) percentages of DC (**C**; n=10) and BC (**D**; n=10) that express IFN-γ and IL-4, respectively. Right panels show mean (±SEM) percentages of DC (**C**) and BC (**D**) expressing IFN-γ and IL-4, respectively, after co-culturing them with Vδ2 T cells in the presence of HMB-PP in the absence (control) or presence of blocking mAb specific for CD86, CD40L, TNF-α, IFN-γ + IFN-γR, IL-4 + IL-4R or with the DC (n=5) or BC (n=3) separated from Vδ2 T cells using transwell inserts (contact). *p<0.05 using a paired t test, compared to DC or BC alone (left panels) or compared to BC control (right panels) and unpaired t test compared to DC control (right panels) except where indicated by horizontal lines.

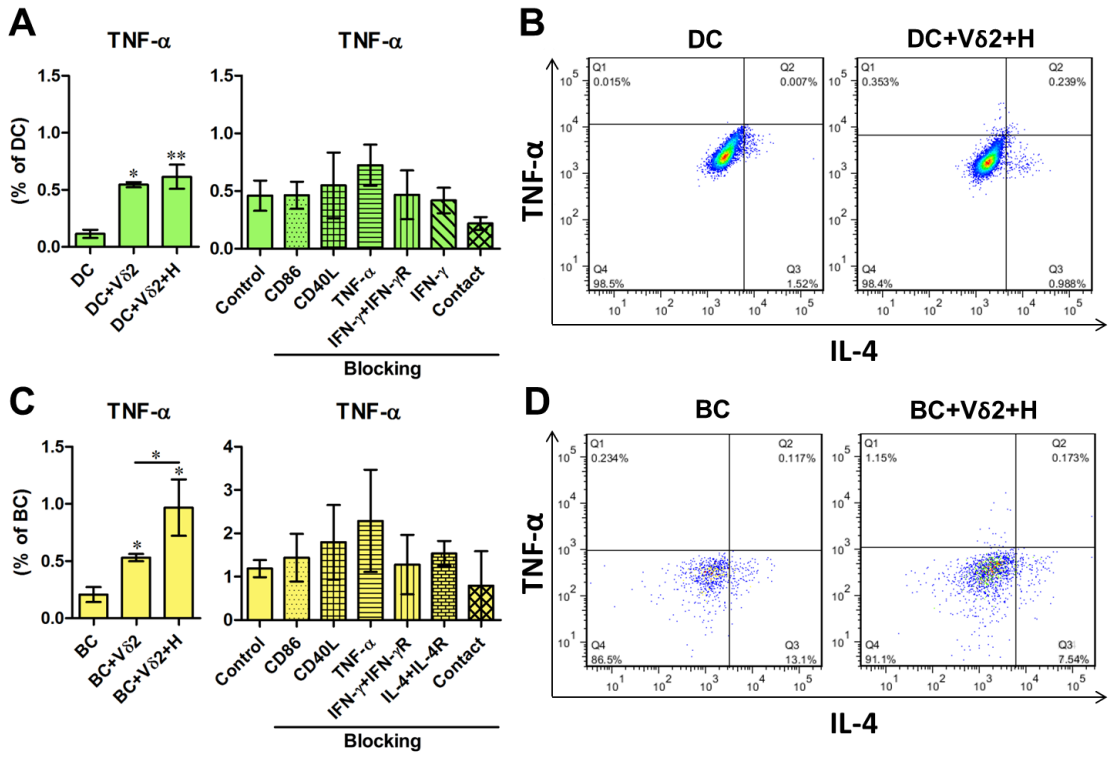

Figure 4.4. TNF-α expression by DC and B cells cultured with Vδ2 T cells. DC or B cells were co-cultured with HMB-PP-expanded Vδ2 T cells in the absence or presence of HMB-PP (denoted H) for 24 h. The cultures were then treated with monensin for a further 16 h and stained for cell surface expression of CD11c or CD19 and intracellular expression of TNF-α by gated $CD11c^+$ cells (DC) and $CD19^+$ cells (BC), respectively. **A**,**C**, left panels show mean (±SEM) percentages of DC (**A**; n=3-9) and BC (**C**; n=3-6) expressing TNF-α; right panels show mean (±SEM) percentages of DC (**A**) and BC (**C**) expressing TNF-α respectively, after co-culturing them with Vδ2 T cells in the absence (control) or presence of blocking mAb specific for CD86, CD40L, TNF-α, IFN-γ + IFN-γR, IL-4 + IL-4R or with the DC or B cells separated from Vδ2 T cells using transwell inserts (contact; n=5 for DC treatments and n=3 for BC treatments). **B**,**D**, representative flow cytometric dot plots of TNF-α expression by DC (**B**) and B cells (**D**). **p*<0.05, ***p*<0.01, using a paired *t* test compared to DC or BC alone (left panels) or compared to BC control (right panels) and unpaired *t* test compared to DC control (right panels) except where indicated by horizontal lines.

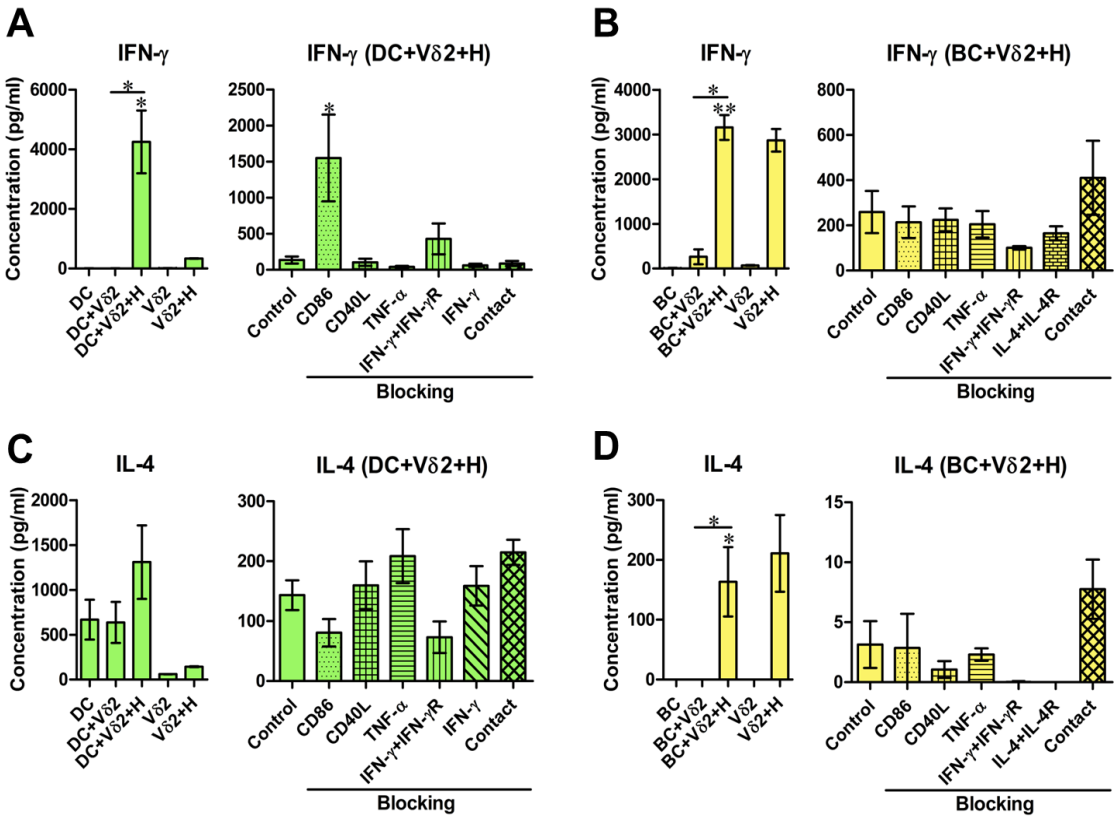

Figure 4.5. Cytokine secretion by cultures of Vδ2 T cells with DC or B cells. DC or B cells (BC) were co-cultured with HMB-PP-expanded human Vδ2 T cells in the absence or presence of HMB-PP (denoted H). After 24 h (for DC) or 72 h (for BC), supernatants were harvested and analysed for levels of IFN-γ and IL-4 by ELISA. Left panels show mean (±SEM) concentrations of IFN-γ in DC (**A**; n=3) and BC (**B**; n=3) co-cultures and IL-4 in DC (**C**; n=3) and BC (**D**; n=6) co-cultures. Right panels show average (±SEM) concentration of IFN-γ and IL-4 from the DC and B cell co-cultures in the presence of HMB-PP in the absence (control) or presence of blocking mAb specific for CD86, CD40L, TNF-α, IFN-γ + IFN-γR, IL-4 + IL-4R or with the DC (n=5) or B cells (n=3) separated from Vδ2 T cells using transwell inserts (contact). **p*<0.05, ***p*<0.01 using a paired *t* test, compared to DC or BC alone (left panels) or compared to BC control (right panels) and unpaired *t* test compared to DC control (right panels) except where indicated by horizontal lines.

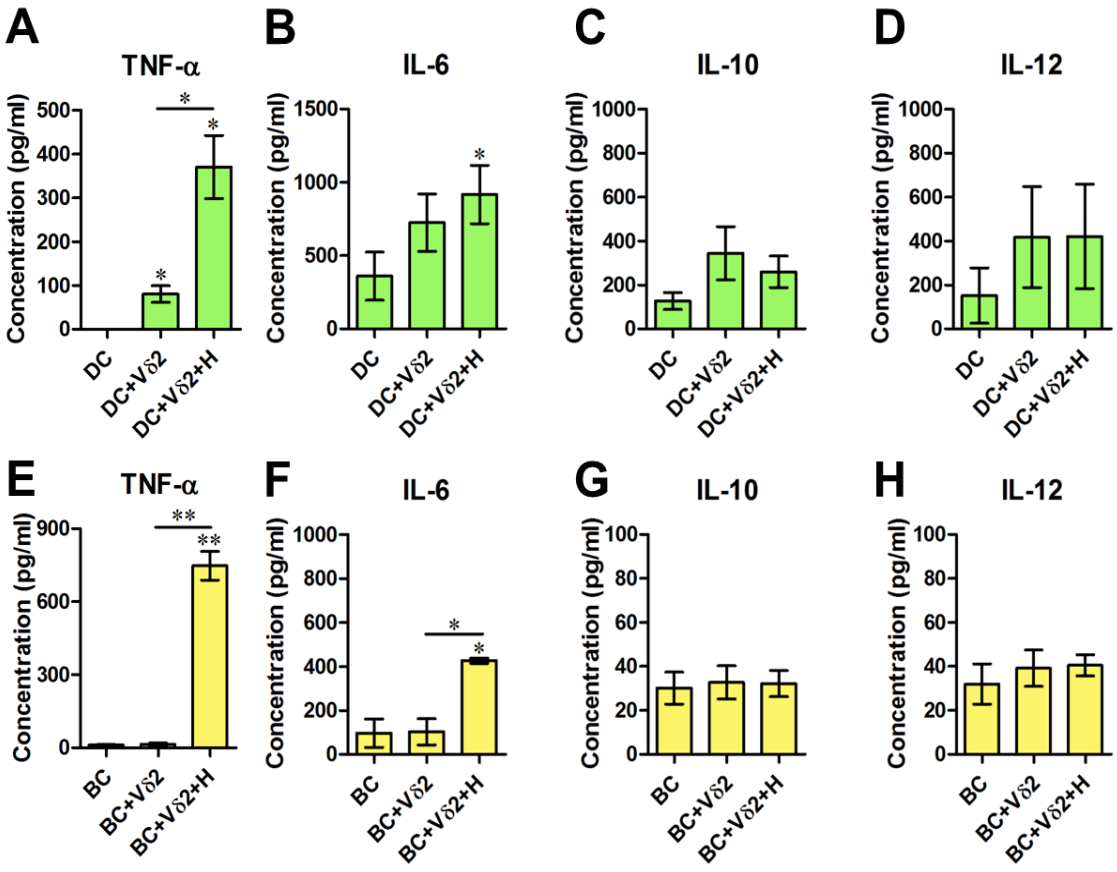

Figure 4.6. Cytokine secretion from cultures of Vδ2 T cells with DC or B cells. Monocyte-derived DC or enriched peripheral blood B cells (BC) were co-cultured with HMB-PP-expanded human Vδ2 T cells in the absence or presence of HMB-PP (denoted H). After 24 h (for DC) or 72 h (for BC), supernatants were harvested and analysed for IL-6, IL-10 and IL-12p70 by ELISA. Results show mean (±SEM) concentration of TNF-α (**A,E**), IL-6 (**B,F**), IL-10 (**C,G**) and IL-12p70 (**D,H**) from the DC (**A-D**; n=4-11) and BC (**E-H**; n=3-7) co-cultures. *p<0.05 using a paired t test compared to DC or BC alone except where indicated by horizontal lines.

4.4.4 Vδ2 T cells induce antibody production by B cells

Previous studies have shown that a subset of Vδ2 T cells can provide help for antibody production by B cells and that it was mediated by CD40L, ICOS and IL-10 (Caccamo, et al., 2012). To investigate whether Vδ2 T cells can induce immunoglobulin production by fresh peripheral B cells *in vitro*, Vδ2 T cells were cultured with B cells for 7 days, and the supernatants were analysed for total IgG, IgA, IgM and IgE. Vδ2 T cells induced IgG (Fig. 4.7A), IgA (Fig. 4.7B), IgM (Fig. 4.7C) but not IgE (Fig. 4.7D) secretion by B cells, while HMB-PP prevented the Vδ2 T cell-induced IgA (Fig. 4.7B) and IgM (Fig. 4.7C) production. The blocking studies revealed that the cytokines and co-stimulatory markers examined and cell contact did not appear to play a part in antibody production by B cells.

4.4.5 Allogeneic and autologous Vδ2 equally activate DC and B cells

The experiments described above indicate that Vδ2 T cells can differentially induce MHC and co-stimulatory molecule expression, cytokine production and T cell allostimulation by allogeneic DC and B cells. We sought to investigate whether the same outcomes could be observed when Vδ2 T cells were cultured with autologous DC or B cells. It was found that Vδ2 T cells could similarly induce CD86 expression (Fig. 4.8A) but not IL-12 secretion (Fig. 4.8C) by autologous and allogeneic DC. IL-4 secretion by B cells was similar where stimulated with autologous or allogeneic Vδ2 T cells (Fig. 4.8D). However, allogeneic Vδ2 T cells induced CD86 expression by B cells but this upregulation was not significant when autologous Vδ2 T cells and B cells were used (Fig. 4.8B). Thus it appears that allogeneic Vδ2 T cells may be substituted for autologous Vδ2 cells as adjuvants for DC or B cells.

4.4.6 Vδ2-matured DC and B cells stimulate proliferation of resting allogeneic T cells

We next investigated whether Vδ2 T cell-matured DC and B cells could induce activation and proliferation of resting αβ T cells. DC or B cells were co-cultured with equal numbers of expanded Vδ2 T cells for 24 h in the absence or presence of HMB-PP. Vδ2 T cell-matured DC or B cells were cultured with 10 times as many CellTrace-labeled resting allogeneic αβ T cells for 6 days and dye dilution due to cell proliferation

was examined (Fig. 4.9A,B). The co-cultures showed that both DC (Fig. 4.9C) and B cells (Fig. 4.9D) that had been cultured with Vδ2 T cells induced significantly more proliferation of resting T cells compared to DC or B cells cultured alone. This induction of allostimulatory activity by Vδ2 T cells did not require HMB-PP.

4.4.7 Vδ2-matured DC but not B cells stimulate cytokine production by resting allogeneic T cells.

We next investigated if DC or B cells, alone or after maturation with Vδ2 T cells, could stimulate cytokine expression and release by alloreactive cells. DC or B cells were matured in the presence of Vδ2 T cells for 24 h. Resting allogeneic αβ T cells were then added at a 10:1 ratio to this culture and incubated for a further 3 days. The supernatants were then assessed for levels of IFN-γ, IL-4, IL-2 and IL-10. Vδ2-matured DC induced IFN-γ (Fig. 4.10A) but not IL-4 (Fig. 4.10B) secretion from cultures with αβ T cells. In contrast, Vδ2-matured B cells did not induce IFN-γ (Fig. 4.10C), IL-4 (Fig. 4.10D), IL-2 (Fig. 4.10E) or IL-10 (Fig. 4.10F) from cultures with αβ T cells.

4.4.8 Vδ2-matured DC induce proliferation of autologous resting T cells irrespective of presence of antigen

The experiments in section 4.4.6 indicate that Vδ2 T cells can augment the allostimulatory capacity of DC and B cells. We then investigated if Vδ2 T cells could augment antigen-specific autologous T cell activation by DC and compared this with the activation of allogeneic T cells. Vδ2-matured DC were cultured with resting autologous αβ T cells in the presence or absence of tetanus toxoid or tuberculin antigen and examined for CellTrace dye dilution. It was found that the presence of antigen resulted in modest proliferation of αβ T cells when presented by Vδ2 T cells or DC alone. However, the presence of antigen did not significantly augment T cell proliferation when autologous Vδ2-matured DC were used compared to when the antigen was absent (Fig. 4.11). In this scenario, maximal proliferation was observed whether or not antigen was added. In all cases, the presence of HMB-PP did not affect αβ T cell stimulation by Vδ2-matured DC.

We also investigated if DC, Vδ2 T cells or Vδ2 T cell-matured DC could induce intracellular IFN-γ and IL-4 secretion by autologous or allogeneic αβ T cells. Figures 4.12 and 4.13 show that the presence of antigen also did not significantly augment intracellular expression of IFN-γ or IL-4 by autologous T cells cultured with Vδ2-matured DC (Fig. 4.12A,B) nor cytokine secretion from the same cultures (Fig. 4.13A,B).

4.4.9 Increasing the ratio of DC to Vδ2 T cells increases cytokine production by DC

The cultures described thus far were carried out using equal ratios of DC and Vδ2 T cells. Since *in vivo* these cells are not found at equal ratios, the effect of skewing the ratios of DC to Vδ2 T cells was assessed by examining cytokine production. It was found that an increase in Vδ2 T cell numbers boosted IFN-γ (Fig. 4.14A), IL-4 (Fig. 4.14B) and TNF-α (Fig. 4.14C) production by DC (left panels), while altering the ratio had no effect on the ability of Vδ2 T cells to produce IFN-γ, IL-4 and TNF-α (right panels).

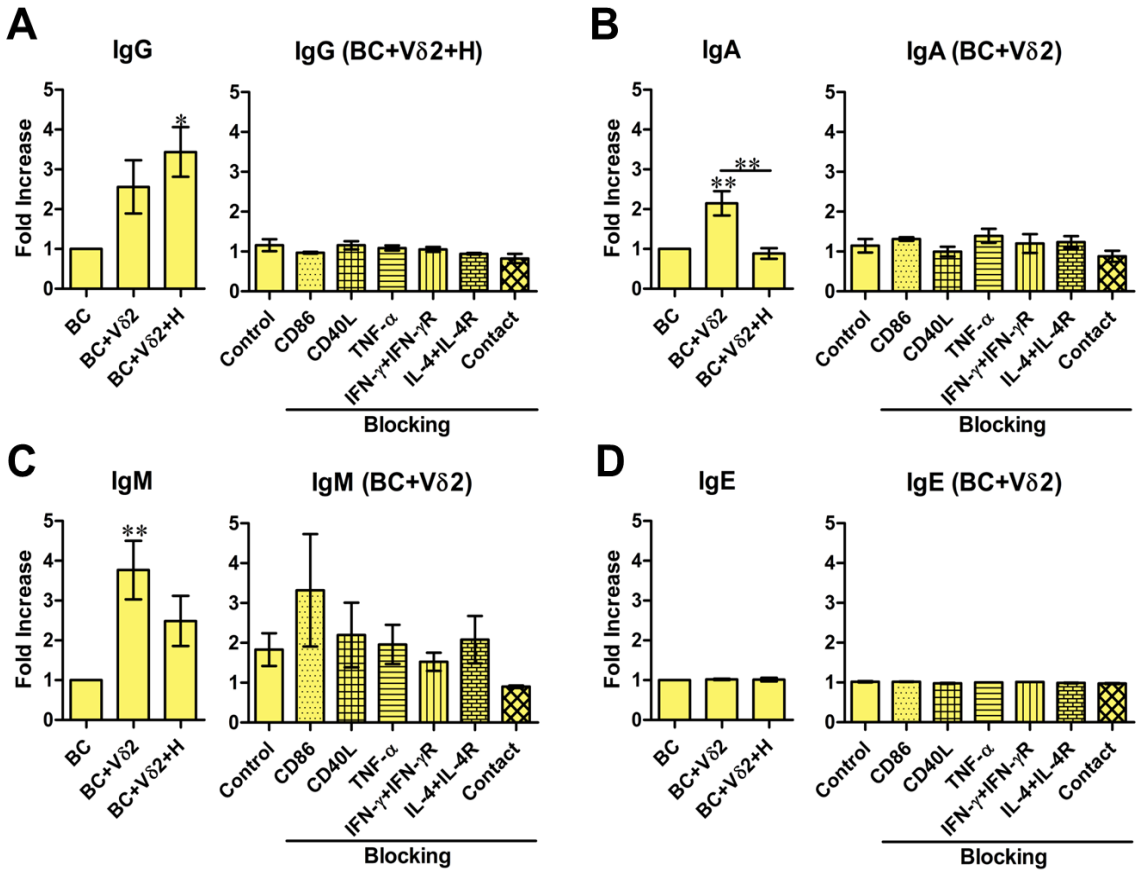

Figure 4.7. Antibody production by B cells cultured with Vδ2 T cells. Enriched peripheral blood B cells (BC) were co-cultured with HMB-PP-expanded human Vδ2 T cells in the absence or presence of HMB-PP (denoted H). After 7 days the supernatants were harvested and analysed for IgA, IgM, IgE and total IgG levels by cytometric bead array and flow cytometry. Left panels show average (±SEM) MFI of staining for IgG (**A**; n=5), IgA (**B**; n=8), IgM (**C**; n=7) and IgE (**D**; n=2). Right panels show average (±SEM) MFI intensities of IgG, IgA, IgM and IgE of B cells after co-culturing them with Vδ2 T cells in the presence of HMB-PP in the absence (control) or presence of blocking mAb specific for CD86, CD40L, TNF-α, IFN-γ + IFN-γR, IL-4 + IL-4R or with the B cells separated from Vδ2 T cells using transwell inserts (contact; n=3). *p<0.05, **p<0.01 using a paired t test, compared to BC alone (left panels) or compared to B cell control (right panels) except where indicated by horizontal lines.

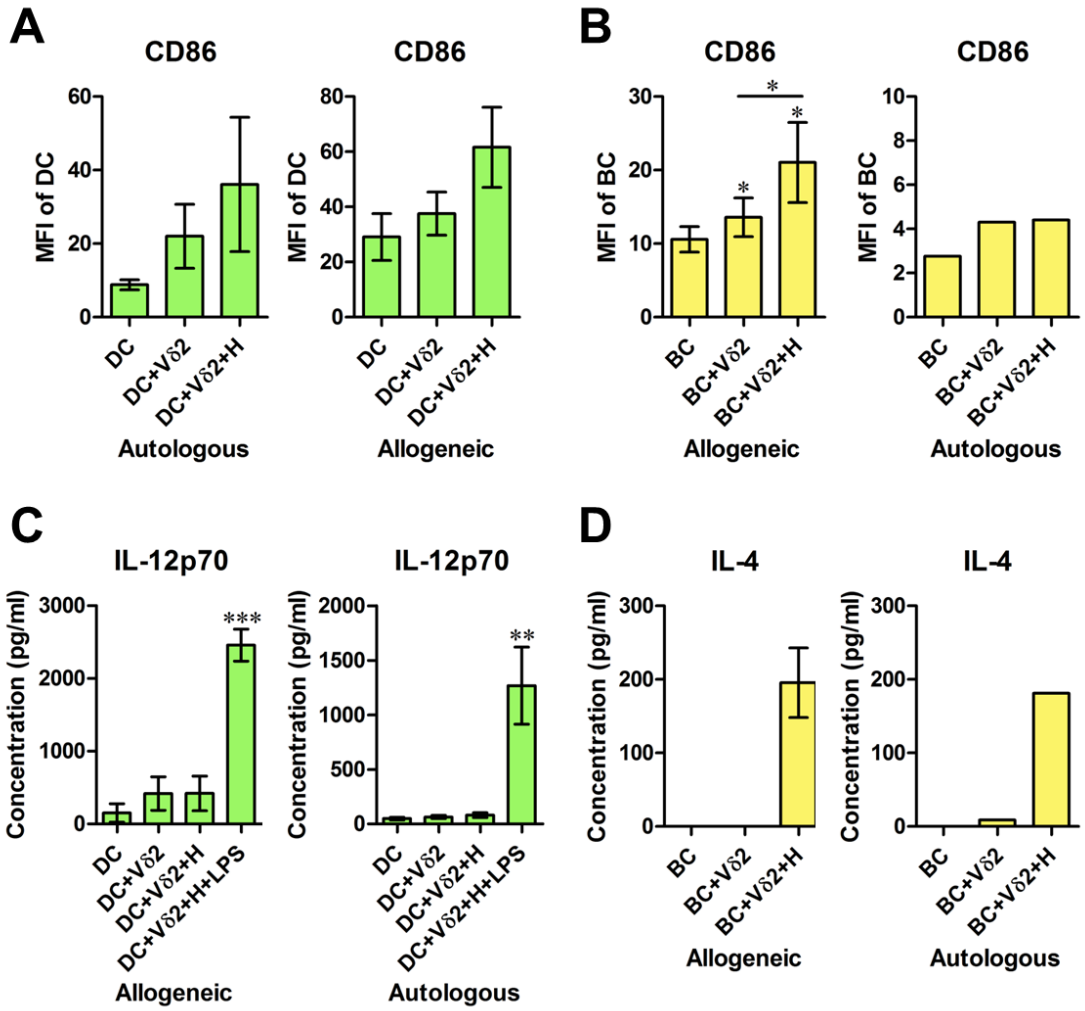

Figure 4.8. Allogeneic and autologous Vδ2 T cells equally activate DC and B cells. DC or B cells (BC) were co-cultured alone or with autologous or allogeneic HMB-PP-expanded human Vδ2 T cells in the absence or presence of HMB-PP (denoted H) or LPS. After 24 h (for DC) or 72 h (for BC), the cells were stained with mAb for CD11c (DC) or CD19 (BC) and CD86 and analysed by flow cytometry. The supernatants were harvested and examined for IL-4 or IL-12p70 secretion by ELISA. **A-B**, average (±SEM) mean fluorescence intensities of staining for CD86 expression by DC (**A**; n=3-8) or B cells (**B**; n=1-12) for allogeneic (left panels) and autologous (right panels) co-cultures. **C**, average (±SEM) concentration of IL-12p70 (n=10-11) released by cultures of DC or DC cultured with allogeneic (left panels) or autologous (right panels) Vδ2 T cells in the absence or presence of HMB-PP or LPS. **D**, average (±SEM) concentration of IL-4 (n=1-2) released from BC cultured alone or with allogeneic (left panels) or autologous (right panels) Vδ2 T cells in the absence or presence of HMB-PP. *p<0.05, **p<0.01, ***p<0.001 using a paired *t* test compared to DC or BC alone except where indicated by horizontal lines.

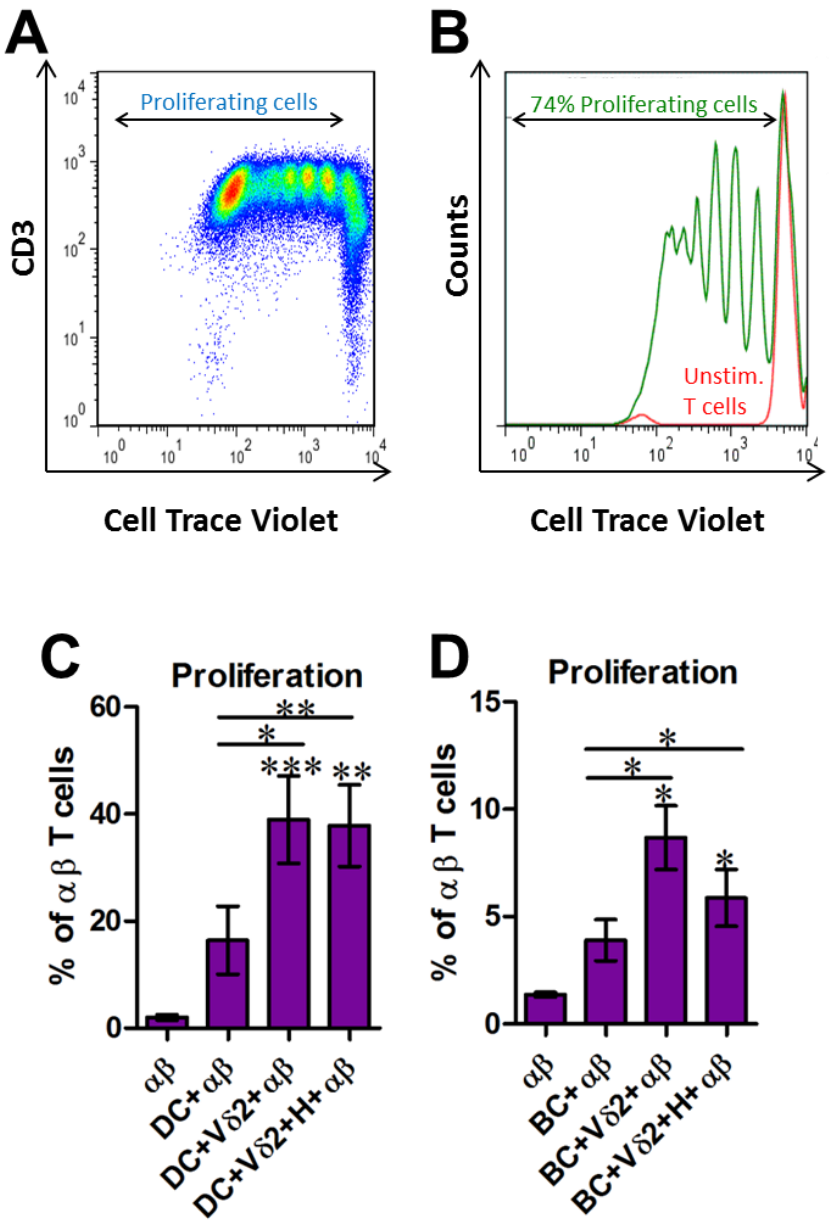

Figure 4.9. Proliferation of resting allogeneic T cells following culture with Vδ2-matured DC and B cells. DC or B cells were co-cultured with HMB-PP-expanded Vδ2 T cells in the absence or presence of HMB-PP (denoted H). After 24 h CellTrace-labeled allogeneic resting αβ T cells were added at a ratio of 10:1 and cultured for 6 days. **A**, representative dot plot showing proliferating T cells. **B**, histogram showing proliferating T cells (green peaks) versus unstimulated T cells (red peak) by flow cytometric analysis of CellTrace dilution. **C**, Average (±SEM) percentage of proliferating T cells when cultured alone, with immature DC or with Vδ2-matured DC in the absence or presence of HMB-PP (n=10) **D**, Average (±SEM) percentage of proliferating T cells when cultured alone, with resting B cells (BC) or with Vδ2-matured B cells (n=4) *p<0.05, **p<0.01, ***p<0.001, paired t test versus T cells except where indicated by horizontal lines.

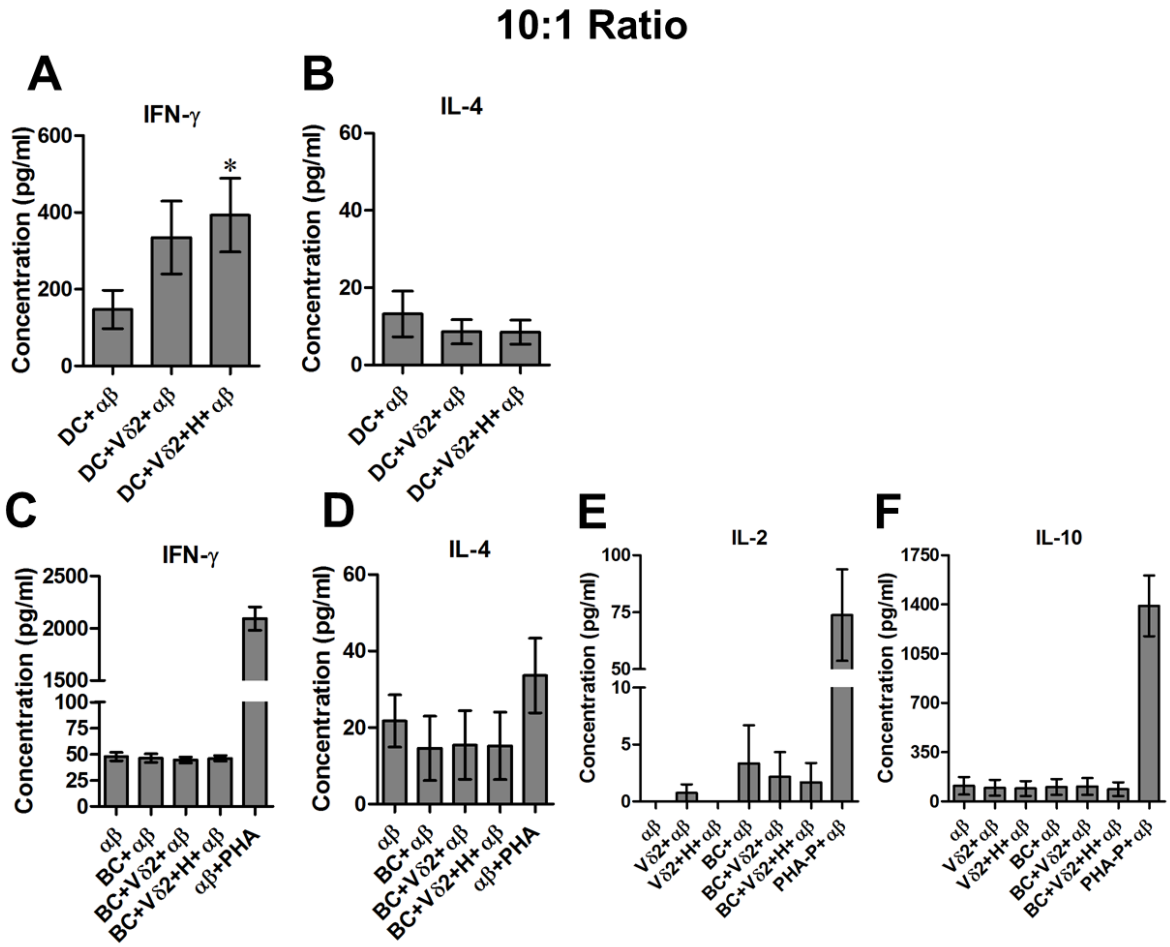

Figure 4.10 Cytokine production by alloreactive T cells cultured with Vδ2 T cell-matured DC or B cells. HMB-PP-expanded human Vδ2 T cells were cultured alone or together with DC or B cells in the absence or presence of HMB-PP (denoted H). After 24 h, resting αβ T cells were added to the culture at a ratio of 10:1. A positive control of αβ T cells treated with PHA and irradiated PBMC was used. After a further 3 days, supernatants were harvested and analysed for IFN-γ, IL-4, IL-2 and IL-10 levels by ELISA. **A-B**, mean (±SEM) concentration of IFN-γ (**A**) and IL-4 (**B**) secreted by the cultures with DC (n=6-10). **C-F**, mean (±SEM) concentration of IFN-γ (**C**), IL-4 (**D**), IL-2 (**E**) and IL-10 (**F**) from the co-cultures (n=4).

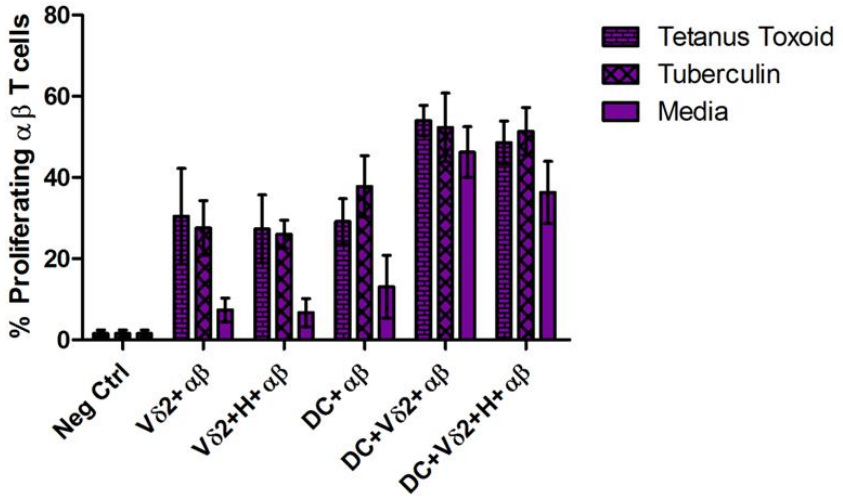

Figure 4.11 Proliferation of resting autologous T cells following culture with Vδ2-matured DC. DC and autologous HMB-PP-expanded Vδ2 T cells were cultured alone or together in the absence or presence of HMB-PP (denoted H). After 24 h, CellTrace-labeled autologous resting αβ T cells were added at a ratio of 10:1 and cultured for 6 days in the presence of tetanus toxoid, tuberculin or medium alone. Average (±SEM) percentage of proliferating T cells following culture with Vδ2-matured DC (n=4).

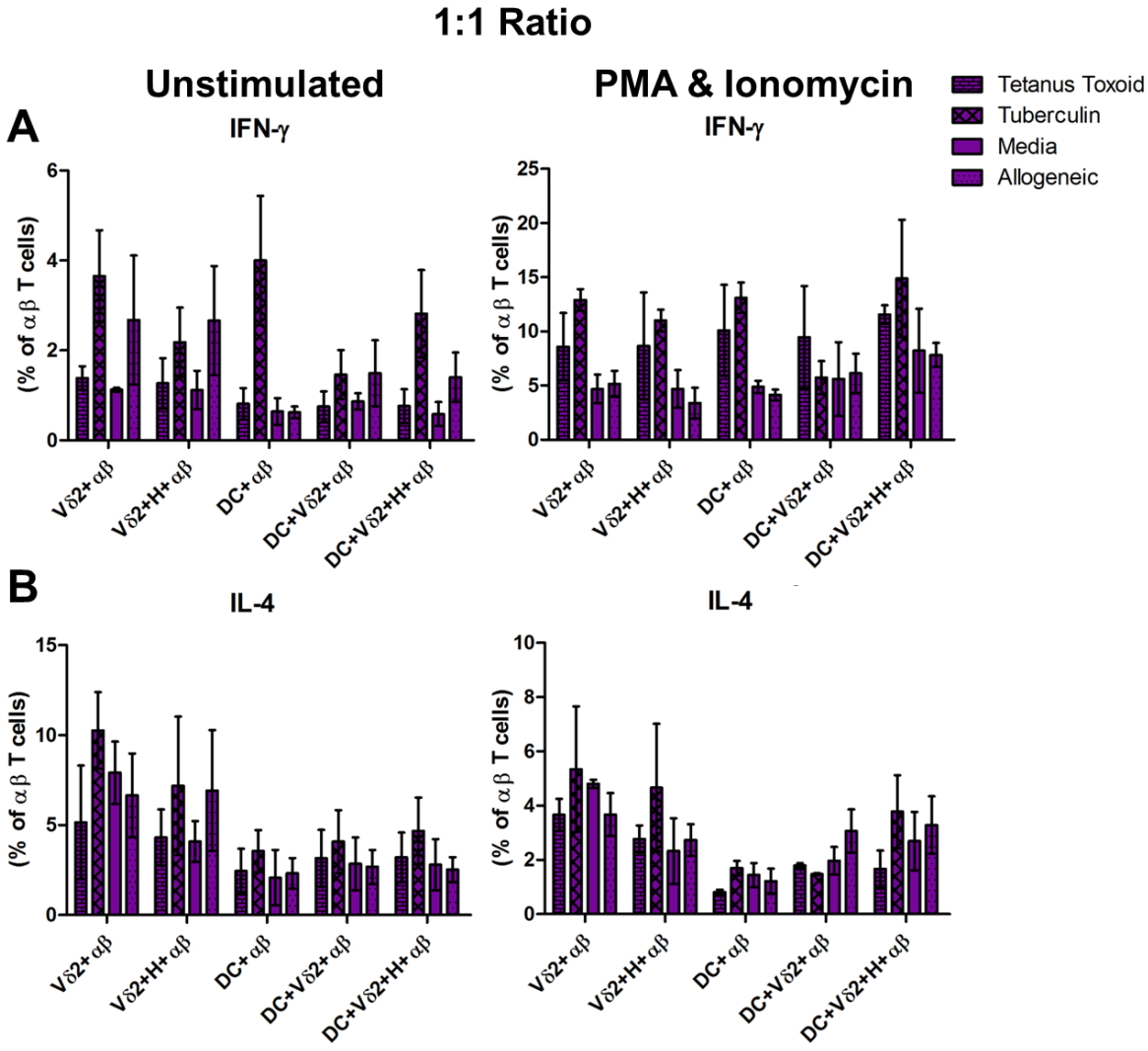

Figure 4.12. Cytokine production by resting αβ T cells cultured with autologous or allogeneic Vδ2-matured DC. DC and autologous or allogeneic HMB-PP-expanded Vδ2 T cells were cultured alone or together in the presence or absence of HMB-PP (denoted H). After 24 h, autologous or allogeneic resting T cells were added at a ratio of 1:1 to the Vδ2-DC culture and incubated for 3 days. The autologous cultures were carried out in the presence of tetanus toxoid, tuberculin or media. The cultures were then stimulated with PMA and ionomycin or left unstimulated for 4 h and examined for IFN-γ and IL-4 expression by flow cytometry. **A-B**, average (± SEM) percentage of αβ T cells expressing IFN-γ (**A**; n=3-5) and IL-4 (**B**; n=2-5) in the presence (right panels) or absence (left panels) of PMA and ionomycin stimulation following culture with Vδ2-matured DC.

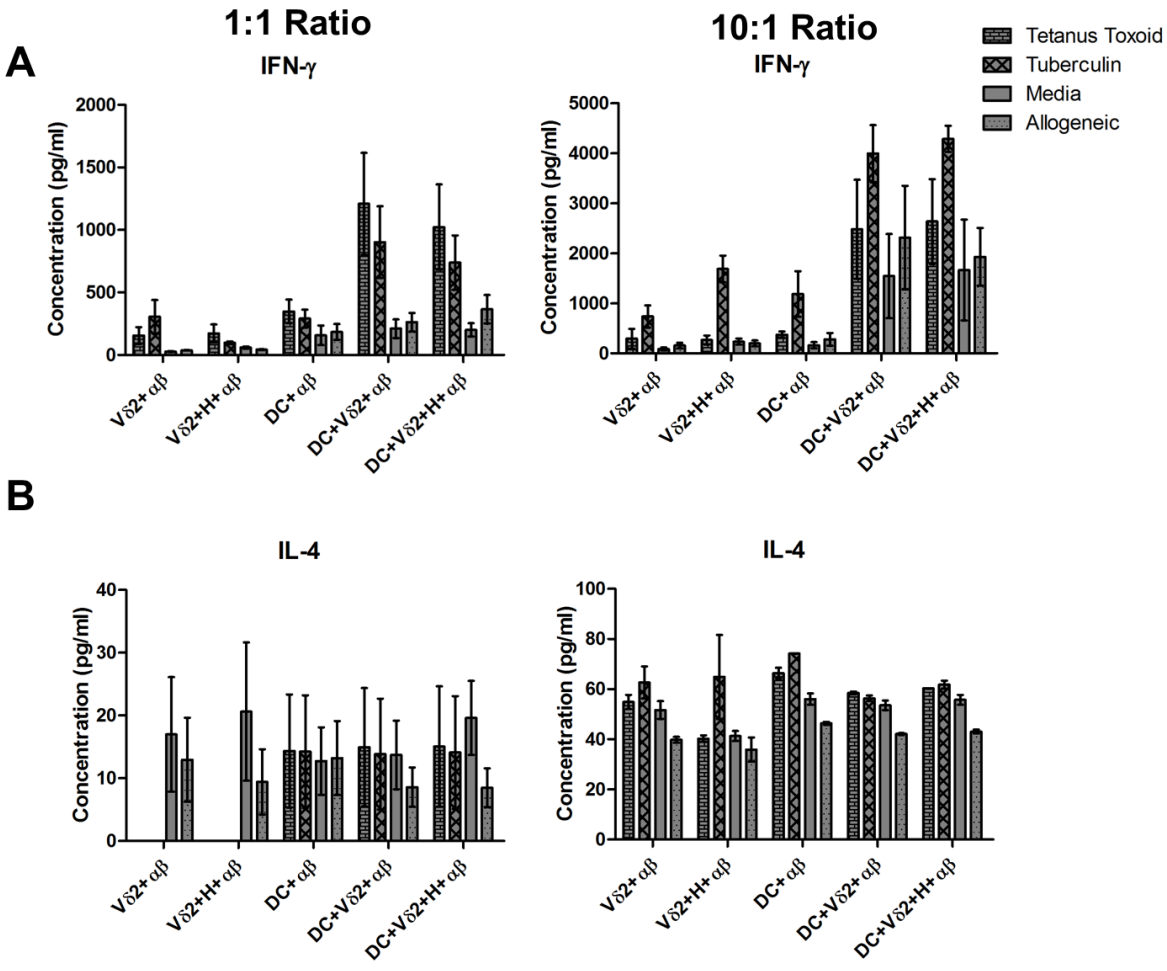

Figure 4.13. Cytokine secretion from cultures of Vδ2-matured DC with resting autologous or allogeneic T cells. DC and autologous or allogeneic HMB-PP-expanded Vδ2 T cells were cultured alone or together in the presence or absence of HMB-PP (denoted H). After 24 h autologous or allogeneic resting T cells were added at 1:1 or 10:1 ratios and cultured for 3 days. The autologous cultures were carried out in the presence of tetanus toxoid, tuberculin or media. The supernatants were harvested and examined for levels of IFN-γ and IL-4 by ELISA. **A-B**, average (±SEM) concentration of IFN-γ (**A**; n=3-5) and IL-4 (**B**; n=2-5) in supernatants of resting T cells cultured with Vδ2 T cells, DC or Vδ2-matured DC in a 1:1 (left panels) or 10:1 (right panels) ratio.

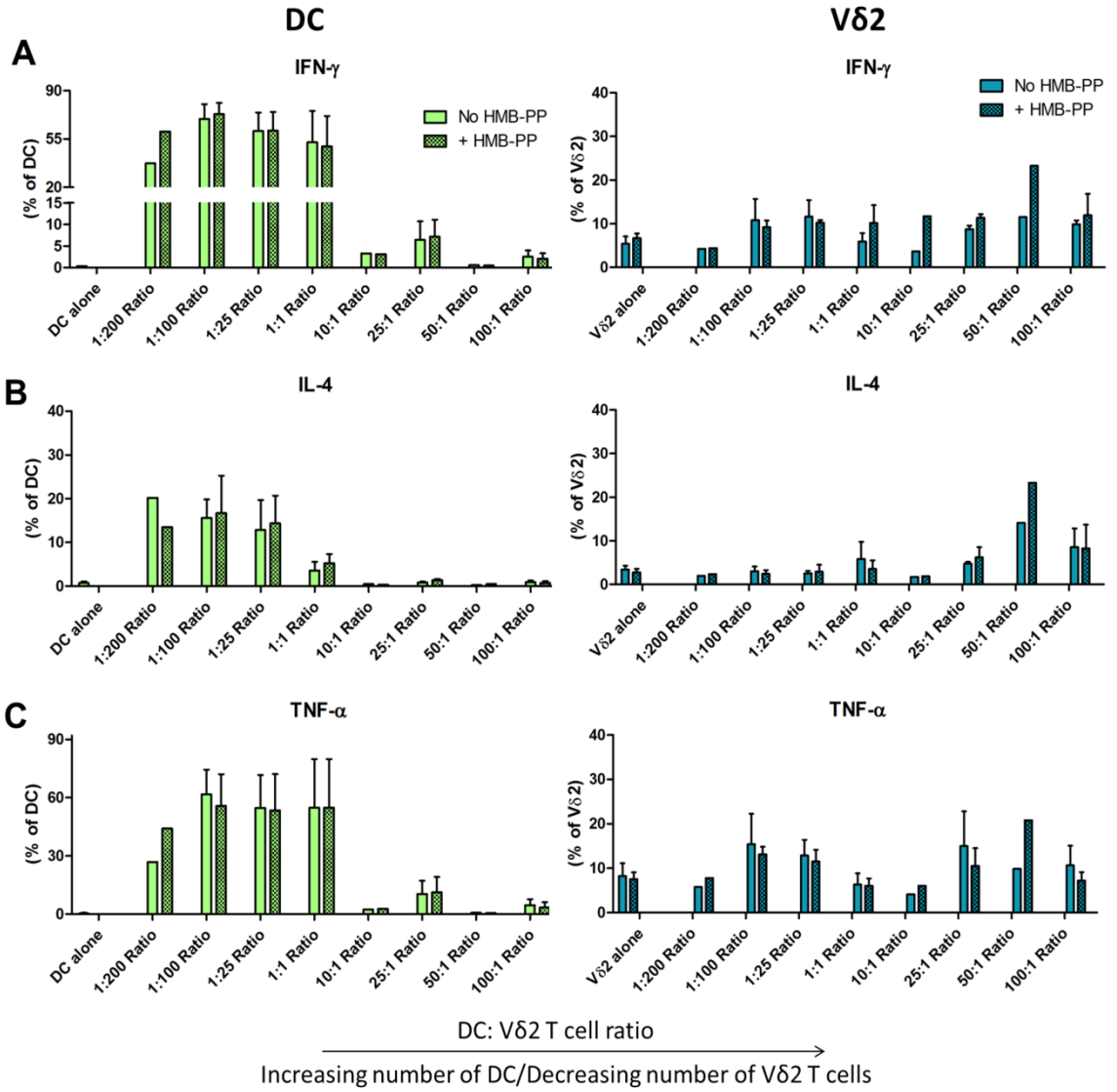

Figure 4.14 Cytokine production from cultures of DC and Vδ2 T cells cultured at various ratios. DC were cultured with HMB-PP-expanded Vδ2 T cells in the presence or absence of HMB-PP for 24 h at various ratios of DC to Vδ2 T cells. The cells were then cultured with monensin for 16 h and then examined for IFN-γ, IL-4 and TNF-α expression by DC and Vδ2 T cells by flow cytometry. **A-C**, average (±SEM) percentage of DC (left panels) or Vδ2 T cells (right panels) expressing IFN-γ (**A**; n=3), IL-4 (**B**; n=3) and TNF-α (**C**; n=3).

4.4.10 The addition of DC to Vδ2-B cell cultures increases co-stimulatory marker and cytokine expression by Vδ2 and B cells

To assess the impact of culturing all three cell types together, DC, B cells and Vδ2 T cells were cultured simultaneously or separately and examined for the effect of the simultaneous culture on costimulatory marker expression and cytokine production by each of the cell types. It was found that the addition of B cells did not augment CD86 (Fig. 4.15A), CD40 (Fig. 4.15B) or HLA-DR (Fig. 4.15C) expression by DC (left panels). In contrast, the addition of DC augmented CD86 (Fig. 4.15A) and CD40 (Fig. 4.15B) expression by B cells (centre panels), although the changes were not significant. Furthermore, the presence of DC augmented CD86 (Fig. 4.15A), CD40 (Fig. 4.15B) and HLA-DR (Fig. 4.15C) expression by Vδ2 T cells (right panels).

When examining the cytokine profiles of these cultures, it was found that the addition of B cells slightly augmented IFN-γ production by DC (Fig. 4.16A) and TNF-α production by Vδ2-matured DC (Fig. 4.16C) but not IL-4 (Fig. 4.16B) expression by DC (left panels). In contrast, the addition of DC augmented IFN-γ (Fig. 4.16A) but not IL-4 (Fig. 4.16B) or TNF-α (Fig. 4.16C) production by B cells (centre panels) and Vδ2 T cells (right panels). The cell culture supernatants were also assayed for levels of IFN-γ, IL-4 and TNF-α by ELISA. The results revealed that the addition of DC but not B cells augmented cytokine secretion by Vδ2 T cells stimulated with HMB-PP (Fig. 4.16D) compared to the levels produced by each cell type when cultured alone.

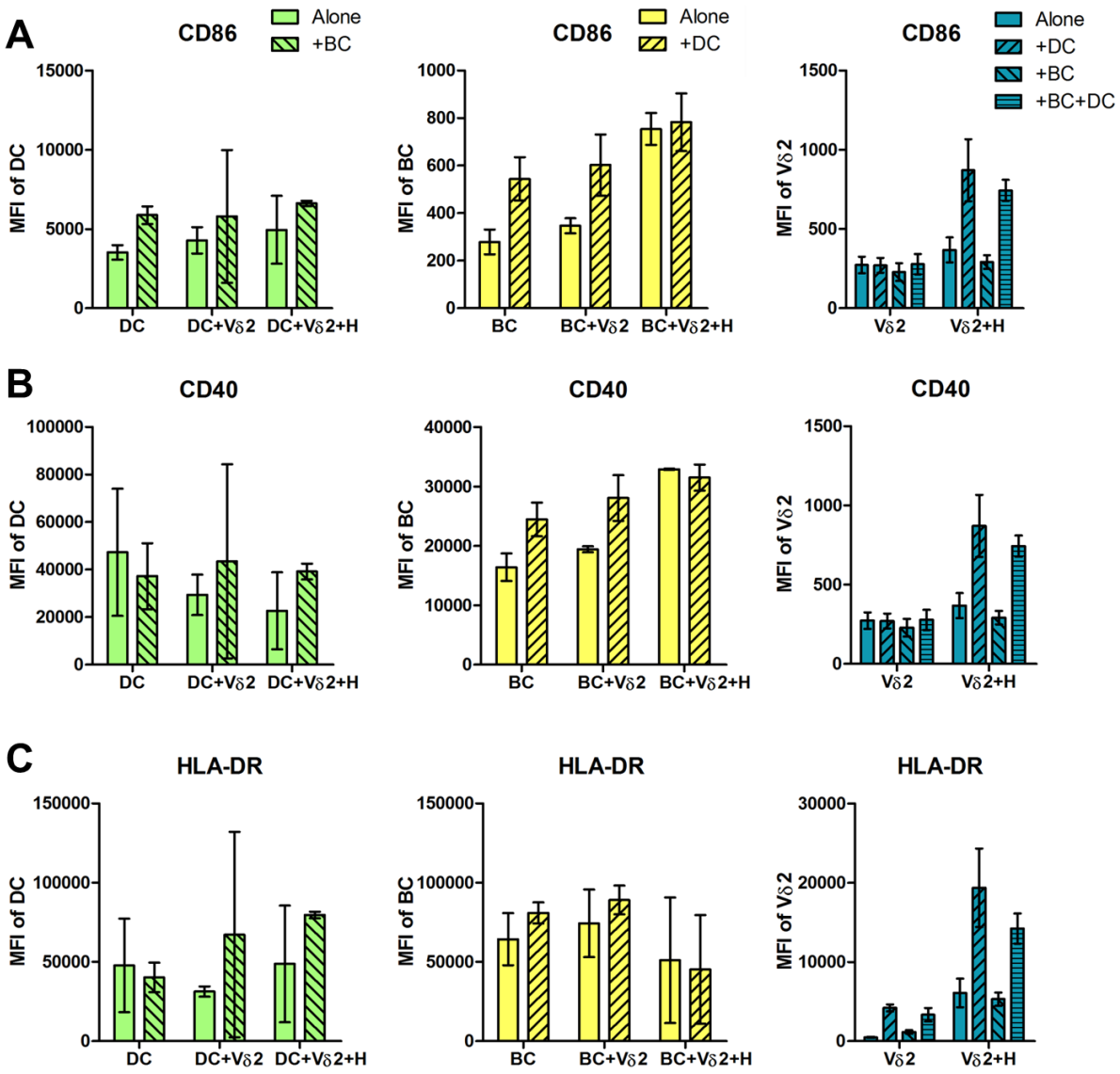

Figure 4.15. Expression of APC markers by DC, B cells and Vδ2 T cells in a triple culture. DC, B cells (BC) and HMB-PP-expanded Vδ2 T cells were cultured separately or together in the presence or absence of HMB-PP (denoted H) for 24 h. The cells were then stained with mAb specific for CD11c (DC), CD19 (BC) and CD3 and Vδ2 (Vδ2 T cells) and examined for expression of CD86, CD40 and HLA-DR. **A-C**, average (±SEM) MFI of expression of each cell type of CD86 (**A**; n=2), CD40 (**B**; n=2) and HLA-DR (**C**; n=2) by DC (left panels), BC (centre panels) or Vδ2 T cells (right panels).

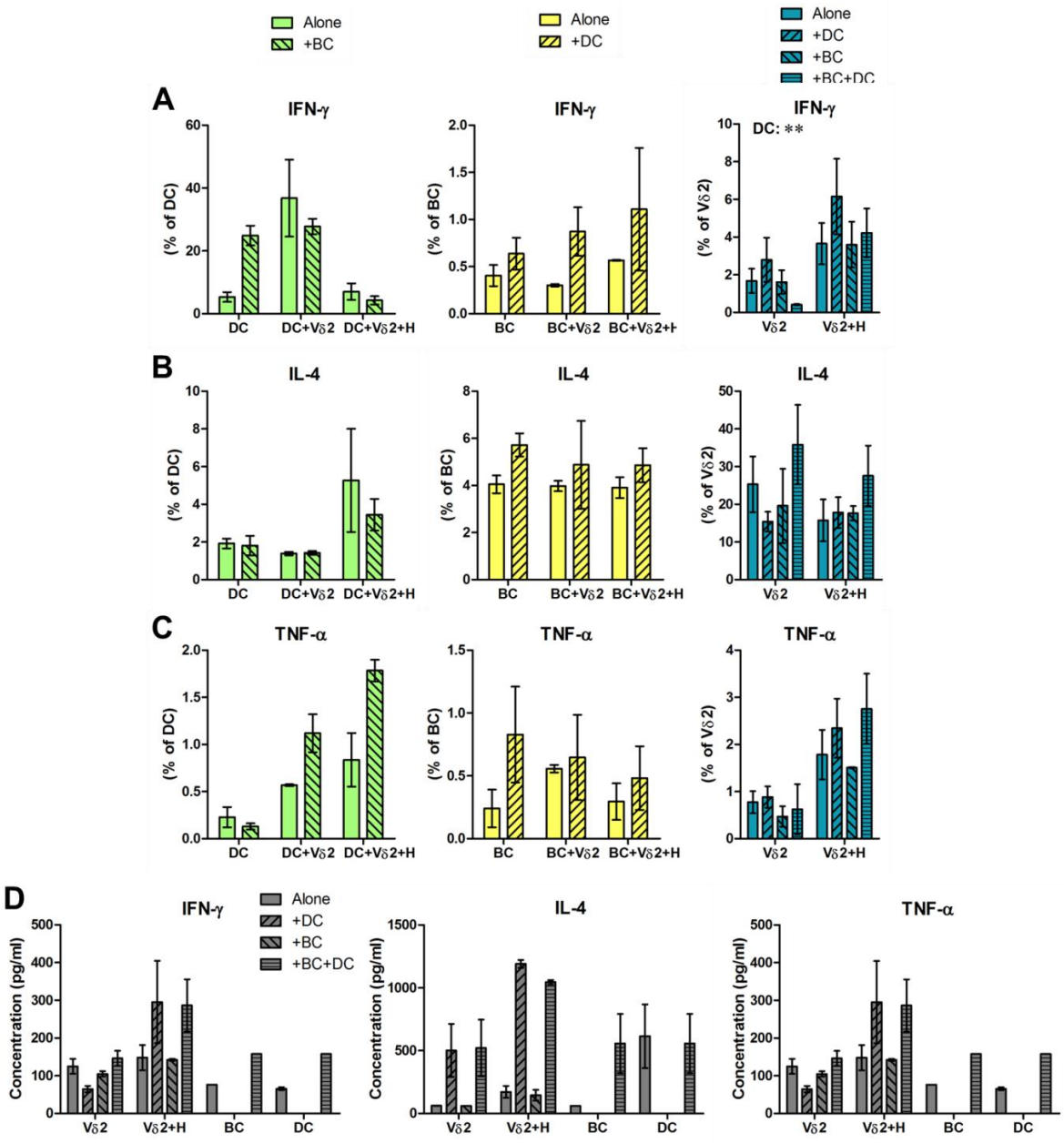

Figure 4.16. Cytokine production by Vδ2 T cells, DC and B cells in triple culture. DC, B cells (BC) and HMB-PP-expanded Vδ2 T cells were cultured separately or together in the presence or absence of HMB-PP (denoted H) for 24 h. The cells were treated with monensin and cultured overnight. The cell cultures were then stained for surface expression of CD11c (DC), CD19 (BC) and CD3 and Vδ2 (Vδ2 T cells). The cells were then fixed, permeabilised and examined for intracellular expression of IFN-γ, IL-4 and TNF-α by flow cytometry. For ELISA, supernatants were harvested immediately after 24 h of culture of DC, B cells and Vδ2 T cells prior to monensin addition and examined for levels of IFN-γ, IL-4 and TNF-α by ELISA. **A-C**, average (±SEM) percentage of DC (left panels), BC (centre panels) and Vδ2 T cells (right panels) expressing IFN-γ (**A**; n=2-10), IL-4 (**B**; n=2) and TNF-α (**C**; n=2). **D**, average (±SEM) levels of IFN-γ (left panel), IL-4 (centre panel) and TNF-α (right panel) from the cultures (n=2). ***p*<0.01 using a two-way ANOVA.

4.4.11 LPS induces co-stimulatory marker expression by DC and B cells

The studies described above were carried out using preparations of immature monocyte-derived DC or resting B cells. We next assessed the effect of LPS on interactions between Vδ2 T cells and DC or B cells, thus simulating inflammatory conditions. DC or B cells were cultured for 24 h with resting or HMB-PP-activated Vδ2 T cells and LPS and examined for expression of co-stimulatory markers. LPS was found to significantly increase expression of CD86 (Fig. 4.17A), CD40 (Fig. 4.17C) and HLA-DR (Fig. 4.17D) but not CD80 (Fig. 4.17B) by DC but these were not higher than levels induced by Vδ2 T cells. In contrast, LPS did not increase CD86 (Fig. 4.17E) or CD80 (Fig. 4.17F) but increased CD40 (Fig. 4.17G) and HLA-DR (Fig. 4.17H) expression by B cells cultured with HMB-PP-activated Vδ2 T cells, but not with resting Vδ2 T cells.

4.4.12 LPS induces cytokine secretion by Vδ2-DC co-cultures and synergises with Vδ2 T cells to induce IL-12 production

We also wanted to investigate the impact of LPS on cytokine expression and secretion by DC and B cells that had been cultured in the absence or presence of Vδ2 T cells. We found that LPS on its own induced significant IL-10 (Fig. 4.18B), IL-6 (Fig. 4.18C) and TNF-α (Fig. 4.18D) secretion but not IL-12 (Fig. 4.18A) secretion by DC. Vδ2 T cells, resting or stimulated with HMB-PP, did not induce significant production of these cytokines, but Vδ2 T cells together with LPS induced the secretion of significantly higher amounts of IL-12 compared to DC exposed to LPS or Vδ2 T cells alone. In contrast, LPS induced IL-12 release from cultures of B cells, and this was not augmented by the presence of Vδ2 T cells (Fig. 4.18E). Neither LPS, Vδ2 T cells, nor LPS and Vδ2 T cells together induced significant IL-10 production by B cells (Fig. 4.18F). Vδ2 T cells were found to induce IL-6 (Fig. 4.18G) and TNF-α (Fig. 4.18H) production by B cells and this was not augmented by LPS, which even inhibited TNF-α production (Fig. 4.18H) secretion by HMB-PP-activated Vδ2 T cells, but did not augment IL-10 (Fig. 4.18F) or IL-6 (Fig. 4.18G), and even inhibited the TNF-α (Fig. 4.18H) secretion upon culture with HMB-PP-activated Vδ2 T cells.

4.4.13 LPS induces an increase in IgG and IgA production by B cells

Lastly, we examined the effect of LPS on antibody production in resting B cells and Vδ2-matured B cells and found that while it did not augment IgM levels (Fig. 4.19C), it augmented IgG (Fig. 4.19A) and IgA (Fig. 4.19B) production by B cells cultured with Vδ2 T cells in the absence of HMB-PP.

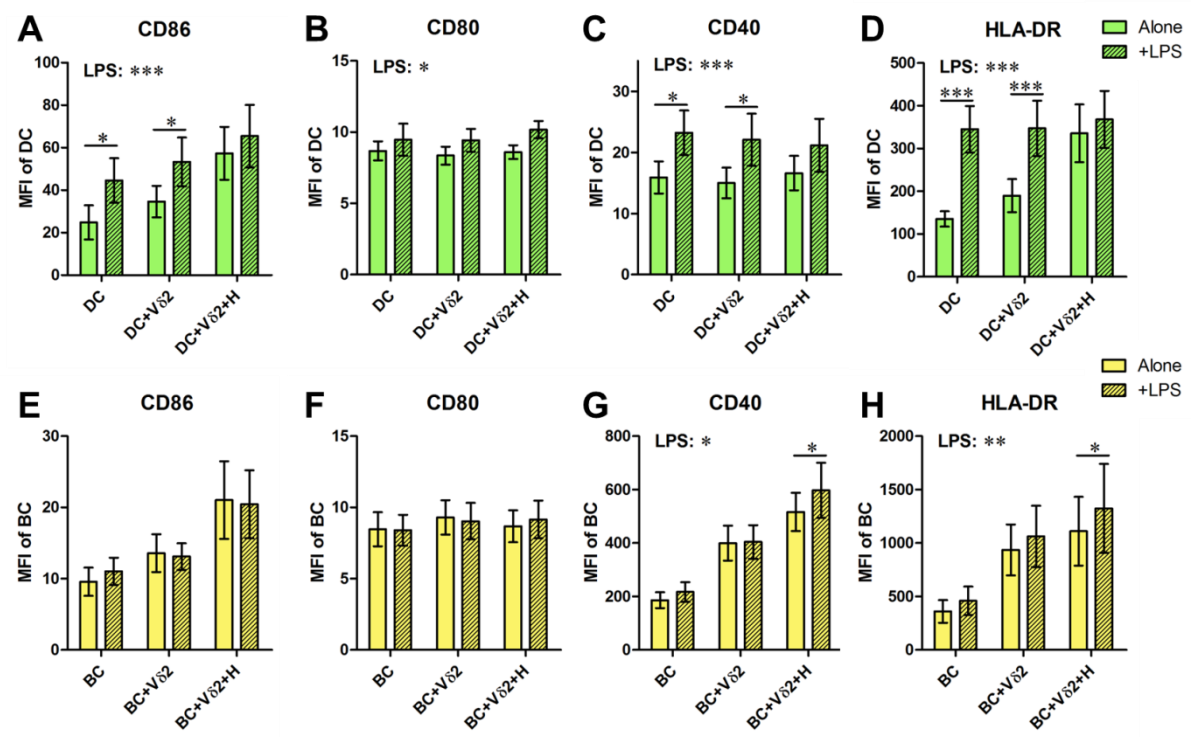

Figure 4.17 Expression of co-stimulatory markers by DC and B cells following LPS stimulation. Monocyte-derived DC or enriched peripheral blood B cells were cultured for 24 h with HMB-PP-expanded Vδ2 T cells and LPS in the absence or presence of HMB-PP (denoted H). Cells were then stained using mAb specific for CD11c or CD19 and CD86, CD80, CD40 and HLA-DR and analysed by flow cytometry. **A-D**, average (±SEM) mean fluorescence intensities (MFI) of staining for CD86 (**A**; n=9), CD80 (**B**; n=7), CD40 (**C**; n=6), HLA-DR (**D**; n=7) by DC. **E-H**, average (±SEM) mean fluorescence intensities (MFI) of staining for CD86 (**E**; n=12), CD80 (**F**; n=14), CD40 (**G**; n=7), HLA-DR (**H**; n=6) by B cells (BC). **p*<0.05, ***p*<0.01, ****p*<0.001 using a two-way ANOVA and Bonferroni post hoc test.

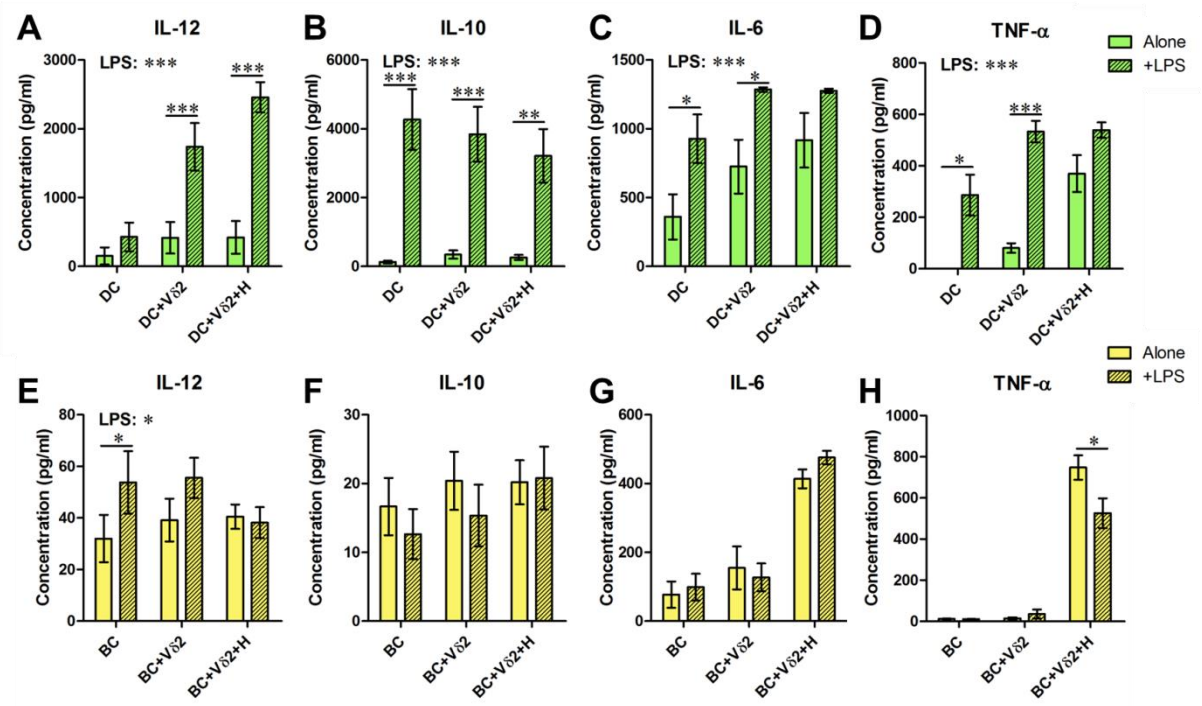

Figure 4.18 Cytokine secretion from cultures of Vδ2 T cells with DC or B cells following LPS stimulation. Monocyte-derived DC or enriched peripheral blood B cells were cultured for 24 h with HMB-PP-expanded Vδ2 T cells and LPS in the absence or presence of HMB-PP (denoted H). The supernatants were then harvested and analysed for levels of IL-12, IL-10, IL-6 and TNF-α by ELISA. **A-D**, average (±SEM) concentration of IL-12 (**A**; n=11), IL-10 (**B**; n=9), IL-6 (**C**; n=4) and TNF-α (**D**; n=4) from DC cultures. **E-H**, average (±SEM) concentration of IL-12 (**E**; n=6), IL-10 (**F**; n=6), IL-6 (**G**; n=5) and TNF-α (**H**; n=3) from B cell (BC) cultures. *p<0.05, **p<0.01, ***p<0.001 using a two-way ANOVA and Bonferroni post hoc test.

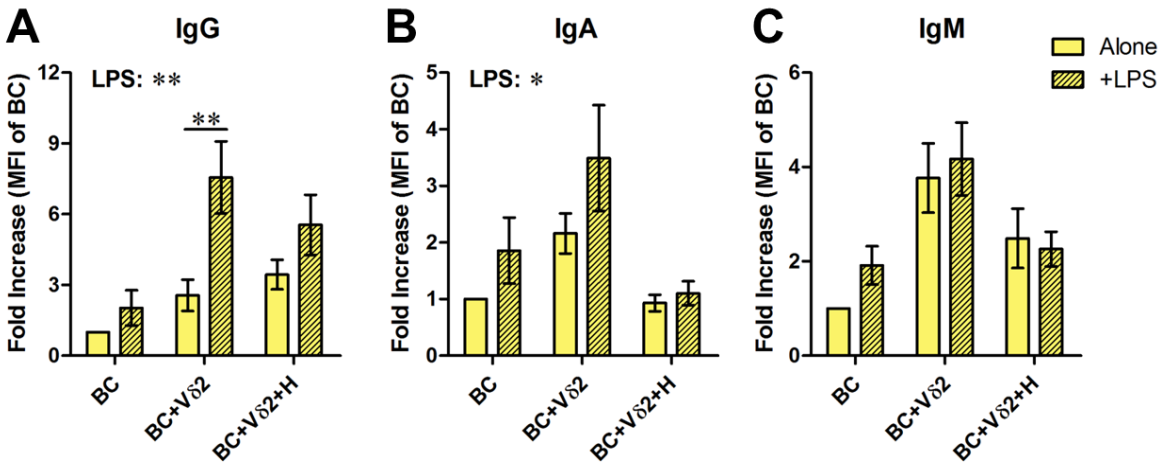

Figure 4.19 Antibody production by LPS-stimulated B cells cultured with Vδ2 T cells. Enriched peripheral blood B cells (BC) were stimulated with LPS and co-cultured with HMB-PP-expanded human Vδ2 T cells in the absence or presence of HMB-PP (denoted H). After 7 days the supernatants were harvested and analysed for IgA, IgM and total IgG levels by cytometric bead array and flow cytometry. **A-C**, average (±SEM) fold increase of IgG (**A**; n=6), IgA (**B**; n=7) and IgM (**C**; n=7) concentration. *p<0.05, **p<0.01 using a two-way ANOVA and Bonferroni post hoc test.

4.5 Discussion

Vγ9Vδ2 T cells exhibit a myriad of effector functions in innate and adaptive immunity. They can kill infected, tumour and stressed target cells, promote inflammation and wound healing, promote the survival, differentiation and activation of monocytes, neutrophils and dendritic cells, provide B cell help for antibody production and prime $CD4^+$ and $CD8^+$ T cells (Kabelitz and He, 2012, Vantourout and Hayday, 2013, Morita, et al., 2007, Chien, et al., 2014). Vγ9Vδ2 T cells can also link innate and adaptive immune responses by promoting differentiation of different types of cells into APC that are capable of initiating antigen-specific T cell responses and long-term immunological memory (Dunne, et al., 2010, Conti, et al., 2005, Devilder, et al., 2006, Ismaili, et al., 2002, Davey, et al., 2014, Eberl, et al., 2009, Martino, et al., 2005, Shrestha, et al., 2005, Kalyan and Chow, 2009, Marcu-Malina, et al., 2014). These findings implicate Vγ9Vδ2 T cells as candidate targets for development of novel therapies and vaccines.

Previous studies have demonstrated that Vγ9Vδ2 T cells can induce maturation of B cells into antibody-secreting plasma cells (Brandes, et al., 2003, Caccamo, et al., 2006, Bansal, et al., 2012, Caccamo, et al., 2012), suggesting that they can promote humoral immune responses *in vivo*. Thus, we sought to further investigate the role of Vδ2 T cells in providing B cell helper activity and the mechanisms involved in this. Furthermore, we compared these findings to those found with culture of Vδ2 T cells with immature DC under the same conditions.

We found that Vδ2 T cells promoted DC maturation, as evidenced by an increase in expression of co-stimulatory markers by DC within 24 h of culture with Vδ2 T cells. In addition, we found that Vδ2-DC co-cultures secreted IFN-γ, TNF-α and IL-6 but not IL-4 and IL-10 after 24 h. While Vδ2 T cells were not potent inducers of IL-12 production by DC, they exhibited a strong synergistic effect with TLR ligand LPS in inducing IL-12 release, which will be discussed in further detail later in this chapter. We also examined the phenotypic changes to B cells that occur in response to co-culturing them with Vδ2 T cells and found that, like for DC, B cells upregulated HLA-DR, CD40 and CD86, suggesting that Vδ2 T cells can drive maturation of B cells into APC. However, analysis of cytokine production revealed that Vδ2-B cell co-cultures could

produce TNF-α, IL-6 and IL-4 but not IFN-γ or IL-12. Thus Vδ2-matured DC and B cells have distinct cytokine profiles, with B cells lacking the T_H1-promoting cytokine bias seen for DC. We also showed that HMB-PP-stimulated Vδ2 T cells can stimulate the production of IgG, IgM and IgA but not IgE by B cells *in vitro* and that further addition of HMB-PP at the time of adding to B cells prevented IgM and IgA production. The Vδ2-induced production of IL-4 by B cells supports their role in antibody production by B cells, as IL-4 is one of the factors required for B cell help (Caccamo, et al., 2006).

Since the mechanisms underlying DC and B cell activation by Vδ2 T cells are poorly understood, we aimed to identify the molecules required to mediate these functional changes. We found that cell contact was found to be important for CD86 expression by DC, while CD86 and T_H1 cytokines were important for HLA-DR and CD40 expression by DC. In contrast, blocking CD40L and separating the B cells from Vδ2 T cells resulted in less upregulation of HLA-DR by B cells, but did not significantly affect CD86 or CD40 expression by Vδ2 stimulated B cells. Thus, while co-stimulatory molecules, pro-inflammatory cytokines and physical contact with Vδ2 T cells were important for DC maturation, co-stimulatory markers and contact played only a minor role in B cell maturation and were not important for antibody production. Our results are in contrast to the study by Caccamo (2006) which showed that blocking IL-4 and CD40L abrogated antibody production by B cells cultured with Vδ2 T cells. They also found this to be abrogated when blocking IL-10 and ICOS. However, they did not investigate the role of these factors on co-stimulatory marker expression and cytokine production by B cells. Thus, the mechanisms responsible for B cell activation need to be further elucidated.

As all these cultures were carried out using allogeneic donors, we compared the outcomes of allogeneic and autologous stimulations. We found that, generally, the same levels of co-stimulatory markers and cytokine production were observed with allogeneic and autologous cultures, as demonstrated by CD86 expression and IL-12 release by DC and IL-4 release by B cells. Thus, this suggests that pure populations of allogeneic Vδ2 T cells may be substituted for autologous Vδ2 T cells as adjuvants for DC- or B cell-based therapies.

Analysis of the capacity of DC matured using Vδ2 T cells to induce proliferation of resting alloreactive T cells found that they induce proliferation and IFN-γ production by these T cells *in vitro*, suggesting that Vδ2 T cell-matured DC also prime antigen-specific T_H1 responses. In comparison, Vδ2 T cell-matured B cells were able to stimulate alloreactive T cells to proliferate but not to produce IFN-γ, IL-2, IL-4 or IL-10. These findings suggest that Vδ2 T cells can drive the differentiation of DC into T_H1-promoting APC and B cells into APC that can stimulate different T cell responses.

Investigation into whether Vδ2 T cell-matured DC could present specific antigen to T cells, revealed that while the presence of tetanus toxoid or tuberculin antigens induced proliferation of resting autologous αβ T cells, this was not more potent than in the absence of these antigens. Furthermore, the presence of these antigens did not induce an increase in cytokine secretion by autologous αβ T cells stimulated by Vδ2-matured DC. The lack of response to the antigens may be due to a low frequency of T cells specific for these antigens.

Another important question was whether DC or B cells reciprocally activate Vδ2 T cells. We found that DC, but not B cells, induced co-stimulatory marker expression and IFN-γ secretion by HMB-PP-activated Vδ2 T cells, while the addition of B cells did not significantly impact on co-stimulatory marker expression or cytokine production by Vδ2 T cells. Furthermore, the simultaneous addition of DC and B cells had no synergistic effect either.

As most of these cultures were carried out in equal ratios, we examined the outcomes of altering the ratios of DC to Vδ2 T cells on cytokine production by DC and Vδ2 T cells. The results showed that an abundance of Vδ2 T cells boosted IFN-γ, IL-4 and TNF-α production by DC, thus confirming that Vδ2 T cells can induce cytokines by DC, whereas increasing the numbers of DC did not augment the ability of Vδ2 T cells to produce these cytokines.

Finally, we investigated the role of LPS in Vδ2 T cell-induced activation DC and B cells, which would reflect a state of inflammation. We found that, in general, LPS activated APC functions by DC and B cells. It upregulated co-stimulatory marker expression by DC and B cells and induced IL-12, IL-10, IL-6 and TNF-α secretion by DC and IgG, IgA

and IL-12 by B cells. Interestingly, while DC and Vδ2 T cells, individually released only small amounts of IL-12, Vδ2 T cells synergised with LPS in inducing high levels of IL-12 secretion by DC. Dunne et al found that IFN-γ augmented IL-12 production by DC in response to LPS (Dunne, et al., 2010b).

Thus, the findings in this chapter (Petrasca and Doherty, 2014) confirm previous reports that Vγ9Vδ2 T cells can induce maturation, MHC and co-stimulatory receptor expression and T_H1 cytokine production by DC (Dunne, et al., 2010, Conti, et al., 2005, Devilder, et al., 2006, Ismaili, et al., 2002, Martino, et al., 2005, Shrestha, et al., 2005) and further show that these matured DC can stimulate proliferation and T_H1 cytokine production by alloreactive αβ T cells. In addition we demonstrated the role of Vδ2 T cells in inducing differentiation of B cells into antibody-secreting plasma cells capable of stimulating alloreactive αβ T cells to proliferate and secreting cytokines that further support antibody production (Brandes, et al., 2003, Caccamo, et al., 2006, Rodriguez-Pinto, 2005, Harris, et al., 2000, Bansal, et al., 2012, Caccamo, et al., 2012). We also demonstrated a role for LPS in DC and B cell activation by Vδ2 T cells and found that DC but not B cells activated Vδ2 T cells. Moreover, our results revealed important factors in DC activation, but did not fully clarify the mechanisms involved in B cell maturation. These results are summarised in figures 4.20 and 4.21.

Several studies have demonstrated a flexibility of DC maturation and their ability to differentiate into APC that selectively promote T_H1, T_H2 or tolerogenic T cell responses (Tisch, 2010, Lutz and Schuler, 2002, Reis e Sousa, 2006, Steinman, et al., 2003). The factors that determine the fate of DC differentiation include the nature of antigen and the presence of TLR ligands and cytokines and it appears that Vγ9Vδ2 T cells contribute by driving T_H1-promoting APC generation. Tolerogenic APC are characterised by the expression of MHC class II and co-stimulatory molecules in the absence of proinflammatory cytokine production and they can present antigen to T cells resulting in the induction of anergy or the expansion of regulatory T cells (Tisch, 2010, Lutz and Schuler, 2002, Reis e Sousa, 2006, Steinman, et al., 2003). Our data suggest that Vδ2 T cell-matured B cells may function as tolerogenic APC, since they display phenotypes of APC but they do not produce proinflammatory cytokines and they stimulate proliferation but not cytokine production by alloreactive T cells.

Furthermore, the ability of Vδ2-matured B cells to produce the anti-inflammatory cytokine IL-4 further supports a tolerogenic phenotype and we speculate that the IL-4 may function in promoting antibody responses. This is supported by the study by Caccamo (2006), which showed that a subset of Vδ2 T cells that produce IL-4 and IL-10 provide help to B cells for antibody production. B cells have previously been shown to present antigen, resulting in tolerogenic T cell responses (Eynon and Parker, 1992, Fuchs and Matzinger, 1992), but future work is required to determine if the T cells stimulated by Vδ2-matured B cells have tolerogenic or immunosuppressive activities.

Vδ2 T cells belong to a family of innate T cells that can differentially promote or regulate T cell and antibody responses through selective interactions with DC and B cells. Whereas Vδ2 T cells promote immunogenic T_H1 responses by inducing maturation of DC into APCs, they appear to promote T_H2 responses or T cell tolerance via their adjuvant activities on B cells, while at the same time promoting antibody production. While Vδ2 T cells are already under investigation as adjuvants for immunotherapies in clinical trials for cancer (Kalyan and Chow, 2009, Meraviglia, et al., 2010, Santolaria, et al., 2013), their distinct effects on DC and B cells must be considered in order to prevent unwanted immunosuppression or autoimmunity.

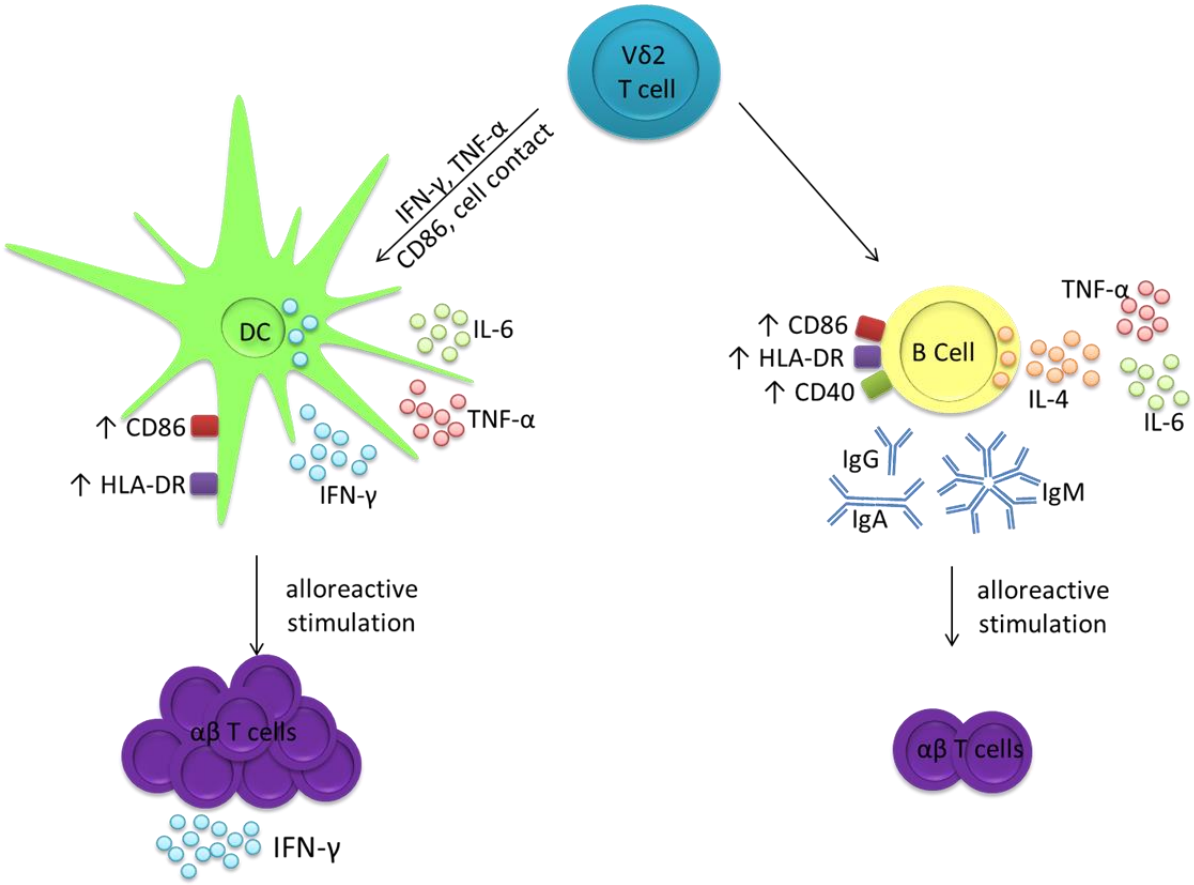

Figure 4.20. Vδ2 T cells have distinct adjuvant effects for DC and B cells. Co-cultures of Vδ2 T cells with either DC or B cells produce IL-6 and TNF-α, but not IL-12 nor IL-10. However, Vδ2 T cells induce IFN-γ production and co-stimulatory marker expression by DC after 24 h and these matured DC induce proliferation of alloreactive T cells which then secrete IFN-γ. In contrast, Vδ2 T cells induce IL-4 production and co-stimulatory molecule expression by B cells after 72 h and immunoglobulin secretion after 7 days. These matured B cells induce proliferation but not IFN-γ nor IL-4 production by alloreactive T cells.

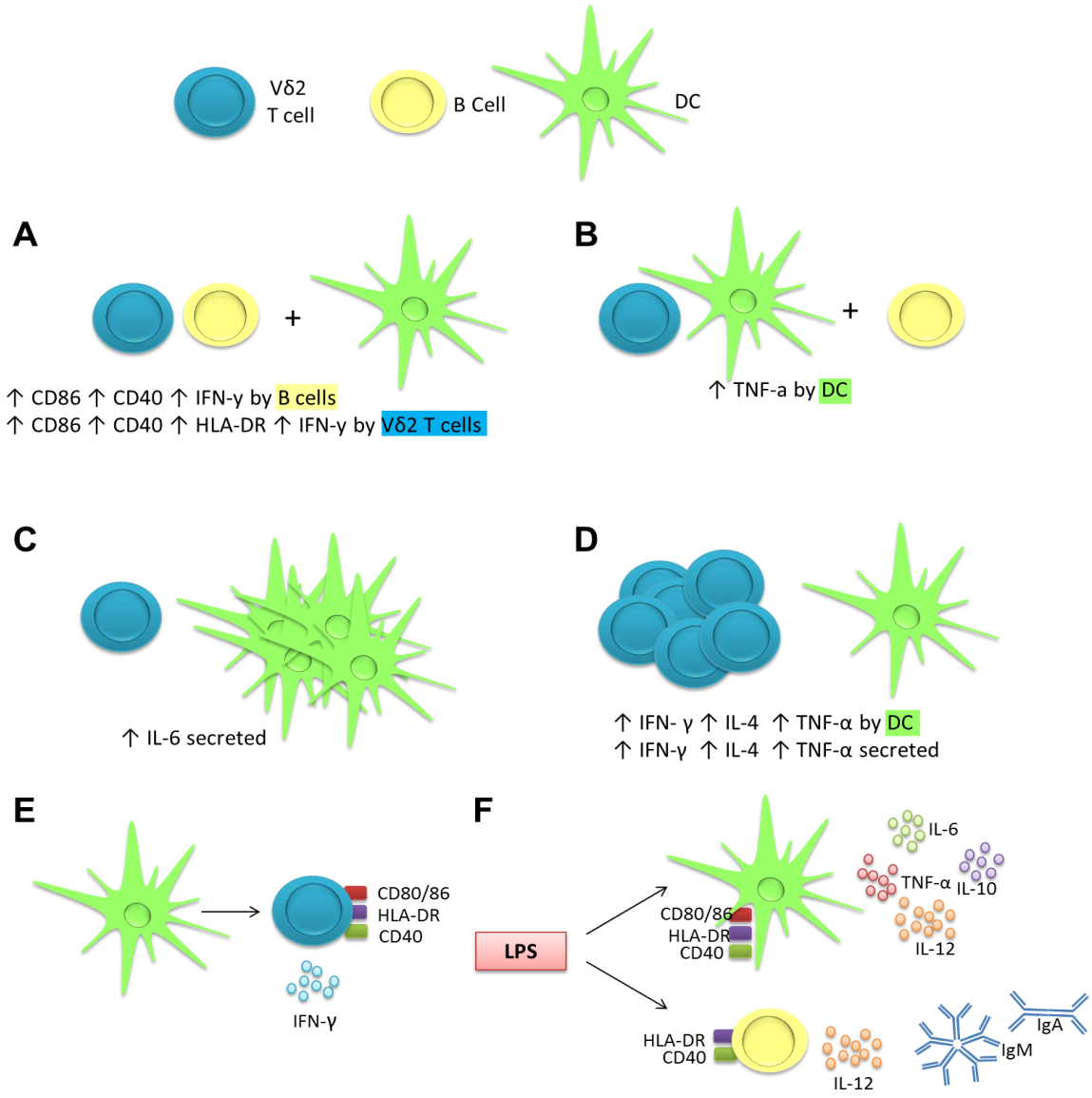

Figure 4.21. Interactions between DC, B cells, Vδ2 T cells and LPS. A-B, DC, B cells and HMB-PP-expanded Vδ2 T cells were cultured separately or together in a triple culture and examined for the resulting cytokine and APC phenotypes. **A**, the addition of DC to a culture of BC and Vδ2 T cells increased co-stimulatory marker expression and IFN-γ by both Vδ2 T cells and B cells. **B**, the addition of B cells to a culture of DC and Vδ2 T cells improved TNF-α production by DC. **C-D**, DC were cultured with HMB-PP-expanded Vδ2 T cells at various ratios and examined for the resulting cytokine profiles. **C**, an abundance of DC resulted in increased IL-6 secretion by Vδ2 T cells. **D**, an increase in Vδ2 T cells augmented cytokine secretion by the cultures and increased cytokine production by the DC. **E**, DC or B cells were cultured with HMB-PP-expanded Vδ2 T cells and examined the resulting APC phenotype and cytokine profiles of Vδ2 T cells. DC induced an increase in IFN-γ production by Vδ2 T cells, while B cells did not activate Vδ2 T cells. **F**, LPS was added to cultures of DC or B cells and the resulting cytokine profiles, APC phenotype and antibody production were assessed. LPS induced an increase in CD40, HLA-DR expression and IL-12, IgA and IgM secretion by B cells. In contrast, LPS induced an increase in CD80, CD86, CD40 and HLA-DR expression by IL-6, IL-10, IL-12 and TNF-α secretion by DC.

Chapter 5

Analysis of antigen recognition and helper function of human Vδ3 T cells

5.1 Introduction

Human Vδ3 T cells constitute a minor subset of peripheral blood lymphocytes and thus far, no Vδ3 T cell specific antigen has been identified. Vδ3 T cells are enriched in the liver (Kenna, et al., 2004) and gut (Dunne, et al., 2013) and they are reported to be expanded in HIV patients (Kabelitz, et al., 1997), B cell chronic lymphocytic leukaemia (Bartkowiak, et al., 2002) and in the peripheral blood of renal stem cell transplant recipients with cytomegalovirus activation (Halary, et al., 2005, Dechanet, et al., 1999, Knight, et al., 2010).

iNKT cells and γδ T cells, including Vδ1 and Vδ2 T cells, and more recently Vδ3 T cells, have been shown to induce DC maturation into APC. Vδ2 T cells and iNKT cells are also capable of inducing cytokine and antibody secretion and APC phenotype by B cells. This prompted us to investigate the potential of Vδ3 T cells for B helper activity and their role in CD1 recognition.

Several innate T cell subsets can recognise autoantigens presented by CD1 molecules (Spada, et al., 2000, de Jong, et al., 2010, Exley, et al., 2002, Uldrich, et al., 2011). There are two distinct groups in the CD1 family of lipid-presenting molecules. The first includes CD1a, CD1b and CD1c which are mainly found on professional APC and developing thymocytes (Dougan, et al., 2007, Pena-Cruz, et al., 2003). The second group includes CD1d, which is widely expressed in tissues (Blumberg, et al., 1991, Canchis, et al., 1993). CD1 molecules are also expressed in some cancers, but expression among patients is varied (Fais, et al., 2005, Lepore, et al., 2014, Metelitsa, et al., 2003). CD1d can be found in multiple myeloma, renal cell carcinoma and medulloblastoma patients (Liu, et al., 2013, Spanoudakis, et al., 2009) and can also be downregulated in viral infections and upregulated under inflammatory conditions (Guo, et al., 2015). CD1d is well characterised as a ligand for iNKT cells. However, since iNKT cells are found at such low frequencies in humans, γδ T cells, which are more abundant and are distinct from murine γδ T cells, may be of superior importance in surveillance of lipid antigens (Bendelac, et al., 2007).

Vδ1 T cells, which are the second most abundant subset of γδ T cells in humans, can respond to CD1c, CD1d, and the stress-inducible molecules MICA and MICB, which are

expressed by virus-infected and tumour cells (Russano, et al., 2007, Bai, et al., 2012, Spada, et al., 2000). Lipid-specific Vδ1 T cells appear to have distinct phenotypes depending on the tissues they reside in. In the circulation, phospholipid-specific Vδ1 T cells exhibit a T_H2 phenotype, while intestinal Vδ1 T cells mediate T_H1 or T_{reg} phenotypes (Russano, et al., 2007, Russano, et al., 2006, Agea, et al., 2005). The lipid repertoire of Vδ1 T cells includes self-lipids such as sulfatide (3-O-sulfogalactosylceramide), which is enriched in kidneys, intestine and neural tissue, but also exogenous pollen-derived phospholipids (Bai, et al., 2012, Russano, et al., 2006, Breimer, et al., 2012, Takahashi and Suzuki, 2012). The role of Vδ1 T cells in tissue homeostasis and repair is in line with their self-ligand detection (Luoma, et al., 2013). Murine γδ T cells have also shown responsiveness to cardiolipin, a phospholipid which plays an important role in the inner mitochondrial membrane of mammals, and can also be found in bacteria (Dieude, et al., 2011).

iNKT cells constitute a subset of unconventional T lymphocytes with a highly restricted repertoire that also co-express cell surface markers of NK cells. iNKT cells recognise antigens presented by CD1d, and are thus CD1d restricted (Bendelac, et al., 2007). The iNKT cells are activated by various endogenous and bacterial glycosphingolipids, but the prototypical antigen for stimulating mouse and human iNKT cells is αGalCer.

A proportion of Vδ3 T cells were shown to recognise CD1d without antigen recognition, and they did not recognise the iNKT ligand αGalCer. Thus, there is a need for identifying ligands for this less prevalent γδ T cell subset.

5.2 Objectives

Following the recent development that Vδ3 T cells can activate DC and have the ability to recognise CD1d, we sought to examine the potential of Vδ3 T cells for B helper activity and CD1 recognition. The main aims were:

- To characterise the interactions between co-cultured allogeneic Vδ3 T cells and B cells by examining co-stimulatory marker expression by both cell types and characterising the resulting cytokine and antibody isotype production

- To confirm that Vδ3 T cells expanded in this study can recognise CD1 molecules on HeLa transfectant cells
- To investigate whether fresh Vδ3 T cells are capable of presenting glycolipids (cardiolipin, ganglioside and sulfatide) via CD1 molecules using CD1a-, CD1b-, CD1c- or CD1d-transfected HeLa cells as APC and to compare these to CD1d recognition by iNKT cells

5.3 Methods

5.3.1 Generation of Vδ3 T cells, iNKT cells and B cells

B cells were obtained by enriching PBMC for $CD19^+$ cells using a negative selection kit (see section 2.6.4). In the absence of a known ligand for Vδ3 T cells and iNKT cells, PHA-P, a mitogen known to induce proliferation of all T cells was used to expand these cells. However, due to the unspecific nature of PHA-P stimulation, pure populations of Vδ3 T cells and iNKT cells were first obtained by flow cytometric cell sorting. Vδ3 T cells were sorted by gating on viable lymphocytes positive for CD3, γδ TCR and the Vδ3 chain, while iNKT cells were sorted by gating on viable lymphocytes positive for CD3, Vα24 and Vβ11. These cells (>99% purity) were then stimulated with PHA and an excess of irradiated PBMC and cultured for 28 days in IL-2-supplemented media (see section 2.6.3). Cell lines of >90% purity following expansion were used for all experiments (Fig. 3.23). Phenotyping of Vδ3 T cells was described in chapter 3.

5.3.2 Analysis of co-stimulatory marker expression by B cells and Vδ3 T cells in co-cultures

We wanted to determine whether, like Vδ2 T cells, Vδ3 T cells could induce B cell maturation. Vδ3 T cells were cultured with B cells in equal numbers in the presence or absence of PHA or LPS for 24 h in cRPMI. LPS was used as a positive control to stimulate B cells, while PHA was used to activate Vδ3 T cells. The cells were stained for expression of CD3, Vδ3 and co-stimulatory markers CD40, CD86 and HLA-DR. Surface expression of these markers was compared by MFI readings obtained using flow cytometry. This is summarised in Fig. 5.1A.

5.3.3 Analysis of cytokine production from co-cultures of Vδ3 T cells and B cells

We next examined the cytokines produced by B cells and Vδ3 T cells upon co-culture. Vδ3 T cells were cultured with equal numbers of B cells for 24 h in the presence of PHA or LPS iIn order to investigate whether resting (unstimulated) or PHA-activated Vδ3 T cells could stimulate cytokine production by B cells. In addition, we examined whether resting or activated Vδ3 T cells could induce maturation of B cells into effector cells that produce cytokines upon re-stimulation with PMA and ionomycin. The supernatants were harvested and assayed for levels of IFN-γ, IL-4 and IL-17 by ELISA, as described in section 2.7.2. The cells of the same co-culture were then treated with monensin for 6 h. PMA and ionomycin-stimulated cell cultures were used as positive controls. The cells were then stained for cell surface expression of CD3, Vδ3 and CD19, as described in section 2.5.2 and were then fixed and permeabilised and examined for intracellular expression of IFN-γ, IL-4, TNF-α and IL-17a for analysis by flow cytometry (as described in section 2.7.3). The assays of B and Vδ3 T cell activation are summarised in Fig. 5.1A. Cytokine production by Vδ3 T cells cultured alone is illustrated in Fig. 3.25.

5.3.4 Measurement of antibody production by B cells

The effect of Vδ3 T cells on antibody production by B cells was investigated by culturing Vδ3 T cells in equal numbers with B cells in the presence or absence of PHA and LPS for 7 days. The supernatants were harvested and analysed using immunoglobulin cytometric bead array kits for IgA, IgM and total IgG levels as described in section 2.7.4. This is summarised in Fig. 5.1A.

5.3.5 Vδ3 T cell responses to CD1 molecules in the absence of glycolipids or in the presence of cardiolipin, sulfatide and ganglioside

A study from our group found that a proportion of Vδ3 T cells were able to recognise CD1d in the absence of glycolipids. Thus, we wished to further investigate the potential of Vδ3 T cells for glycolipid reactivity.

The cytolytic degranulation of Vδ3 T cells in response to CD1-transfected HeLa cells (Fig. 2.9) was examined by flow cytometric analysis of CD107a expression by Vδ3 T

cells. Mock-transfected HeLa cells or HeLa cells transfected with CD1a, CD1b, CD1c or CD1d (section 2.6.7) were cultured with Vδ3 T cells at equal ratios for 4 h in the presence or absence of PMA and ionomycin. The cultures were stained with CD107a PE-Cy7 and after 1 h, the cultures were treated with monensin to prevent proteolysis of the mAb conjugate upon re-internalisation of CD107a. The cells were then stained for CD3 and Vδ3 expression and analysed by flow cytometry. This is summarised in Fig. 5.1B.

To determine whether Vδ3 T cells within freshly-prepared PBMC respond to mock-transfected HeLa cells or HeLa cells expressing transfected CD1a, CD1b, CD1c or CD1d with the help of Masters student Robyn Bruen, they were cultured in the absence of added glycolipid or presenting cardiolipin, ganglioside or sulfatide. Glycolipids were thawed, vortexed, heated and sonicated and diluted in cDMEM. HeLa transfectant cells were cultured overnight in 96-well plates in the absence or presence of glycolipids. γδ T cell-enriched PBMC were cultured for 4 h with the glycolipid-pulsed HeLa transfectants. The cultures were then examined for cytotoxicity as described above.

5.3.6 Analysis of cytokine production by Vδ3 T cells cultured with CD1-transfected HeLa cells with or without glycolipids

In order to investigate whether the presence of HeLa CD1 transfectants could induce cytokine secretion by Vδ3 T cells, Vδ3 T cells were cultured for 4 h in equal ratios with mock-transfected HeLa cells or HeLa cells transfected with CD1a, CD1b, CD1c, or CD1d which had been pulsed overnight with medium alone, sulfatide, gangliosides or cardiolipin in the presence of monensin. Cells were examined for intracellular expression of IFN-γ, IL-4, TNF-α and IL-17a by Vδ3 T cells using flow cytometry (see section 2.7.3). This experiment is summarised in Fig. 5.1B.

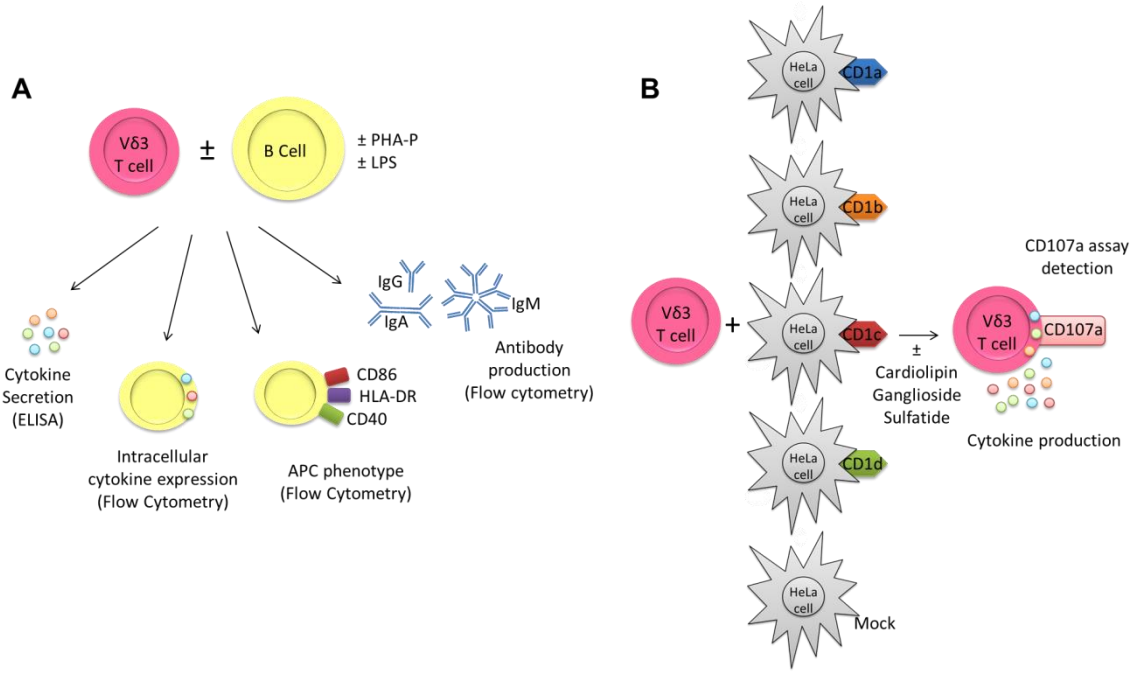

Figure 5.1 Investigation of B cell helper activity and CD1 recognition by human Vδ3 T cells. A, Vδ3 T cells were cultured with enriched peripheral blood B cells and cultured for 24 h and analysed for APC phenotype, cytokine and antibody production. **B**, Vδ3 T cells were cultured with mock-transfected HeLa cells or HeLa cells transfected with CD1a, CD1b, CD1c or CD1d in the presence or absence of cardiolipin, ganglioside and sulfatide and examined for cytotoxicity and cytokine profiles.

5.4 Results

5.4.1 Vδ3 T cells induce co-stimulatory marker expression by B cells

To investigate whether Vδ3 T cells could induce B cell maturation, expanded Vδ3 T cells were cultured with freshly-isolated allogeneic B cells and examined for expression of co-stimulatory markers CD86, CD40 and HLA-DR. It was found that both resting and PHA-activated Vδ3 T cells induced an increase in CD86 (Fig. 5.2A), HLA-DR (Fig. 5.2B) and CD40 (Fig. 5.2C) and this increase was significantly higher when the Vδ3 T cells were stimulated with PHA. LPS activation did not enhance this Vδ3-induced maturation of B cells. Likewise, the addition of B cells to Vδ3 T cells resulted in an increase in CD86 (Fig. 5.3A), HLA-DR (Fig. 5.3B) and CD40 (Fig. 5.4C) expression by Vδ3 T cells. Interestingly, PHA stimulation induced HLA-DR but not CD86 or CD40 upmodulation by Vδ3 T cells, while LPS did not activate Vδ3 T cells, nor synergise with B cells. Thus, there is reciprocal activation between B cells and Vδ3 T cells.

5.4.2 Vδ3 T cells do not induce cytokine production by B cells

Next, we examined the cytokine profile of B cells when cultured with Vδ3 T cells. Expanded Vδ3 T cells were cultured with B cells for 24 h, and monensin was added for a further 6 h. The cells were then examined for expression of IFN-γ (Fig. 5.4A), TNF-α (Fig. 5.4B) and IL-4 (Fig. 5.4C). It was found that Vδ3 T cells did not induce an increase in cytokine production by B cells compared to background levels produced by B cells alone. While PMA and ionomycin stimulation induced an increase in TNF-α expression by B cells, it resulted in decreased IFN-γ and IL-4 expression by B cells. Thus, although PHA-activated Vδ3 T cells appeared to induce an increase in IFN-γ production, these levels were the same as background IFN-γ levels produced by B cells and thus there was no net increase. Furthermore, PHA and LPS did not enhance IFN-γ or TNF-α production. However, LPS enhanced IL-4 production by B cells. These results were also confirmed by ELISA, which showed that PHA-activated Vδ3 T cells produced IFN-γ (Fig. 5.5A) and IL-4 (Fig. 5.5B) but B cells and Vδ2 T cells could not stimulate cytokine secretion by each other.

5.4.3 B cells induce IL-17 and IL-4 production by Vδ3 T cells in the presence of PMA and ionomycin

We also sought to investigate whether B cells can induce cytokine production by Vδ3 T cells. The presence of B cells alone did not augment cytokine production by Vδ3 T cells, while in the presence of PMA and ionomycin, B cells induced production of IL-17, but not IFN-γ, IL-4 or TNF-α by Vδ3 T cells, whereas PMA and ionomycin alone induced a significant increase in IFN-γ (Fig. 5.6A), IL-4 (Fig. 5.6B) and TNF-α (Fig. 5.6C) but not IL-17 (Fig. 5.6D). Thus, IL-17 was produced only when B cells synergised with PMA and ionomycin. While PHA did not augment cytokine production by Vδ3 T cells, together with PMA and ionomycin, it augmented IL-4 production compared to when PHA was absent. Furthermore, LPS had no effect on cytokine production by Vδ3 T cells. This agrees with the ELISA data above which confirmed that the addition of B cells to the cell culture did not increase IFN-γ and IL-4 secretion (Fig. 5.5).

5.4.4 Vδ3 T cells do not induce antibody production by B cells

Next we wanted to test whether Vδ3 T cells can induce antibody production by B cells. Expanded Vδ3 T cells were cultured with B cells for 7 days and the supernatants were analysed for levels of IgA (Fig. 5.7B), IgM (Fig. 5.7C) and total IgG (Fig. 5.7A). PHA appeared to inhibit antibody production, which is consistent with the HMB-PP-induced inhibition of antibody production in Vδ2-matured B cells (Fig. 4.7). LPS did not augment antibody production either. Vδ3 T cells did not induce an increase in IgA or IgG levels, but induced a significant increase in IgM production by B cells.

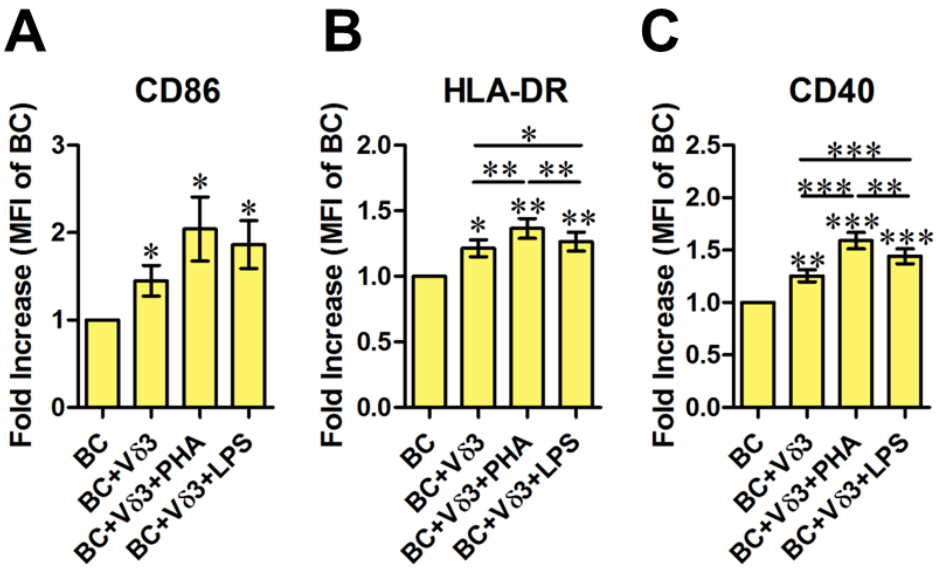

Figure 5.2 Expression of APC markers by B cells cultured with Vδ3 T cells. Enriched peripheral blood B cells (BC) were cultured for 24 h with PHA-expanded Vδ3 T cells in the presence or absence of PHA or LPS. The cells were then stained using mAb specific for CD19 (B cells), CD3 and Vδ3 (Vδ3 T cells), CD86, CD40 and HLA-DR and analysed by flow cytometry. **A-C**, average (±SEM) fold increase (based on MFI of B cells) of CD86 (**A**), HLA-DR (**B**) and CD40 (**C**) (n=8). **p*<0.05, ***p*<0.01, ****p*<0.001 using a paired *t* test compared to BC.

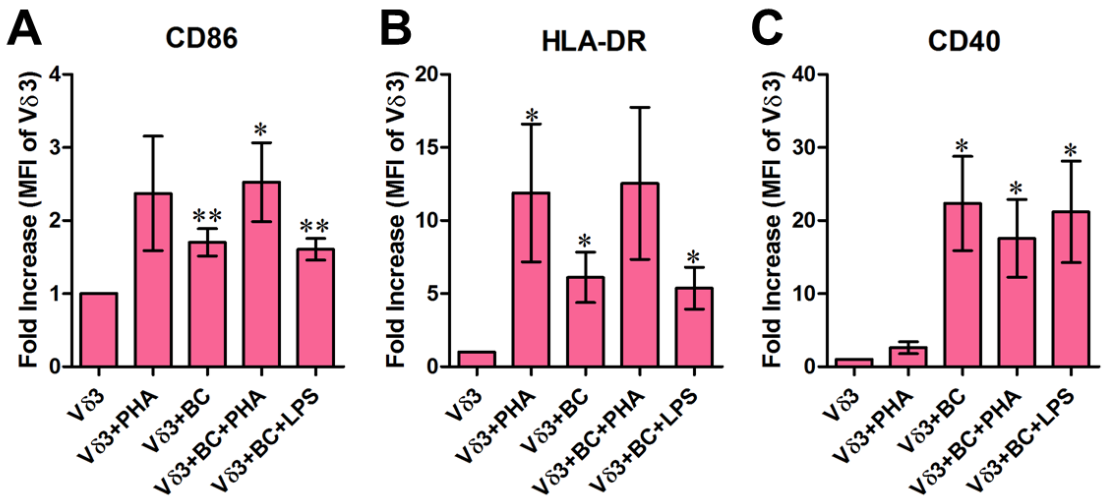

Figure 5.3 Expression of APC markers by Vδ3 T cells cultured with B cells. Enriched peripheral blood B cells (BC) were cultured for 24 h with PHA-expanded Vδ3 T cells in the presence or absence of PHA or LPS. The cells were then stained using mAb specific for CD3, Vδ3, CD86, CD40 and HLA-DR and analysed by flow cytometry. **A-C**, average (±SEM) fold increase (based on MFI of Vδ3 T cells) of CD86 (**A**), HLA-DR (**B**) and CD40 (**C**) (n=8). *p<0.05, **p<0.01 using a paired t test compared to Vδ3 T cells alone.

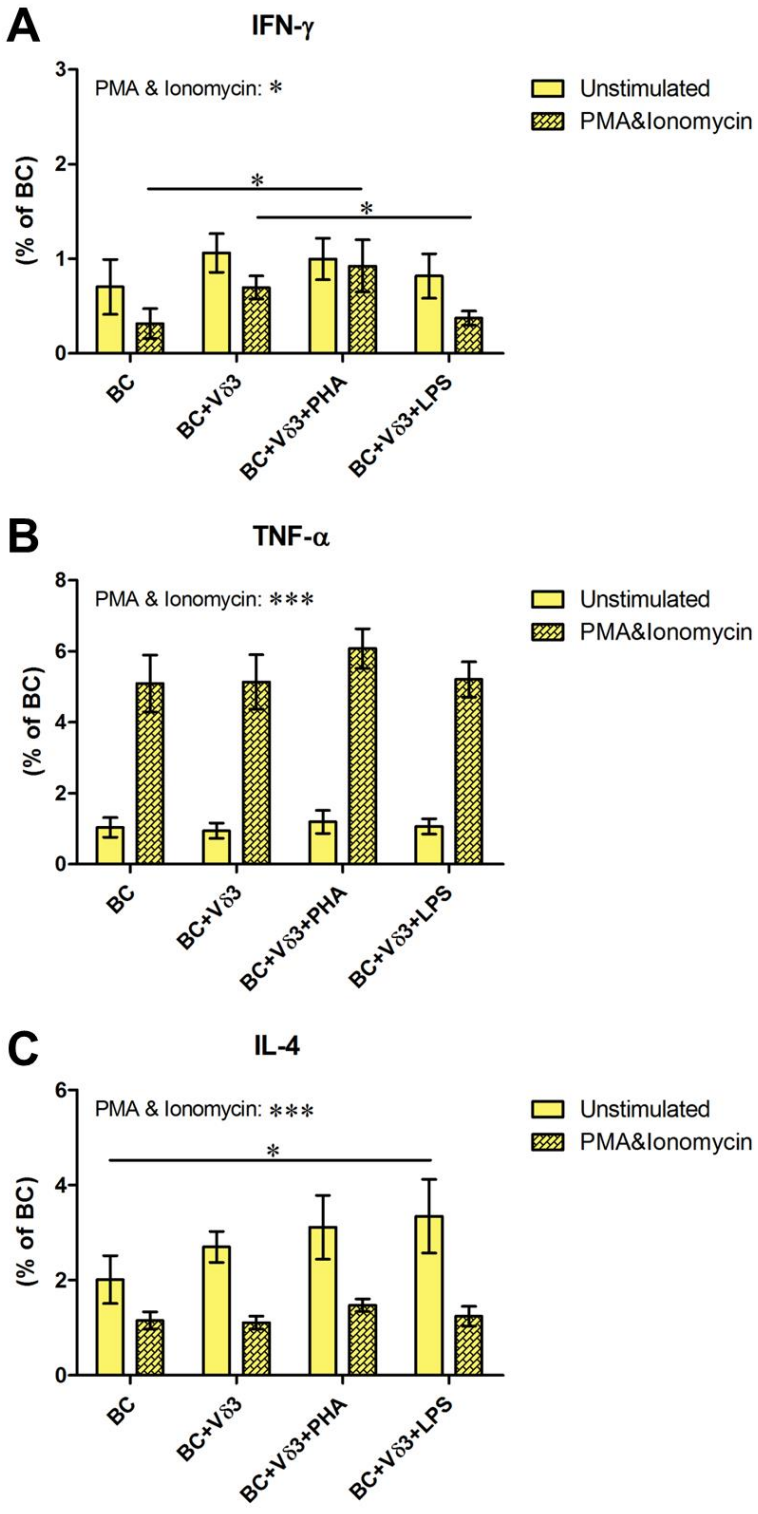

Figure 5.4. Cytokine production by B cells cultured with Vδ3 T cells. Enriched peripheral blood B cells (BC) were culture with PHA-expanded Vδ3 T cells in the presence or absence of PHA or LPS for 24 h. The cultures were then left unstimulated or stimulated with PMA and ionomycin in the presence of monensin for a further 6 h and then stained for cell surface expression of CD19, CD3 and Vδ3 and intracellular expression of IFN-γ, TNF-α and IL-4. **A-C**, mean (± SEM) percentages of B cells expressing IFN-γ (**A**), TNF-α (**B**) or IL-4 (**C**) (n=4). *p<0.05, ***p<0.001 using a paired t test.

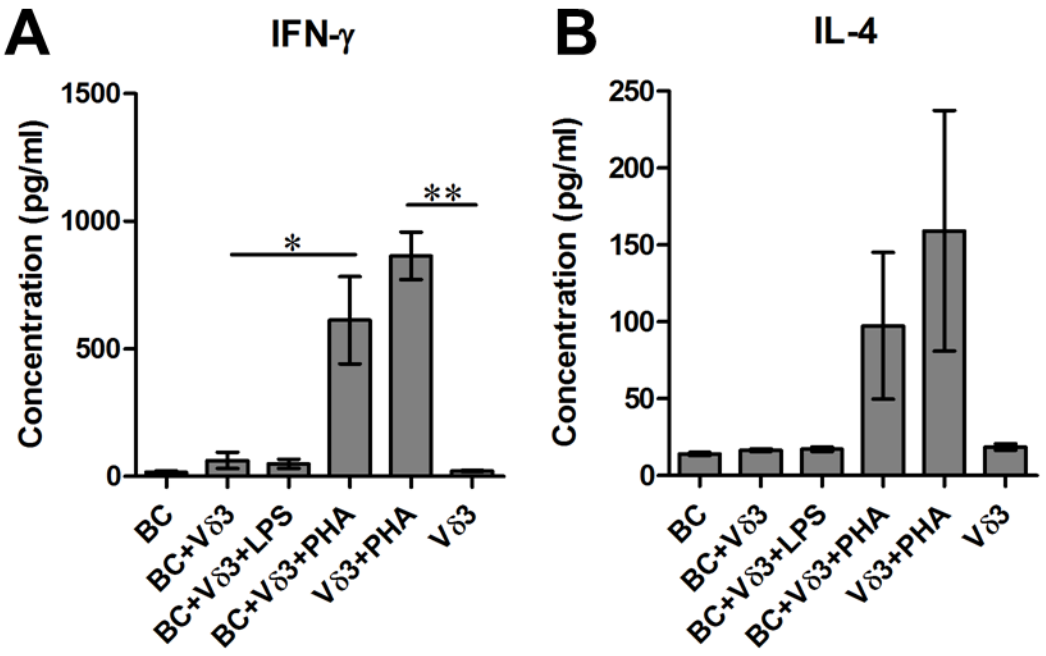

Figure 5.5. Cytokine secretion by cultures of Vδ3 T cells with B cells. Enriched peripheral blood B cells (BC) were cultured with PHA-expanded Vδ3 T cells in the presence or absence of PHA or LPS for 24 h. The supernatants were harvested and analysed for levels of IFN-γ and IL-4 by ELISA. **A-B**, average (±SEM) concentration of IFN-γ (**A**) and IL-4 (**B**) in supernatants (n=4). **p*<0.05, ***p*<0.001, ****p*<0.001 using a paired *t* test.

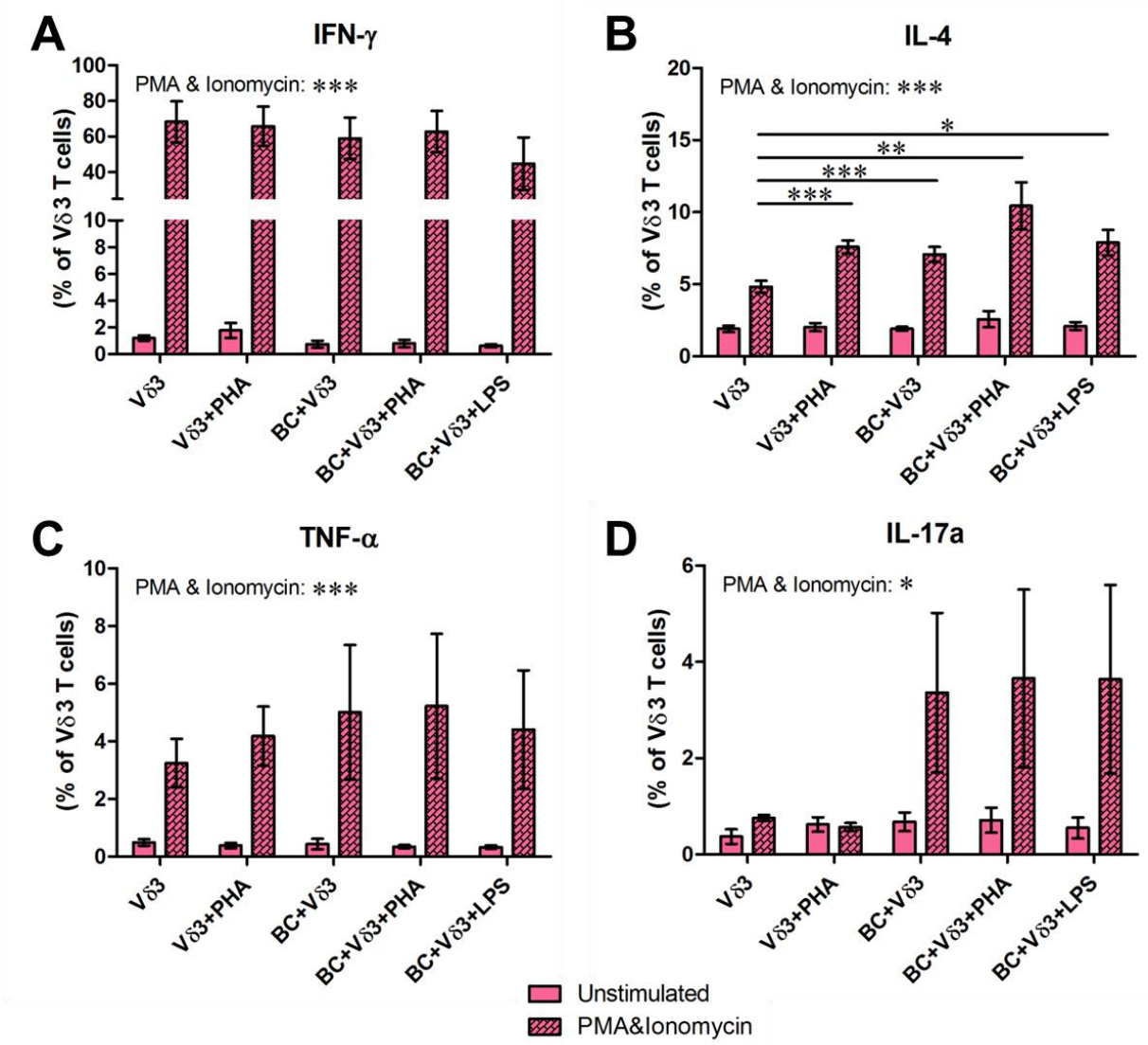

Figure 5.6. Cytokine production by Vδ3 T cells cultured with B cells. Enriched peripheral blood B cells (BC) were cultured with PHA-expanded Vδ3 T cells in the presence or absence of PHA or LPS for 24 h. The cultures were then left unstimulated or stimulated with PMA and ionomycin and cultured with monensin for a further 6 h and analysed for expression of IFN-γ, IL-4, TNF-α and IL-17a by flow cytometry. **A-D**, average (±SEM) percentage of Vδ3 T cells (n=4) expressing IFN-γ (**A**), IL-4 (**B**), TNF-α (**C**) or IL-17a (**D**). *p<0.05, **p<0.001, ***p<0.001 using a two-way ANOVA and paired *t* test.

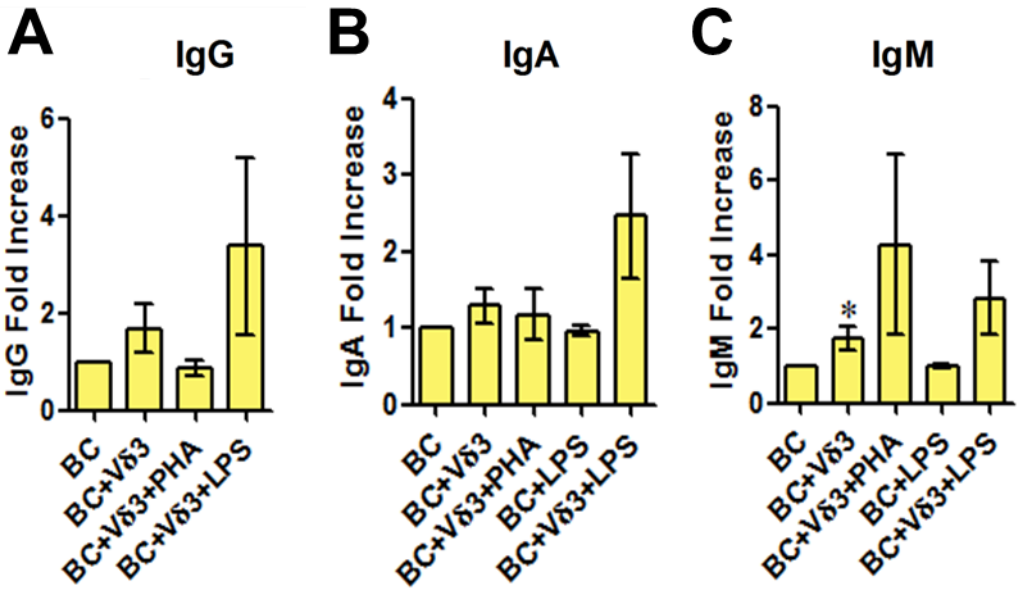

Figure 5.7. Antibody production by B cells cultured with Vδ3 T cells. Enriched peripheral blood B cells were co-cultured with equal numbers of PHA-expanded human Vδ3 T cells in the absence or presence of PHA and LPS. After 7 days, the supernatants were harvested and analysed for IgG (n=4), IgM (n=7-10) and IgA (n=8-10) levels by cytometric bead array and flow cytometry. **A-C**, average (±SEM) fold increase of IgG (**A**), IgA (**B**) and IgM (**C**).

5.4.5 Vδ3 T cells do not recognise CD1a, CD1b, CD1c or CD1d

A number of human T cell types including subsets of γδ T cells and αβ T cells can recognise glycolipid antigens presented by CD1 molecules. Humans have 4 CD1 isotypes that are capable of presenting lipid antigens to iNKT cells and Vδ1 T cells. It was recently demonstrated that Vδ3 T cells can recognise CD1d molecules in the absence of lipid antigens. Thus, we wished to test the ability of Vδ3 T cells to recognise lipids via CD1 molecules, but first we wanted to confirm that our Vδ3 T cell lines can recognise CD1 molecules in the absence of added glycolipid. HeLa cervical cancer cells transfected with CD1a, CD1b, CD1c or CD1d or mock-transfected were cultured with Vδ3 T cells and examined for expression of CD107a, a marker of degranulation and indirectly, cytotoxicity, by Vδ3 T cells. Externalisation of CD107a would indicate the ability of Vδ3 T cells to recognise HeLa cells due to their expression of CD1 molecules. We found that stimulating Vδ3 T cells with PMA and ionomycin resulted in degranulation, regardless of whether HeLa cells or CD1 molecules were present (Fig. 5.8). However, there was no increase in CD107a expression with any of the HeLa cell lines used, suggesting that Vδ3 T cells from these donors do not have the ability to recognise CD1a, CD1b, CD1c or CD1d.

This was also shown with the help of Masters student Robyn Bruen using fresh Vδ3 T cells (Fig. 5.9), which did not induce degranulation in response to HeLa cells transfected with CD1a (Fig. 5.9A), CD1b (Fig. 5.9B), CD1c (Fig. 5.9C) or CD1d (Fig. 5.9D). The lipids sulfatide, cardiolipin and ganglioside were presented to Vδ3 T cells via HeLa transfectant cells. However, the presence of these did not induce any reactivity towards the HeLa transfectants, thus indicating that most Vδ3 T cells cannot recognise and bind these lipids. While there appeared to be some reactivity with HeLa mock cells, this was not significant. In contrast, expanded iNKT cells (Fig. 5.10A) showed reactivity against HeLa CD1d cells (Fig. 5.10B,C) and this was markedly increased upon pulsing with αGalCer.

5.4.6 Vδ3 T cells do not produce cytokines upon culture with HeLa transfectants

We also examined the cytokine profile of Vδ3 T cells following culture with HeLa transfectants and found that HeLa cells transfected with CD1a, CD1b, CD1c or CD1d

did not induce IFN-γ (Fig. 5.11A,E), IL-4 (Fig. 5.11B,E), TNF-α (Fig. 5.11C,F) or IL-17a (Fig. 5.11D,F) production by Vδ3 T cells. These results showed that while PMA and ionomycin induced IFN-γ, IL-4 and TNF-α expression by Vδ3 T cells, the presence of HeLa cells or CD1 molecules did not augment cytokine production. Furthermore, there was no increase in IL-17 expression by Vδ3 T cells with either stimulus. Analysis using fresh Vδ3 T cells with the help of Ms Bruen confirmed these findings, where HeLa cells transfected with CD1a (Fig. 5.12A), CD1b (Fig. 5.12B), CD1c (Fig.12C) or CD1d (Fig. 12D) did not induce IFN-γ expression by freshly isolated Vδ3 T cells.

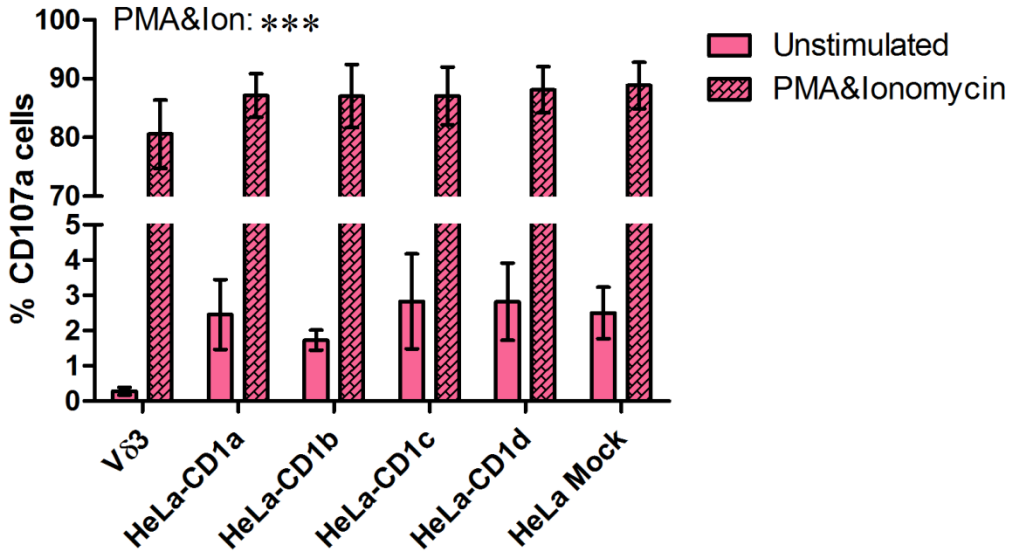

Figure 5.8. CD107a expression by Vδ3 T cells following culture with CD1-transfected or mock-transfected HeLa cells. Mock-transfected HeLa cells or HeLa cells transfected with CD1a, CD1b, CD1c or CD1d were cultured with PHA-expanded Vδ3 T cells for 4 h in the presence of monensin and anti-CD107a mAb and left unstimulated or stimulated with PMA and ionomycin. The cells were then stained with mAb specific for CD3 and Vδ3 and examined by flow cytometry. Average (±SEM) percentage of $CD107a^+$ Vδ3 T cells (n=5). ***p<0.001 using a two-way ANOVA.

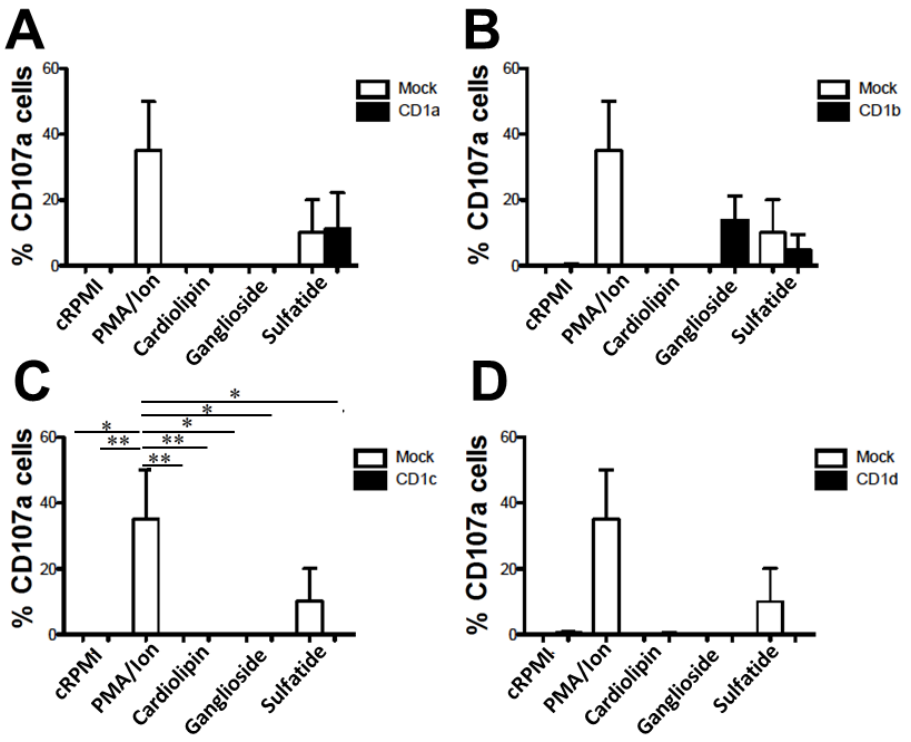

Figure 5.9 CD107a expression by Vδ3 T cells following culture with CD1-transfected or mock-transfected HeLa cells. PBMC were enriched for γδ TCR$^+$ cells to obtain enriched levels of Vδ3 T cells. Mock-transfected HeLa cells or HeLa cells transfected with CD1a, CD1b, CD1c or CD1d were were cultured overnight with cardiolipin, ganglioside or sulfatide. The following day, fresh Vδ3 T cells were added for 4 h in the presence of monensin and anti-CD107a mAb. PMA and ionomycin (PMA/Ion) was used as a positive control to stimulate degranulation. The cells were then stained with mAb specific for CD3 and Vδ3 and examined by flow cytometry. **A-D**, average (±SEM) percentage of CD107a$^+$ depicting cytolytic granulation of fresh Vδ3 T cells in response to CD1a- (**A**), CD1b- (**B**), CD1c- (**C**) or CD1d-transfected (**D**) HeLa cells presenting cardiolipin, ganglioside or sulfatide (n=3). *p<0.05, **p<0.01 using a paired t test.

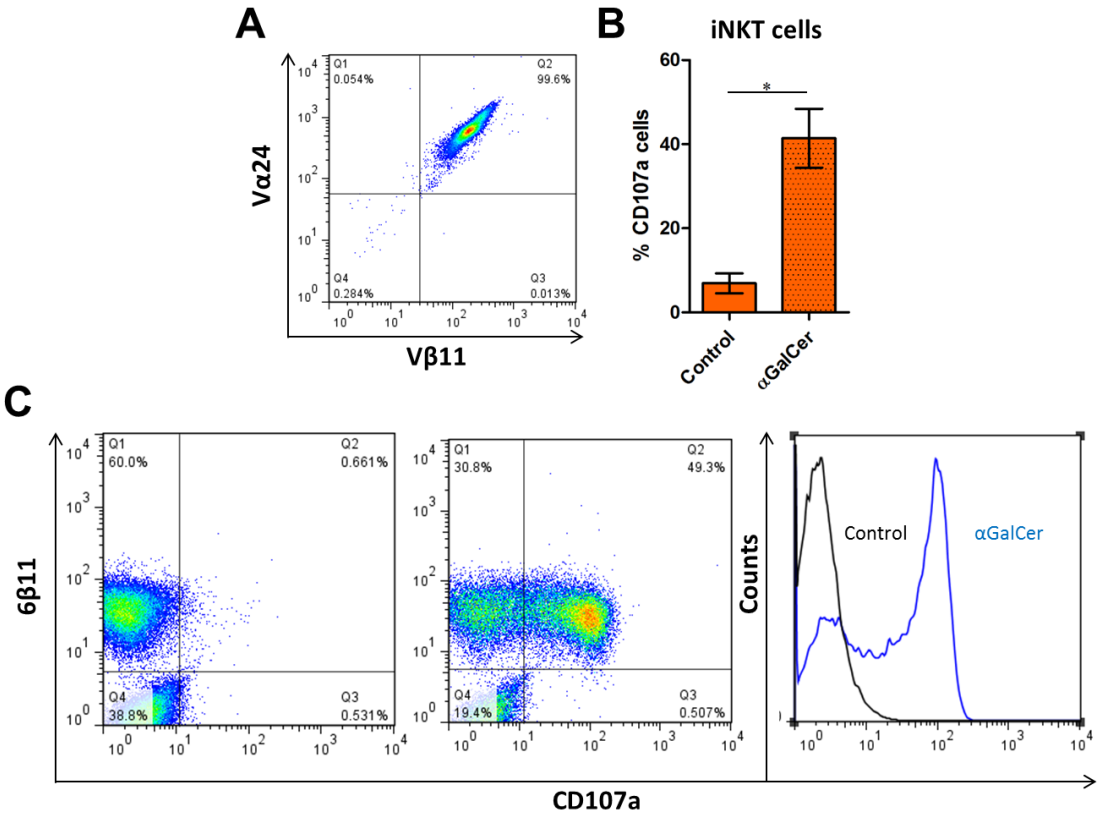

Figure 5.10 CD107a expression by iNKT cells following culture with CD1-transfected or mock-transfected HeLa cells. Mock-transfected HeLa cells or HeLa cells transfected with CD1d were cultured with PHA-expanded iNKT cells for 4 h in the presence of monensin and anti-CD107a mAb and the presence or absence of αGalCer. The cells were then stained with mAb specific for CD3 and 6β11 and examined by flow cytometry. **A**, purity of expanded iNKT cells detected using mAb against the Vα24 and Vβ11 chains. **B**, average (±SEM) percentage of $CD107a^+$ iNKT cells (n=5). **C**, flow cytometric dot plots of CD107a expression by iNKT cells in the absence (left panel) or presence (centre panel) of αGalCer; right panel, histogram of CD107a expression by iNKT cells in the absence (control) or presence of αGalCer. *p<0.05 using a paired t test.

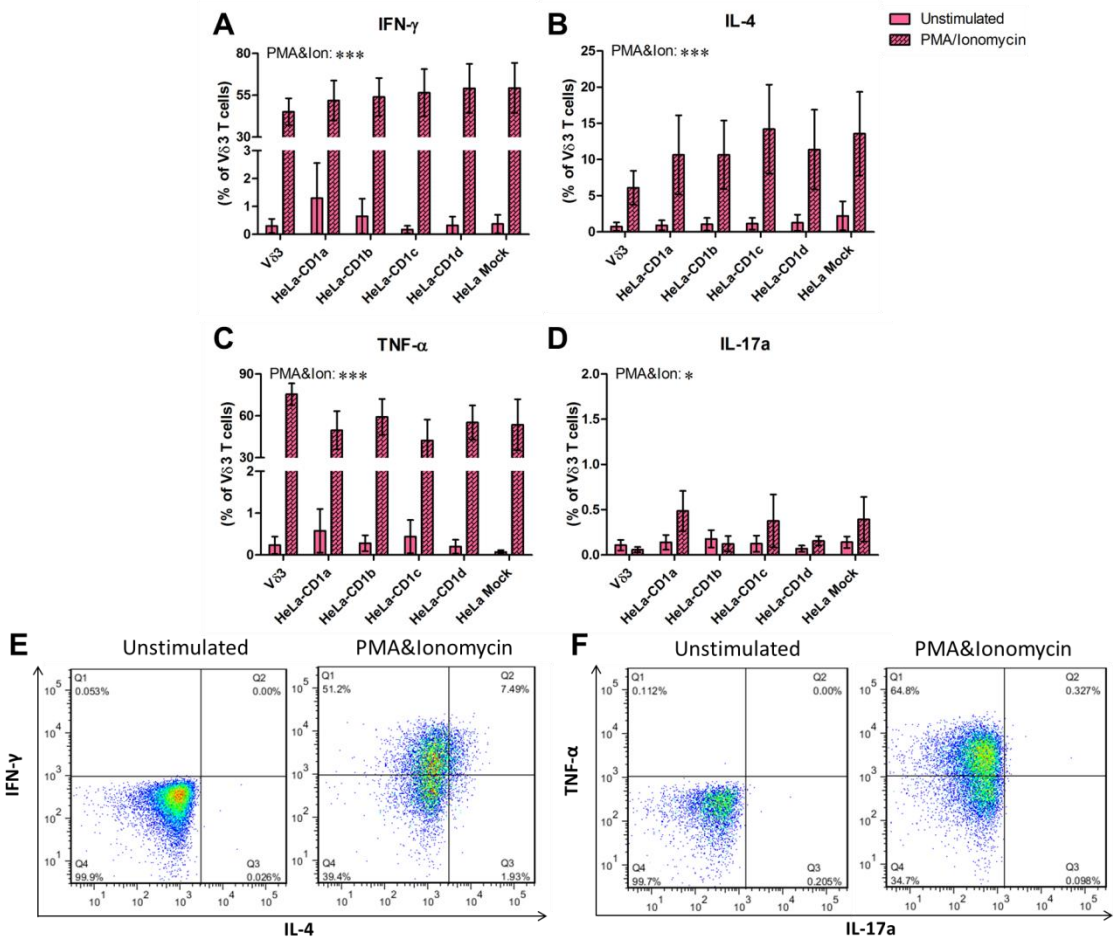

Figure 5.11 Cytokine production by Vδ3 T cells cultured with CD1-transfected or mock-transfected HeLa cells. Mock-transfected HeLa cells or HeLa cells transfected with CD1, CD1b, CD1c or CD1d were cultured with PHA-P-expanded Vδ3 T cells for 4 h in the presence of monensin and left unstimulated or stimulated with PMA and ionomycin. The cells were then stained for cell surface expression of CD3 and Vδ3 and intracellular expression of IFN-γ, IL-4, TNF-α and IL-17a and examined by flow cytometry. **A-D**, average (±SEM) percentage of Vδ3 T cells (n=3) expressing IFN-γ (**A**), IL-4 (**B**), TNF-α (**C**) and IL-17a (**D**).*p<0.05, ***p<0.001 using a two-way ANOVA.

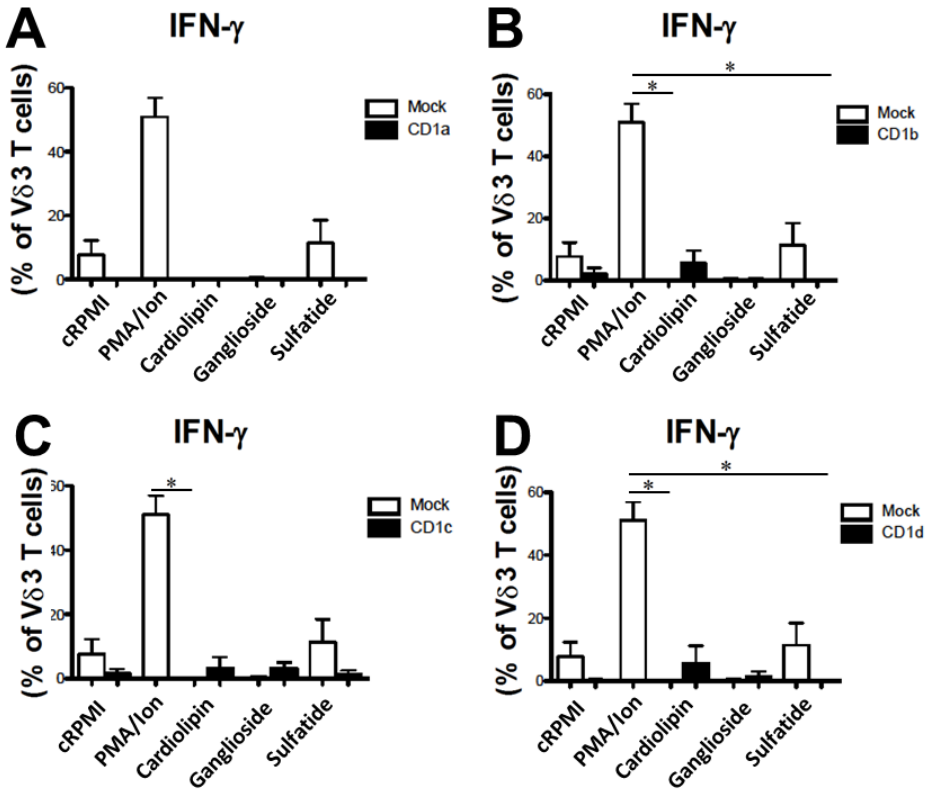

Figure 5.12 Intracellular IFN-γ expression by fresh Vδ3 T cells in response to mock-transfected HeLa cells and HeLa cells expressing transfected CD1a, Cd1b, CD1c or CD1d presenting cardiolipin, ganglioside or sulfatide. PBMC were enriched for γδ TCR^+ cells to obtain enriched levels of Vδ3 T cells. Mock-transfected HeLa cells or HeLa cells transfected with CD1a, CD1b, CD1c or CD1d were cultured with fresh Vδ3 T cells for 4 h in the presence of monensin and the presence or absence of cardiolipin, ganglioside or sulfatide. The cells were then stained for cell surface expression of CD3 and Vδ3 and intracellular expression of IFN-γ for analysis by flow cytometry. **A-D**, average (±SEM) percentage of IFN-γ expression by fresh Vδ3 T cells in response to CD1a- (**A**), CD1b- (**B**), CD1c- (**C**) or CD1d-transfected (**D**) HeLa cells presenting cardiolipin, ganglioside or sulfatide (n=3). **p*<0.05 using a paired *t* test.

5.5 Discussion

Human Vδ3 T cells have recently gained interest due to a report of their CD1d restriction. They are also reported to be capable of maturing DC into cytokine-producing APC, a function which was found to be CD1d-dependent (Mangan, et al., 2013).

This study, which is the first to explore the reciprocal interaction between Vδ3 T cells and B cells, demonstrates that Vδ3 T cells can drive maturation of B cells. We have shown that co-culturing Vδ3 T cells and B cells resulted in upregulated CD86, CD40 and HLA-DR expression on both Vδ3 T cells and B cells within 24 h. This feature is unique to Vδ3 T cells, as B cells do not upregulate co-stimulatory marker expression by Vδ2 T cells, as was shown in chapter 4 (Fig. 4.15). In addition, while Vδ2 T cells induce IL-4 production by B cells, no T_H1, T_H2 or T_H17 cytokines were observed from cultures of Vδ3 T cells with B cells. Interestingly, however, B cells stimulated with PMA and ionomycin induced a small increase in IL-17 production by Vδ3 T cells. Furthermore, while Vδ2 T cells induce IgA, IgM and total IgG production by B cells, this was not seen with Vδ3 T cells, which only induced moderate levels of IgM. Thus, while Vδ2 T cells have been established to play an important role in orchestrating immune responses through their interaction with DC, B cells and neutrophils in peripheral blood, Vδ3 T cells may have a more important role in tissues, such as lipid surveillance, which is discussed below.

Several studies have described the ability of innate T cells, including Vδ2 T cells and iNKT cells, to drive the differentiation of iDC into APC (Bendelac, et al., 2007, Dunne, et al., 2010, Fujii, et al., 2007), which resulted in their exploration as adjuvants in DC-based therapies (Fournie, et al., 2013, Motohashi, et al., 2011). A previous study from our group (Mangan, et al., 2013) investigated the relationship between Vδ3 T cells and DC and discovered that within 3 days, Vδ3 T cells induced an upregulation in expression of several APC markers by DC including CD40, CD83, CCR7 and HLA-DR to levels comparable to LPS-stimulated DC. Interestingly, they found that this was CD1d-dependent, as blocking CD1d also blocked the co-stimulatory marker expression. In comparison, some studies found that DC activation by Vδ2 T cells was TNF-α-

dependent (Conti, et al., 2005, Ismaili, et al., 2002), thus suggesting that different γδ T cell subsets use distinct methods to activate APC.

Several types of human innate T cells, including γδ T cells, are capable of recognising antigens presented by CD1a, CD1b, CD1c or CD1d molecules (Russano, et al., 2007, Bai, et al., 2012, Spada, et al., 2000, de Jong, et al., 2010, Exley, et al., 2002, Uldrich, et al., 2011, de Lalla, et al., 2011). We examined whether Vδ3 T cell lines can recognise and kill target cells expressing CD1 isotypes by culturing them with CD1- or mock-transfected HeLa cells and measuring the expression of the degranulation marker CD107a. The results showed that the 5 Vδ3 T cell donors assessed in this study failed to degranulate in response to HeLa cells expressing CD1d. In addition, Vδ3 T cells did not respond to CD1a, CD1b or CD1c either. These data are in contrast to the study by Mangan et al., which using the same methods of expansion and experimental setup found that Vδ3 T cells from 5 donors were able to recognise CD1d but not CD1a, CD1b or CD1c. In addition, they found a CD1d-dependent release of IFN-γ and IL-17 by Vδ3 T cells which was blocked by anti-CD1d treatment (Mangan, et al., 2013). In chapter 3, we showed that our Vδ3 T cell lines did not produce IL-17.

Sulfatide is a sulfoglycolipid which is synthesised endogenously and plays important roles in homeostasis, thrombosis, infection, insulin secretion and the nervous system. Cardiolipin is a phospholipid constituting an important component of the mitochondrial membrane and bacterial membranes. Gangliosides are glycosphingolipids found on cell surfaces and are involved in cell-to-cell communication. We examined the ability of freshly isolated Vδ3 T cells to recognise these lipids presented by HeLa cells via CD1 molecules. However, we found that Vδ3 T cells from 3 donors were not capable of responding to these lipids, and these lipids did not induce IFN-γ production by Vδ3 T cells.

Thus the Vδ3 T cell lines examined do not appear to recognise glycolipids that stimulate iNKT cells. Since DC can express CD1d on their surface, they could be used to present lipids to Vδ3 T cells (Cao, et al., 2002). We propose that strategies which could directly detect CD1d-restriction, such as by using antigen-loaded CD1d tetramers, should be used to further investigate CD1d restriction by Vδ3 T cells.

CD1 molecules were among the first γδ T cell ligands described, yet NKT cell discovery has diverted the focus from γδ T cells in CD1-lipid recognition (Luoma, et al., 2013). Vδ1^{+} γδ T cells comprise nearly half of IEL (Cheroutre, 2005), which together with Vδ3 T cells, would make these the largest lipid-reactive population in humans (Luoma, et al., 2013). The ability of γδ T cells in recognising self and foreign antigens such as pyrophosphates or lipids which result in activation of DC and B cells makes them crucial players in immunosurveillance and immunoregulation. The findings discovered in this chapter are summarised below (Fig. 5.13).

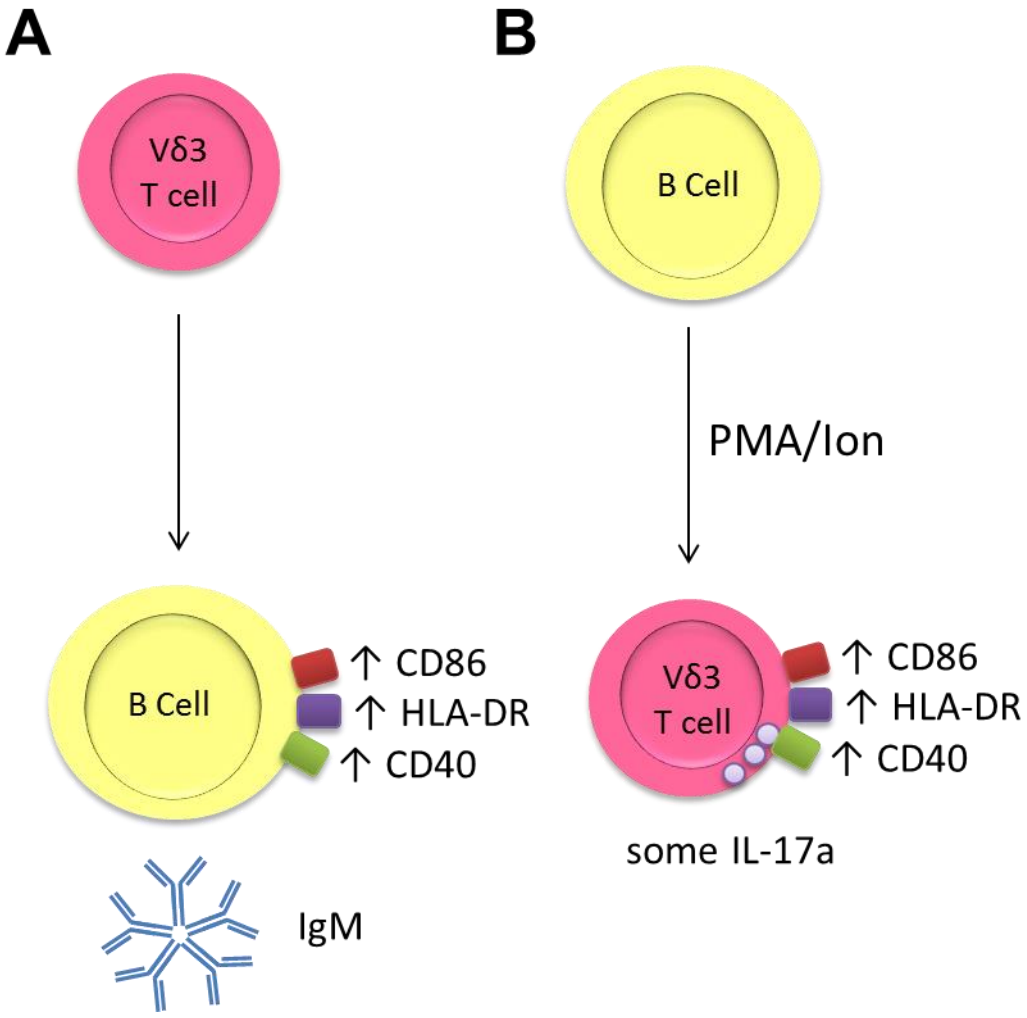

Figure 5.13. Vδ3 T cells induce an APC phenotype by B cells. A, Vδ3 T cells induce an increase in CD86, CD40 and HLA-DR expression by B cells and moderate IgM production. **B**, B cells also induce an increase in APC marker expression by Vδ3 T cells. In addition, B cells enhance IL-4 and IL-17a production by Vδ3 T cells in the presence of PMA and ionomycin only.

Chapter 6

Preliminary studies investigating the role of Vδ2 T cells in mediating immunity against *Clostridium difficile*

6.1 Introduction

Clostridium difficile is a Gram-positive, spore-forming bacterium found mainly in the gastro-intestinal (GI) tract. It is the main culprit for nosocomial antibiotic-associated diarrhoea among hospital patients and in severe cases it can result in pseudo-membranous colitis and can even be fatal (Poxton, et al., 2001, Wren, 2006). *C. difficile* infection often follows antibiotic treatment, which causes disruption of the normal gut flora, thus giving the bacterium the opportunity to thrive. Worryingly, it is increasingly found in young adults and patients with no recent antibiotic exposure (Jafari, et al., 2013).

Pathogenesis is mediated via toxin production by the bacterium (Babcock, et al., 2006, Lyras, et al., 2009). *C. difficile* secretes toxins A (tcdA), an enterotoxin and B (tcdB), a cytotoxin, (Elliott, et al., 2007, Kelly, et al., 1994, Voth and Ballard, 2005), but also a third binary toxin which can increase tcdA and tcdB production and resistance (Indra, et al., 2006, Kuijper, et al., 2007). They can also produce cytolethal distending toxins (CDT) A and B, which cause cells to arrest in the G2M phase of the cell cycle (Lara-Tejero and Galan, 2001). In addition, they express surface layer proteins (SLP) which form a surface layer (S-layer) composed of a paracrystalline surface protein array (Ni Eidhin, et al., 2008). There are two SLP, termed high molecular weight (HMW) and low molecular weight (LMW) which cover the surface of the bacterium and are thought to mediate binding of *C. difficile* to the GI tract (O'Brien, et al., 2005).

There has been a recent emergence in hyper-virulent strains, characterised by an increase in toxin production, increased resistance to treatment and increased fatality (Carroll and Bartlett, 2011). One of these strains is known as North American pulsed-field gel electrophoresis type I (NAP1) or ribotype (RT) 027 (Brazier, et al., 2008, Indra, et al., 2009, Loo, et al., 2005, Pepin, et al., 2004). Other highly virulent and lethal strains include RT 001 and RT 017, with RT 078 having similar severity to RT 027 (Arvand, et al., 2009). Reil and colleagues found that among 355 *C. difficile* isolates, RT 001 was most prevalent and found in 70% of isolates, followed by RT 027 and RT 078/126 which were found in 4.8% and 4.7% of isolates, respectively (Reil, et al.,

2011). The high toxin levels in RT 027 were associated with an increased mortality (McDonald, et al., 2005).

Current treatment strategies involve administration of metronidazole, vancomycin and fidaxomicin (Koo, et al., 2010, Soriano, et al., 2013, Zar, et al., 2007), but while these are effective in most patients, 15-35% relapse following treatment (Barbut, et al., 2000). Probiotics and faecal microbiota transplantation are under investigation and have shown cure rates of over 90% (Brandt, 2013, McFarland, 2009). Vaccines are also in various stages of clinical trials (Donald, et al., 2013, Sougioultzis, et al., 2005, Tian, et al., 2012).

PRR such as TLR allow pathogen detection by cells of the innate immune system. TLR4, which detects LPS is important in several bacterial infections such as *Mycobacterium tuberculosis*. Since *C. difficile* does not produce LPS, Ryan et al tested the hypothesis that SLP in *C. difficile* is important in pathogen recognition and whether this was mediated by TLR4 (Ryan, et al., 2011). They found that SLP and LPS induced DC maturation in a similar manner, and induced similar cytokine profiles by DC and the effect of SLP on DC was dose dependent. Thus, TLR4 plays an important role in immunity against *C. difficile*, and suggests an important role for SLP in generation of the immune responses necessary for bacterial clearance.

Isoprenoid biosynthesis is a pathway essential for cell survival and is used by eukaryotes, bacteria and archaea. All isoprenoids are derived from IPP, which can be produced via one of two pathways: the mevalonate pathway, which is used by eukaryotes and some bacteria, or the MEP pathway, which is employed by most human pathogens. An intermediate of the MEP pathway, HMB-PP is the most potent antigen for activating Vγ9Vδ2 T cells, and thus expansion of Vγ9Vδ2 is seen upon infection with a broad range of microbial pathogens.

Genetic studies were carried out to determine which pathways are used by common pathogens (Table 1.2). It was revealed that *C. difficile*, among other pathogens such as *Escherichia coli, Bacillus anthracis* and other *Clostridium* species, appear to utilise the MEP pathway of isoprenoid biosynthesis, as evidenced by possession of MEP pathway genes, while other pathogens such as *Staphylococcus aureus, Streptomyces*

pneumonia and *Legionella pneumophila* use the mevalonate pathway (Heuston, et al., 2012). This would suggest the ability of *C. difficile* to produce HMB-PP and induce Vγ9Vδ2 T cell activation. In support of this, several studies have demonstrated that MEP utilising bacteria can stimulate Vγ9Vδ2 T cells (Heuston, et al., 2012). In addition, *Staphylococcus* and *Streptococcus* were unable to stimulate Vγ9Vδ2 T cells, due to lack of HMB-PP which is only produced via the MEP pathway (Jomaa, et al., 1999). However, it has not been shown if *C. difficile* can activate Vγ9Vδ2 T cells.

6.2 Objectives

We sought to investigate whether *C. difficile* extracts could activate Vδ2 T cells, which would suggest that *C. difficile* produces HMB-PP or another phosphoantigen.

The main aims were:

- To investigate whether *C. difficile* can activate human Vδ2 T cells by stimulating PBMC or Vδ2 T cells with extracts of *C. difficile* and examining Vδ2 T cell numbers
- To examine the cytokine profile induced by Vδ2 T cells in response to *C. difficile* and compare this to the profile induced by HMB-PP

6.3 Methods

6.3.1 Method for deriving *C. difficile* supernatant and lysate

Strains of *C. difficile* prepared by Dr. Micheál MacAogáin were recovered from frozen stock cultures. Strain RT 001 produces toxins A and B, while strain RT 027 is also capable of producing cytolethal distending toxins A and B (Cdt A/B). Strain RT 060 (ATCC), in contrast, produces neither toxin. 5 µl of the stock culture was inoculated onto cefoxitin-cycloserine-egg yolk agar (Brazier's CCYE media) and incubated for 24-48 h at 37°C under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) within a Don Whitley anaerobic workstation. Plate-grown colonies were used to seed liquid cultures of *C. difficile* (with starting OD_{600} = 0.05) in pre-reduced brain heart infusion (BHIS) media supplemented with 0.5% yeast extract, 0.1% cysteine, 0.1% sodium thioglycolate and 0.05% thaurocholate. Liquid cultures were grown until an OD_{600} of

>2 was recorded and approximately 9 h growth under anaerobic conditions, in stoppered Wheaton flasks (20 ml). Once strains obtained the required OD, cultures were centrifuged at 4000 rpm to pellet viable cells/spores. Culture supernatants were removed and passed through a 0.22 µm filter. Following this initial filtration step, supernatants were passed through a low molecular weight ultra-filter with a 3 kDa cut off (Merck Millipore Ltd, Cork, Ireland) by centrifugation for 30 min at 4000 g. To obtain the *C. difficile* lysate, the bacteria were homogenised to lyse the cells. The presence of toxins A and B in 0.22 µm-filtered supernatants prior and post ultra-filtration was assessed using the Premier toxin A and B enzyme immunoassay (Meridian Bioscience Inc, Cincinnati, USA). This toxin-free ultracentrifuged *C. difficile* supernatant was added to immune cells in various concentrations to assess their responses.

6.3.2 Expansion of Vδ2 T cells from PBMC using *C. difficile*

Fresh human PBMC or PBMC enriched for γδ T cells were stimulated with BHIS media (negative control), *C. difficile* supernatant, *C. difficile* lysate or HMB-PP (positive control) and cultured in IL-2-supplemented cRPMI media for 14 days at a ratio of 1:9 or 1:1. It was determined that 1:9 ratio was best, as it was not toxic towards the cells, whereas the 1:1 ratio was toxic and thus the subsequent experiments were carried out at a 1:9 ratio of BHIS or *C. difficile* to cRPMI. The cells were then examined for CD3 and Vδ2 expression by flow cytometry. These methods are summarised in Fig. 6.1A.

6.3.3 Cytokine production by Vδ2 T cells expanded using *C. difficile* or HMB-PP

HMB-PP or *C. difficile*-expanded Vδ2 T cells were taken out of IL-2 media overnight and then stimulated with RPMI, *C. difficile*, HMB-PP or PMA and ionomycin in the presence of monensin for 4-6 h. The cells were then stained for cell surface expression of CD3 and Vδ2 and intracellular expression of IFN-γ, IL-4, TFN-α, IL-13, IL-10 and IL-17 (see section 2.6.3 for full procedure). These methods are summarised in Fig. 6.1A.

6.3.4 Investigation of the roles of B cells, monocytes and αβ T cells in Vδ2 T cell expansion in response to *C. difficile*

γδ T cells, αβ T cells, monocytes and B cells were isolated from the same donor and cultured separately or together in the presence of HMB-PP or *C. difficile* supernatant for 14 days in IL-2-supplemented RPMI media. The cells were then examined for viability, cell counts and cell surface expression of CD3 and Vδ2. These methods are summarised in Fig. 6.1B.

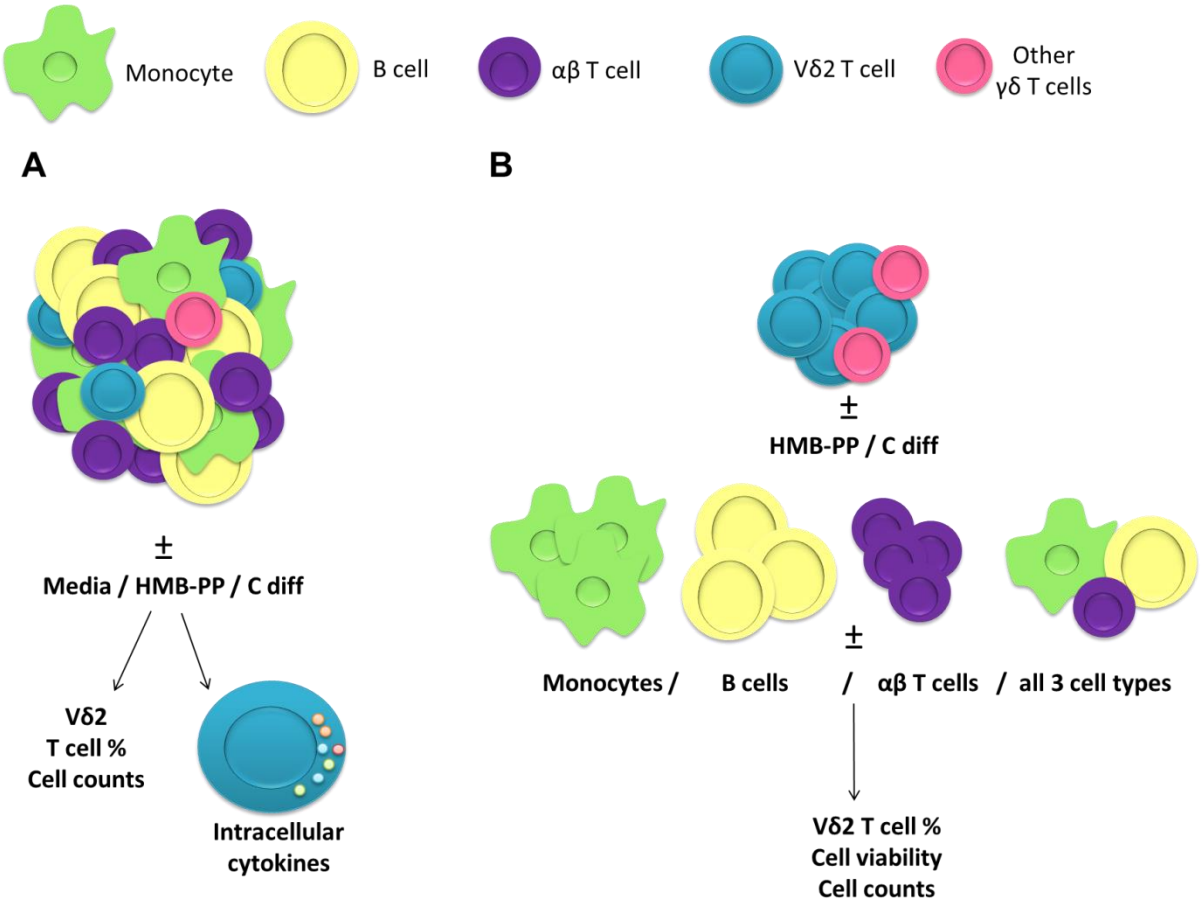

Figure 6.1 Analysis of Vδ2 T cell responses to *C. difficile in vitro.* A, PBMC were stimulated in the presence or absence of HMB-PP or *C. difficile* (denoted C diff) supernatant or lysate and cultured for 2 weeks. The cells were then examined for Vδ2 T cell expansion and cytokine production by flow cytometry. **B**, PBMC were enriched for γδ T cells (to ~70% purity) and stimulated with HMB-PP or *C. difficile* supernatant and cultured in the presence or absence of autologous monocytes, B cells or αβ T cells for 2 weeks and examined for Vδ2 expression and cell viability by flow cytometry.

6.4 Results

6.4.1 *C. difficile* induces Vδ2 T cell proliferation

Since it is not known whether *C. difficile* produces HMB-PP or another Vδ2-stimulating agent, we wanted to investigate this by stimulating PBMC or Vδ2 T cells with *C. difficile* extracts of strain RT 001, and compare these to stimulation with the media used for growing *C. difficile,* known as BHIS. The cells were cultured in a 1:1 or 1:9 ratio of BHIS media or *C. difficile* to cRPMI. The two ratios were set up to determine the ratio that would yield the highest Vδ2 T cell numbers. As expected, *C. difficile* induced expansion of Vγ9Vδ2 T cells, while BHIS did not (Fig. 6.2A,D). Also, there was no difference between *C. difficile* lysate or *C. difficile* supernatant, suggesting the stimulating agent is present in both. However, at high concentration, i.e. when *C. difficile* in BHIS media made up 50% of the culture volume, it did not expand Vδ2 T cells, suggesting the presence of an inhibitor, which may be a component of the BHIS media, which at high concentration killed the cells. When the *C. difficile* supernatant or lysate comprised only 10% of the total culture volume, the cell count was not negatively affected. Thus, in subsequent investigations, we stimulated the cells with the lower concentration of *C. difficile* or BHIS media.

6.4.2 *C. difficile* supernatant from 3 different strains induces Vδ2 T cell expansion

Next we wanted to examine the effects of 3 distinct *C. difficile* strains on Vδ2 T cell expansion. The strains used were the toxin-producing strains RT 001, RT 027 and the toxin-free strain ATCC. However, the toxins produced by RT 001 and RT 027 were excluded through a 3 kDa filter. The only factors present would be small molecules, less than 3 kDa in size, and phosphoantigens fall in this category (Harly, et al., 2014) and HMB-PP is 514 Da. The results showed that all 3 strains induced similar levels of Vδ2 T cell expansion (Fig. 6.3), but in the PBMC donors used, these were not as potent as HMB-PP. The results indicate that *C. difficile* can induce Vδ2 T cell expansion but that the media used to grow *C. difficile* (BHIS) displays toxicity towards PBMC.

6.4.3 *C. difficile* induces moderate levels of cytokine production by Vδ2 T cells

We were also interested in defining the cytokine profile by Vδ2 T cells upon stimulation with *C. difficile*. PBMC were stimulated with *C. difficile* SN or BHIS and examined for expression of T_H1 cytokines IFN-γ and TNF-α and the T_H2 cytokines IL-4 and IL-13, as well as the T_H17 cytokine IL-17. It was found that *C. difficile* induced IFN-γ (Fig. 6.4A,E) but also moderate levels of TNF-α (Fig. 6.4D,G) but not IL-4 (Fig. 6.4B,E,F), IL-13 (Fig. 6.4C,F) or IL-17 (Fig. 6.4G) by Vδ2 T cells within PBMC, which matches the cytokine profile induced by HMB-PP.

We also assessed whether *C. difficile* could induce cytokine production by expanded Vδ2 T cells. We found that when HMB-PP-expanded Vδ2 T cells were stimulated with *C difficile* supernatant or BHIS media, there was no significant increase in IFN-γ (Fig. 6.5A,E,G), IL-4 (Fig. 6.5B,E), IL-13 (Fig. 6.5C,H), TNF-α (Fig. 6.5D,F), IL-17 (Fig. 6.5F,H) or IL-10 (Fig. 6.5G). These experiments show that *C. difficile* can induce cytokine production by Vδ2 T cells in PBMC but not by expanded Vδ2 T cells.

6.4.4 *C. difficile* appears to be toxic to fresh γδ T cells, but this inhibition is prevented by addition of monocytes, B cells and αβ T cells.

The above studies show that *C. difficile* can induce IFN-γ production by Vδ2 T cells within fresh PBMC but not by expanded Vδ2 T cells. We also attempted Vδ2 T cell expansion with *C difficile* using PBMC enriched for γδ T cells, which contained ~60% Vδ2 T cells. Interestingly, we found that when an enriched population of fresh γδ T cells was simulated with *C. difficile* supernatant, cell death was observed, and this was not exclusive for Vδ2 T cells (Fig. 6.6B). This effect was not observed with BHIS media. This is in contrast to when fresh PBMC were stimulated with *C. difficile* supernatant (Fig. 6.2C). Thus, to elucidate whether one or more cell populations that are normally found in PBMC are protective of Vδ2 T cells against this toxic effect, γδ T cells, αβ T cells, B cells and monocytes were isolated from the same donor and subsequently cultured together or separately with enriched γδ T cells and stimulated with HMB-PP or *C. difficile* supernatant. It was found that while the separate addition of αβ T cells (Fig. 6.7C), monocytes (Fig. 6.7D) or B cells (fig. 6.7E) to γδ T cells was not sufficient to prevent the toxic effect, the addition of all 3 cell types to γδ T cells prevented the *C.*

difficile supernatant-mediated cell death (Fig. 6.7A,F). This inhibition is distinct from that seen with BHIS when used at high ratios to Vδ2 T cell media (Fig. 6.2) and suggests that although *C. difficile* can expand Vδ2 T cells, components of *C. difficile* can kill PBMC but this is overcome when T cells, monocytes or B cells are present. Absolute numbers of Vδ2 T cells show that the addition of T cells, monocytes and B cells did not significantly increase Vδ2 T cell counts (Fig. 6.7I). Therefore, these cells protect from overall cell death and do not help increase Vδ2 T cell numbers.

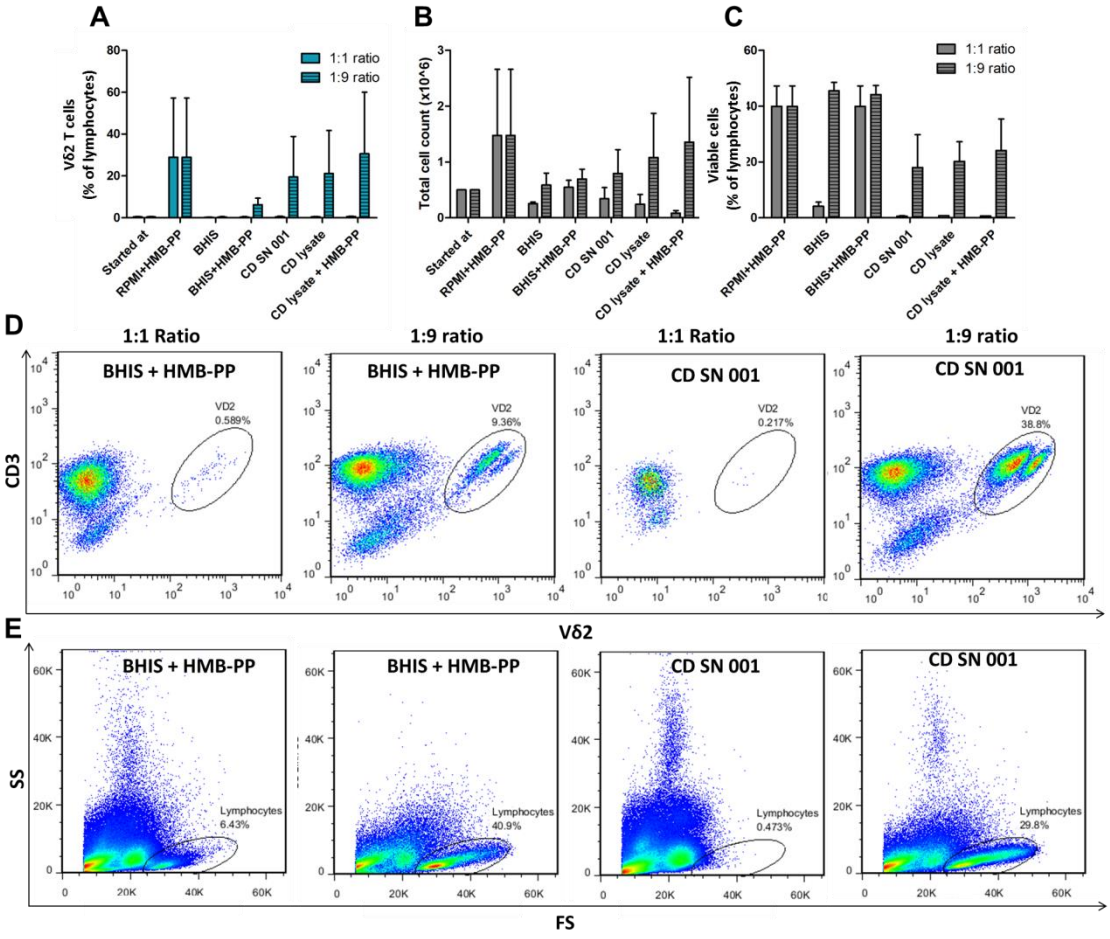

Figure 6.2 *Clostridium difficile* can induce expansion of Vδ2 T cells from PBMC. PBMC were stimulated with *C. difficile* supernatant (denoted CD SN 001), *C. difficile* lysate or *C. difficile* media (BHIS) at a 1:1 or 1:9 ratio to IL-2-supplemented RPMI and cultured for 7 days in the presence or absence of HMB-PP. The cells were then examined for CD3 and Vδ2 expression by flow cytometry. **A**, mean (±SEM) Vδ2 percentage following culture with a 1:1 or 1:9 ratio of *C. difficile* supernatant, lysate or media to RPMI (n=2). **B**, mean (±SEM) total cell counts following culture at the two different ratios (n=2). **C**, mean (±SEM) percentage of live cells (also confirmed by a viability stain) following culture at the two different ratios (n=2). **D**, representative flow cytometric dot plots depicting Vδ2 percentage. **E**, representative flow cytometric dot plots depicting viable lymphocytes.

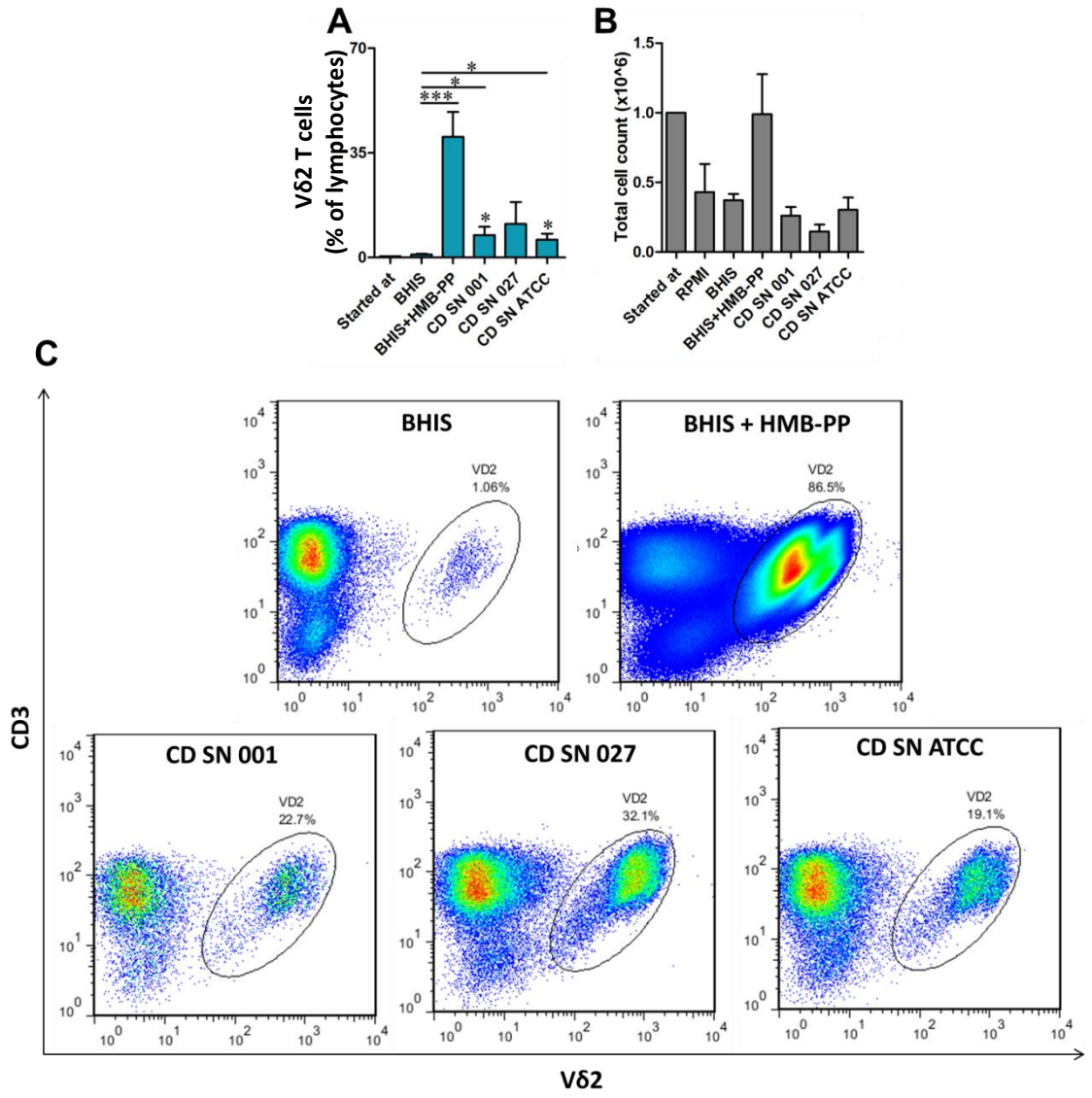

Figure 6.3 All 3 strains of *Clostridium difficile* induced expansion of Vδ2 T cells. PBMC were stimulated with *C. difficile* supernatant (denoted CD SN) of three different *C. difficile* strains (001, 027 and ATCC) at a 9:1 ratio of RPMI to BHIS or *C. difficile* SN. The cells were expanded for 2 weeks in the presence or absence of HMB-PP and examined for CD3 and Vδ2 expression by flow cytometry. **A**, mean (±SEM) Vδ2 percentage following 2 weeks of culture (n=4-12). **B**, mean (±SEM) of total cell counts after 2 weeks of culture (n=4-12). **C**, representative flow cytometric dot plots depicting Vδ2 percentage under the different stimuli. *p<0.05, ***p<0.001 using a paired t test compared to starting Vδ2 percentage except where indicated by horizontal lines.

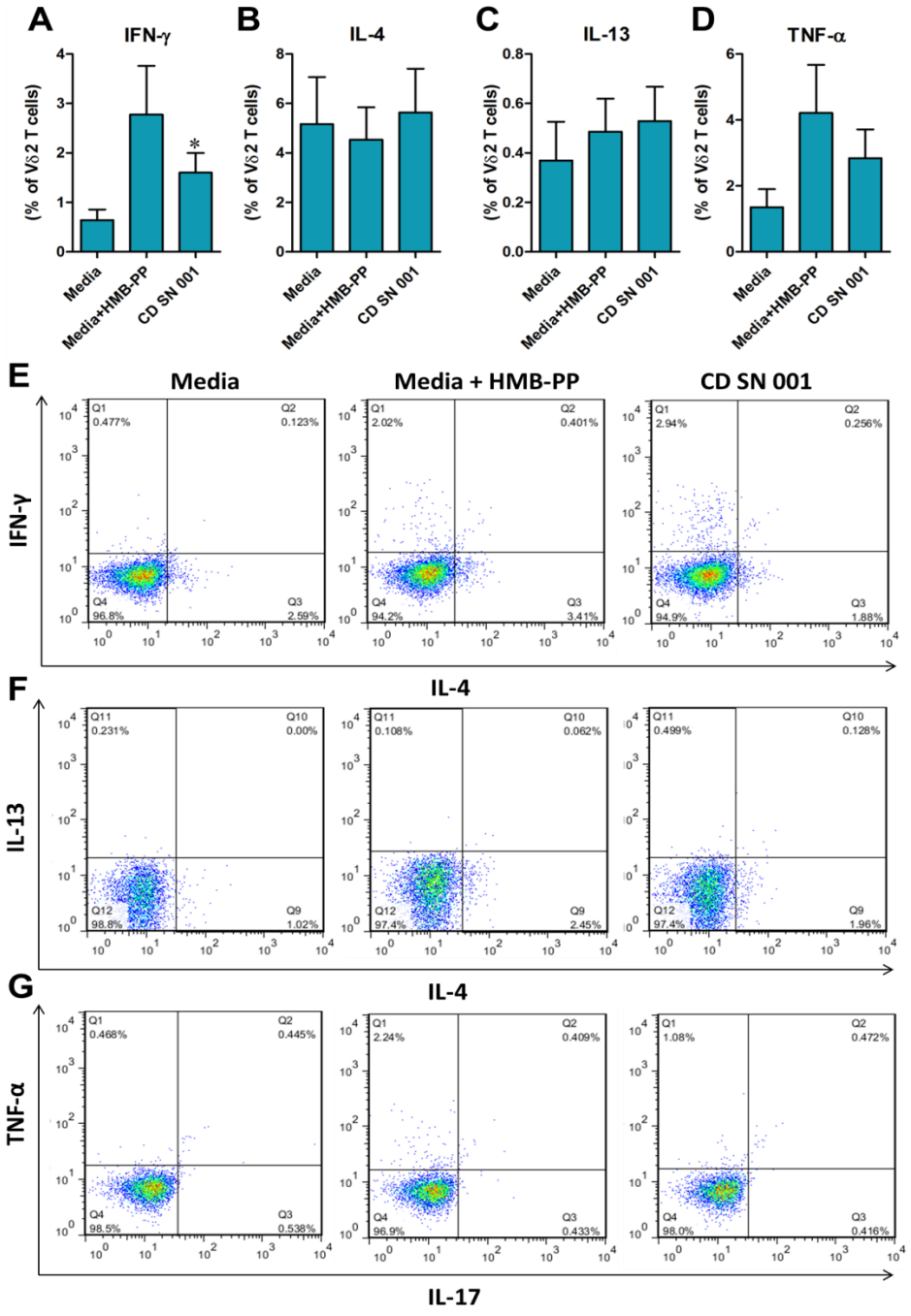

Figure 6.4 *Clostridium difficile* induces T_H1 cytokine production by Vδ2 T cells. PBMC were stimulated with BHIS media alone or containing HMB-PP or *C. difficile* supernatant strain 001 (denoted CD SN 001) overnight at a 9:1 ratio of RPMI to BHIS or *C. difficile* SN. The cells were then cultured with monensin for 4 h and examined for CD3 and Vδ2 and intracellular expression of IFN-γ, IL-4, TNF-α, IL-13 and IL-17 by flow cytometry. **A-D**, mean (±SEM) percentage of Vδ2 T cells (n=4) expressing IFN-γ (**A**), IL-4 (**B**), IL-13 (**C**) and TNF-α (**D**). **E-G**, representative flow cytometric dot plots depicting IFN-γ (**E**), IL-4 (**E,F**), IL-13 (**F**), TNF-α (**G**) and IL-17 (**G**) expression by Vδ2 T cells in the presence of media alone (left panels), HMB-PP (centre panels) or CD SN 001 (right panels). *p<0.05 using a paired t test compared to media.

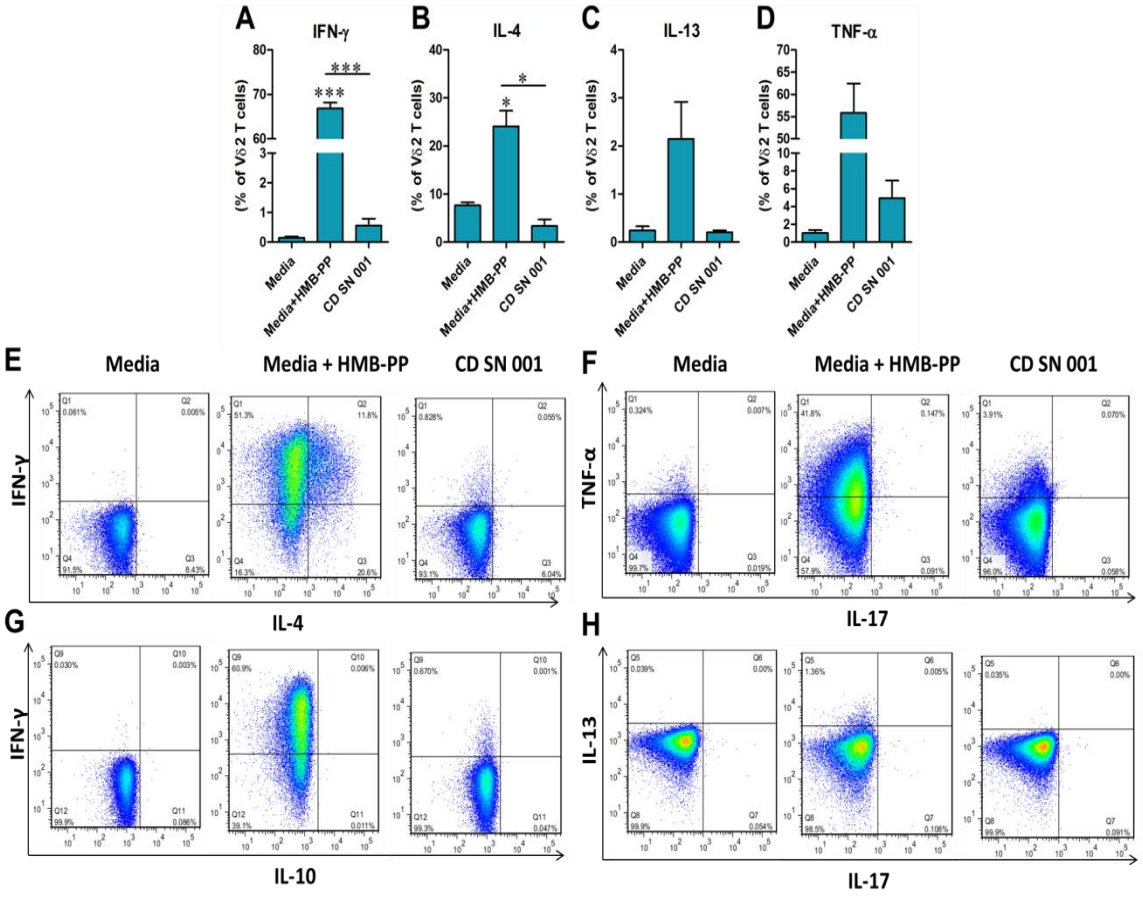

Figure 6.5 *Clostridium difficile* does not induce cytokine production by HMB-PP-expanded Vδ2 T cells. Vδ2 T cell lines were generated by stimulating γδ-enriched PBMC with HMB-PP and culturing with IL-2 for 14 days. The cells were taken out of IL-2 overnight and the following day, they were re-stimulated with *C. difficile* supernatant strain 001 (denoted CD SN 001), BHIS media (denoted media) or HMB-PP at a 9:1 ratio of RPMI to BHIS or *C. difficile* SN and cultured in the presence of monensin for 6 h. The cells were then examined for CD3 and Vδ2 and intracellular expression of IFN-γ, IL-4, TNF-α, IL-10, IL-13 and IL-17 by flow cytometry. **A-D**, mean (±SEM) percentage of Vδ2 T cells (n=3) expressing IFN-γ (**A**), IL-4 (**B**), IL-13 (**C**) and TNF-α (**D**). **E-H**, representative flow cytometric dot plots depicting IFN-γ (**E,G**), IL-4 (**E**), TNF-α (**F**), IL-17 (**F,H**), IL-19 (**G**) and IL-13 (**H**) expression by Vδ2 T cells in the presence of media alone (left panels), HMB-PP (centre panels) or CD SN 001 (right panels). **p*<0.05, ****p*<0.001 using a paired *t* test compared to media.

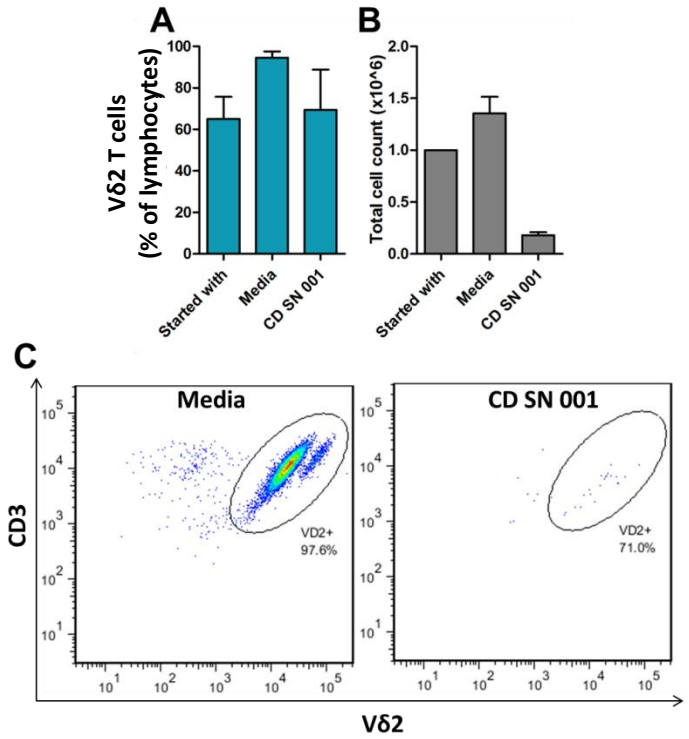

Figure 6.6 *Clostridium difficile* is toxic to fresh γδ T cells. PBMC were enriched for γδ T cells until the sample consisted of ~60% Vδ2 T cells. These cells were then stimulated with BHIS media (denoted media) or *C. difficile* supernatant strain 001 (denoted CD SN 001) at a 9:1 ratio of RPMI to BHIS or *C. difficile* SN and cultured for 2 weeks in IL-2-supplemented RPMI and examined for CD3 and Vδ2 expression by flow cytometry. **A**, mean (±SEM) Vδ2 percentage following 2 weeks of culture (n=2). **B**, mean (±SEM) of total cell counts after 2 weeks of culture (n=2). **C**, representative flow cytometric dot plots depicting Vδ2 percentage following stimulation with BHIS media (left panel) or *C. difficile* (right panel).

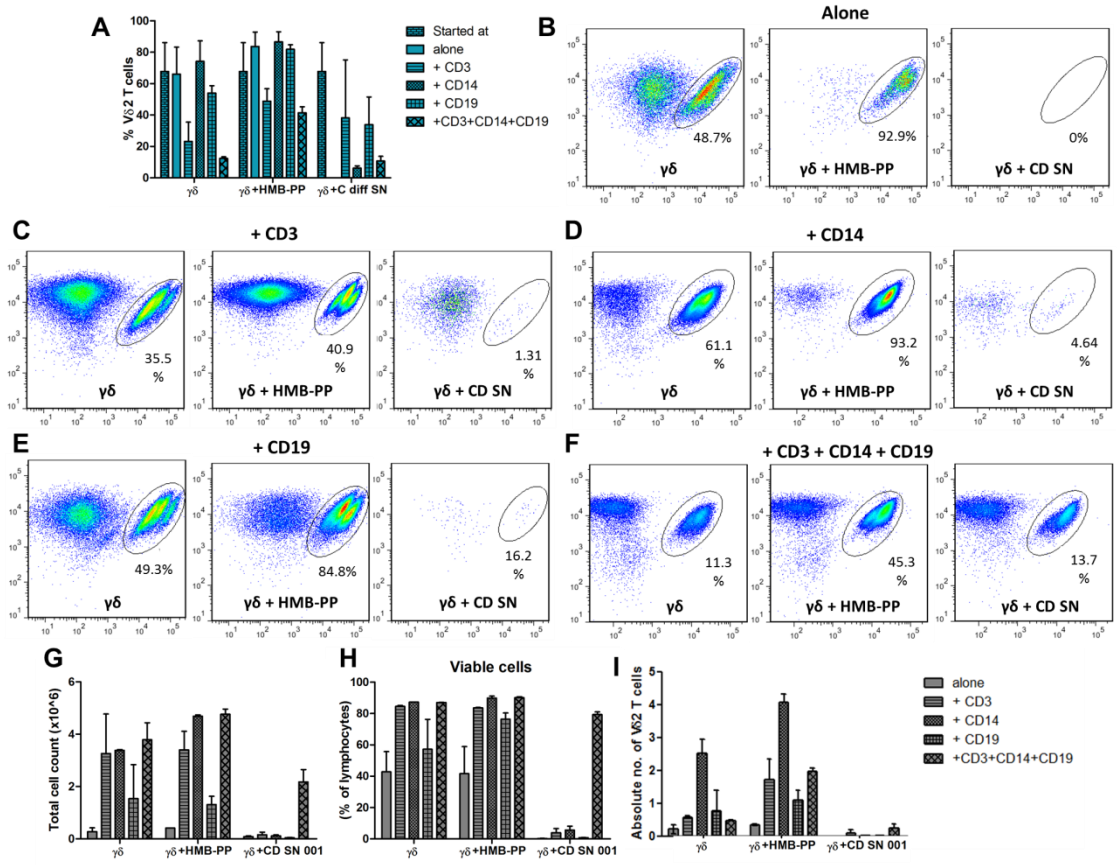

Figure 6.7 *Clostridium difficile* toxicity against fresh γδ T cells is reversed by the presence of monocytes, B cells and αβ T cells. PBMC were enriched for γδ T cells until the sample consisted of >60% Vδ2 T cells. Monocyte ($CD14^+$), B cell ($CD19^+$) and αβ T cell ($CD3^+$ γδ TCR^-) populations were also isolated from the same donors. γδ T cells were either left unstimulated or stimulated with HMB-PP or *C. difficile* supernatant strain 001 (denoted CD SN) and cultured in the presence or absence of monocytes, B cells or αβ T cells for 2 weeks in IL-2-supplemented RPMI. The cultures were then examined for cell counts and CD3, CD11c (monocyte-derived immature DC), CD19 and Vδ2 expression by flow cytometry. **A**, mean (±SEM) Vδ2 percentage following 2 weeks of culture (n=2). **B-F**, representative flow cytometric dot plots of γδ T cells stimulated with HMB-PP (centre panels) or CD SN (right panels) or unstimulated (left panels) in the absence of any other cells (**B**), the presence of αβ T cells (**C**), monocytes (**D**), B cells (**E**) or all three cell types (**F**). **G**, mean (±SEM) of total cell counts after 2 weeks of culture (n=2). **H,** mean (±SEM) percentage of viable cells (n=2). **I**, absolute numbers of Vδ2 T cells (n=2).

6.4.5 *C. difficile* induces IFN-γ and TNF-α production by *C. difficile*-expanded Vδ2 T cells

Earlier we examined the cytokines produced by Vδ2 T cells within fresh PBMC (Fig. 6.4) and HMB-PP-expanded Vδ2 T cells (Fig. 6.5), and found that *C. difficile* induced T_H1 cytokine production by Vδ2 T cells in PBMC, but not by expanded Vδ2 T cells. We also examined the cytokine profile of *C. difficile*-expanded Vδ2 T cells when restimulated with *C. difficile* and compared these to HMB-PP-expanded Vδ2 T cells, to see whether *C. difficile* can influence cytokine production by Vδ2 T cells. For this experiment, a fresh batch of strain RT 001 was isolated, and its bioactivity was confirmed. Thus, we found that this new batch of *C. difficile* supernatant did not induce significant Vδ2 T cell expansion (Fig. 6.8A,C) and there was no inhibition at the low *C. difficile* supernatant concentration (Fig. 6.8B). We also found that when these cells were re-stimulated with the original stimulus used for the expansion, *C. difficile* induced the production of the same cytokines by Vδ2 T cells as HMB-PP. Both *C. difficile* and HMB-PP induced IFN-γ (Fig. 6.9A,G) and TNF-α (Fig. 6.9E,H) but not IL-4 (Fig. 6.9 B,G), IL-10 (Fig. 6.9C), IL-13 (Fig. 6.9D,H) or IL-17 (Fig. 6.9E). This cytokine profile is very distinct from that induced by the aminobisphosphonate zoledronate (Fig. 3.22), thus further supporting the hypothesis of the presence of a pAg within the *C. difficile* supernatant.

6.4.6 *C. difficile* supernatant induces proliferation of both CD4 and CD8 subsets of Vδ2 T cells

We examined whether *C. difficile* has an impact on CD4 or CD8 subsets and found that it induced the same Vδ2 T cell CD4 and CD8 subsets as HMB-PP and did not specifically expand any of the subsets (Fig. 6.10).

6.4.7 *C. difficile*-induced activation of Vδ2 T cells is donor specific

Finally, since we found that not all Vδ2 T cell donors responded equally well to *C. difficile*, we examined Vδ2 T cell expansion in response to *C. difficile* in an additional number of donors. When combining all the 32 donors used, we found great variability between them (Fig. 6.11A). *C. difficile* induced up to 20 fold increase in Vδ2 T cell

percentages in some donors, while in other donors the percentage increase was only one fold (Fig. 6.11B).

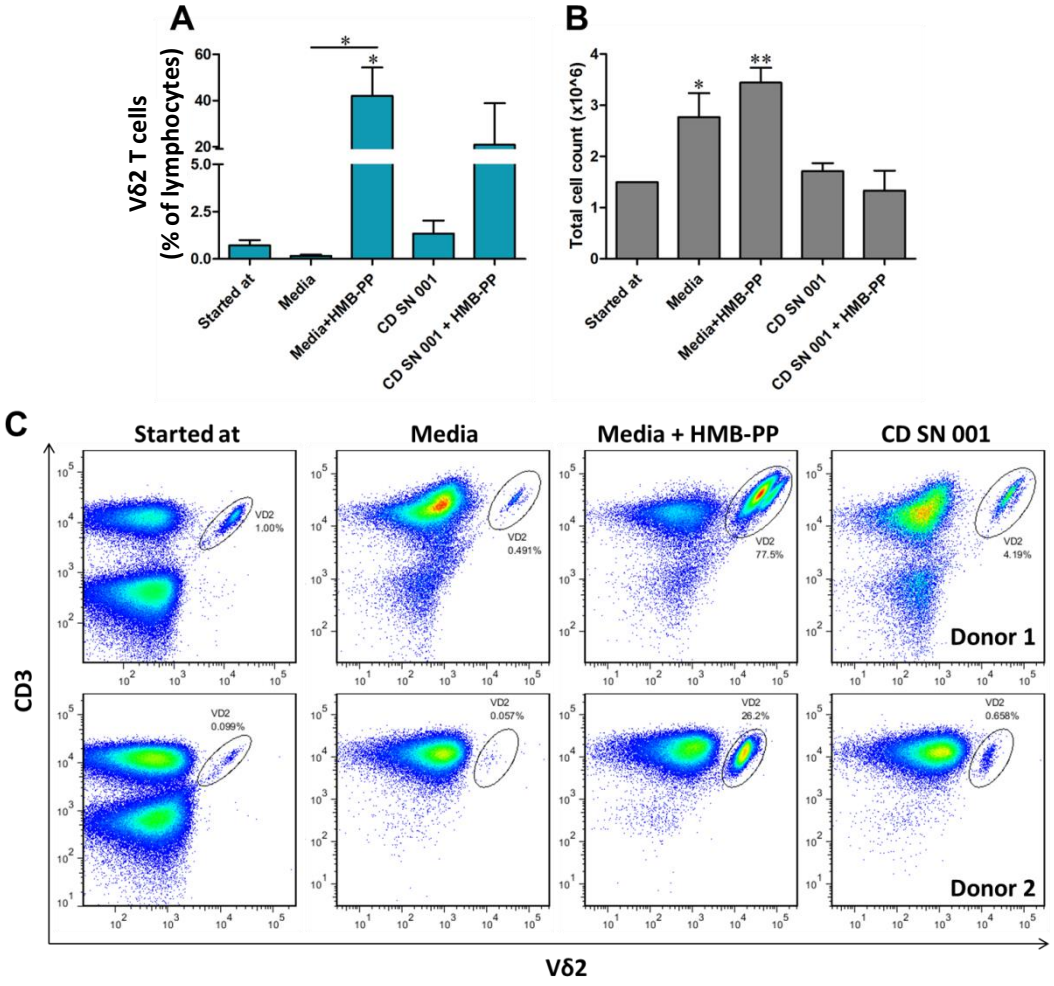

Figure 6.8 *Clostridium difficile* induces expansion of Vδ2 T cells in some donors. PBMC were stimulated with a fresh batch of *C. difficile* supernatant strain 001 (denoted CD SN 001) or BHIS media (denoted media) and cultured for 2 weeks in IL-2-supplemented RPMI and examined for CD3, CD4, CD8 and Vδ2 expression by flow cytometry. **A**, mean (±SEM) Vδ2 percentage following 2 weeks of culture (n=6). **B**, mean (±SEM) of total cell counts after 2 weeks of culture (n=6). **C**, representative flow cytometric dot plots depicting Vδ2 percentage under the different conditions for two different donors. **p*<0.05, ***p*<0.01 using a paired *t* test compared to the starting percentage or cell count except where indicated by the horizontal bars.

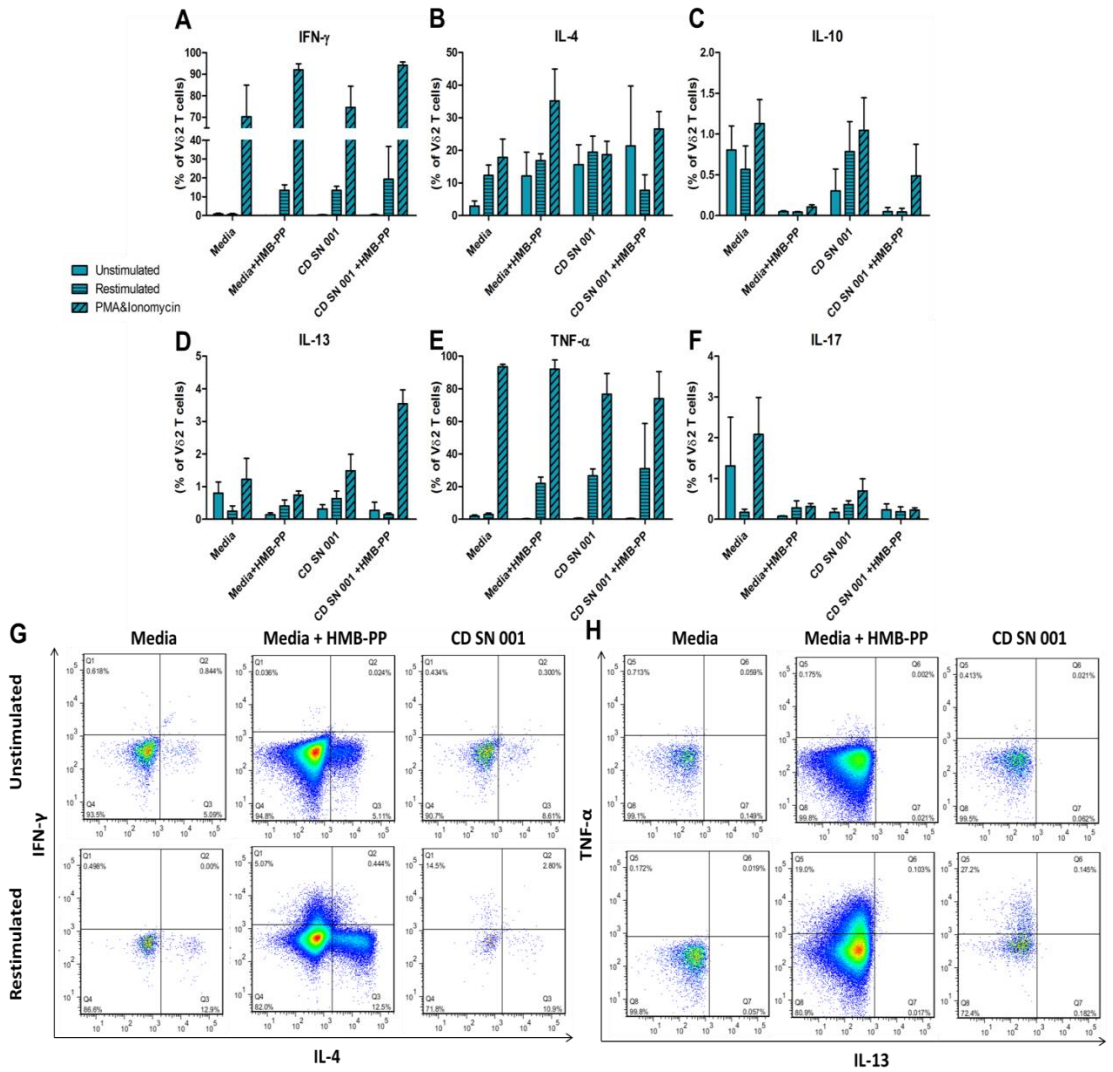

Figure 6.9 *Clostridium difficile* induces the same cytokine profile by Vδ2 T cells as HMB-PP. PBMC (Fig. 6.9) were expanded using *C. difficile* supernatant strain 001 (denoted CD SN 001) or BHIS media (denoted media) in the presence or absence of HMB-PP for 2 weeks in IL-2-supplemented RPMI. Cells were taken out of IL-2 overnight and then cultured in the presence of monensin for 4 h and left unstimulated or restimulated with the same stimulus as originally expanded with, and as a positive control, using PMA and ionomycin. The cells were the examined for CD3 and Vδ2 and intracellular expression of IFN-γ, IL-4, TNF-α, IL-10, IL-13 and IL-17 by Vδ2 T cells. **A-F**, mean (±SEM) percentage Vδ2 T cells expressing IFN-γ (**A**), IL-4 (**B**), IL-10 (**C**) IL-13 (**D**) TNF-α (**E**) and IL-17 (**F**) (n=6). **G-H**, representative flow cytometric dot plots showing IFN-γ and IL-4 (**G**) and TNF-α and IL-13 (**H**) expression by unstimulated (top panels) or restimulated (bottom panels) Vδ2 T cells.

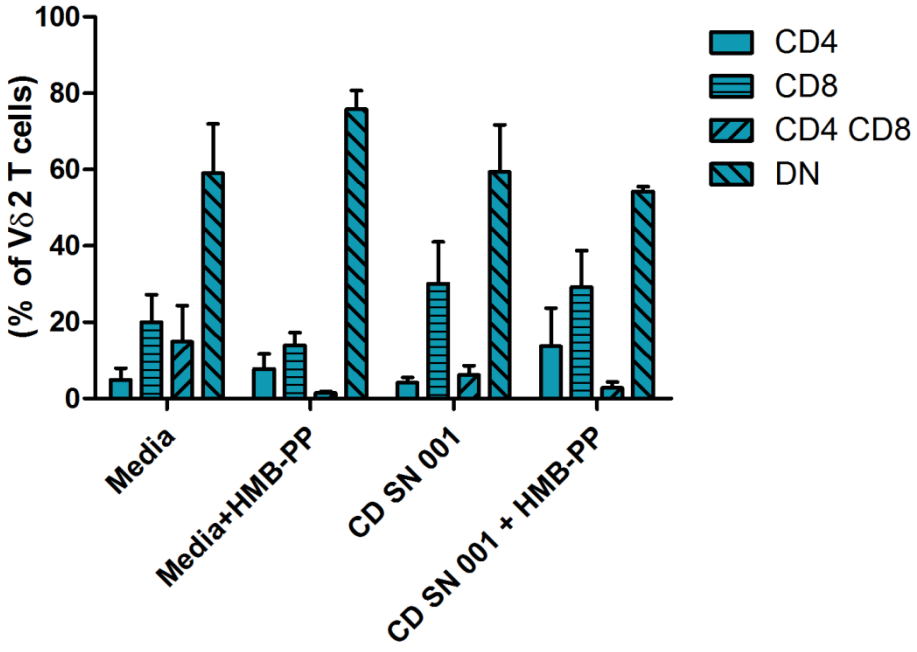

Figure 6.10 *C. difficile* induces expansion of both CD4 and CD8 subsets of Vδ2 T cells. PBMC were stimulated with a fresh batch of *C. difficile* supernatant strain 001 (denoted CD SN 001) or BHIS media (denoted media) and cultured for 2 weeks in IL-2-supplemented RPMI and examined for CD3, CD4, CD8 and Vδ2 expression by flow cytometry. Graph shows CD4 and CD8 subsets of Vδ2 T cells expanded with media, CD SN 001 or HMB-PP (n=6).

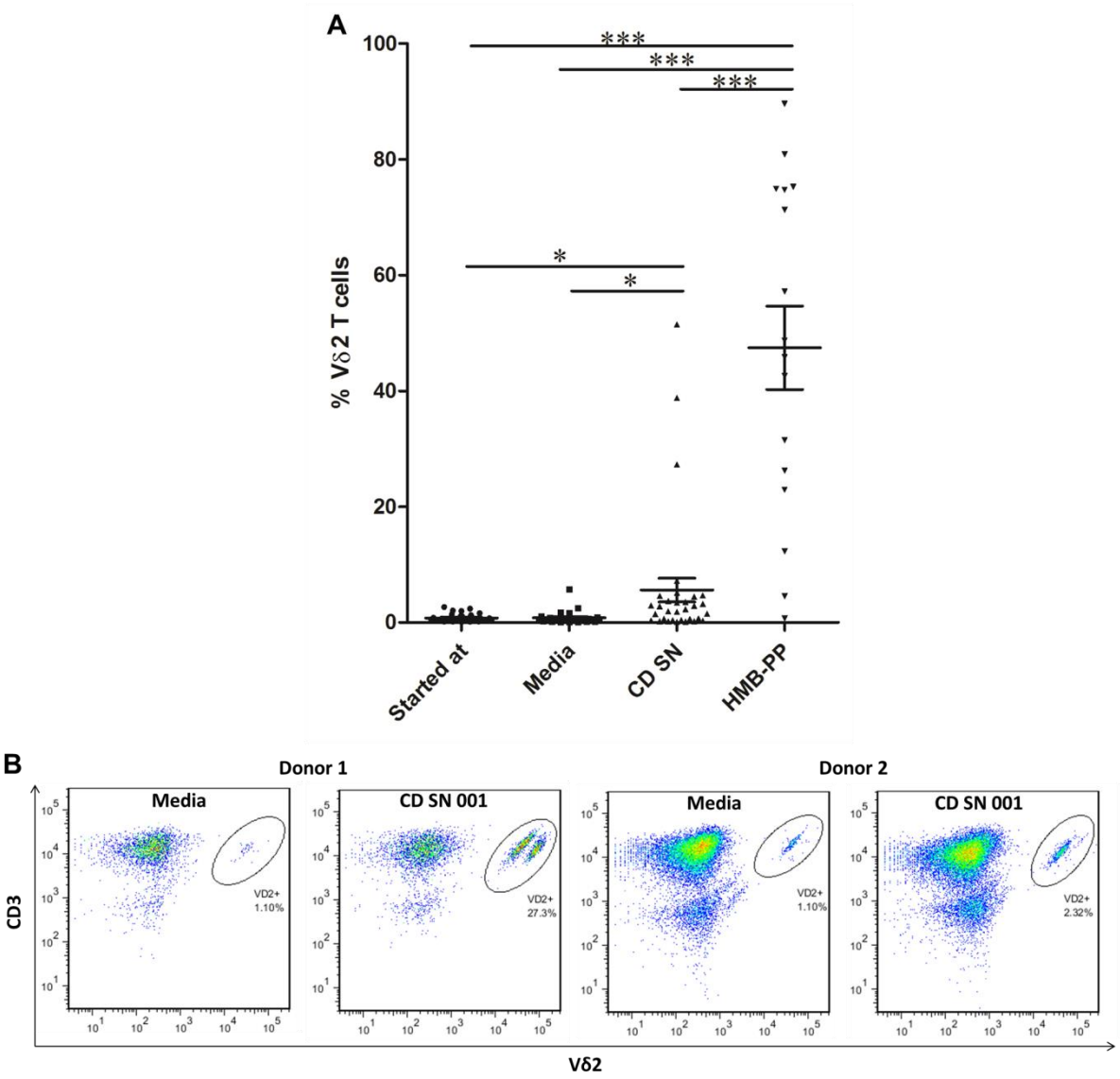

Figure 6.11 *Clostridium difficile* induced Vδ2 T cell expansion in a large proportion of donors tested. All PBMC donors which were stimulated with *C. difficile* supernatant strain 001 (denoted CD SN), BHIS media (denoted media) or HMB-PP and cultured for 2 weeks in IL-2-supplemented RPMI and examined for CD3 and Vδ2 expression by flow cytometry were pooled together. **A**, scatterplot shows mean (±SEM) Vδ2 percentage following 2 weeks of culture with media (n=27), CD SN (n=32) or HMB-PP (n=16) compared to the starting percentage (n=32). **B**, representative flow cytometric dot plots depicting Vδ2 percentage following stimulation with media or *C. difficile* supernatant for two different donors.

6.5. Discussion

C. difficile infection is a major cause of mortality from healthcare-associated infections in economically-developed countries. Expression of MEP pathway genes by *C. difficile* indicates that *C. difficile* utilises the MEP pathway of isoprenoid biosynthesis. This would suggest its ability to produce HMB-PP, an intermediate of the MEP pathway and as a result could potentially activate Vγ9Vδ2 T cells, which are the only cells to recognise this antigen.

To date, no studies have previously investigated the ability of *C. difficile* to activate Vγ9Vδ2 T cells. We found that an unidentified factor secreted by *C. difficile* strain RT 001 was able to induce Vδ2 T cell expansion from PBMC, while the media did not. However, the effect was not as striking as that observed with HMB-PP-stimulated PBMC. We speculated there may only be a low concentration of stimulus in the *C. difficile* supernatant. However, when a higher concentration of *C. difficile* supernatant was used, the *C. difficile* media BHIS appeared to inhibit Vδ2 T cell expansion. Furthermore, examination of the ability of another two *C. difficile* strains RT 027 and ATCC in Vδ2 T cell activation revealed no difference in the stimulating capacities between the different strains. The *C. difficile* supernatants were devoid of toxins, as they were filtered using a 3 kDa filter, which excluded toxins and SLP. Thus, the stimulating agent in the *C. difficile* supernatant would have to be a small molecule. We further examined the role of *C. difficile* in cytokine secretion by Vδ2 T cells following *C. difficile* supernatant stimulation and compared these to HMB-PP-stimulated cells. We found that *C. difficile* induced the same cytokines as HMB-PP, the proinflammatory T_H1 cytokines IFN-γ and TNF-α, while neither induced T_H2 or T_H17 cytokines.

Interestingly, we found that Vδ2 T cells were only expanded by *C. difficile* when used at 10% of culture volume. This preparation did not have inhibitory effects on Vδ2 T cells found within fresh PBMC but in enriched PBMC containing a high proportion of Vδ2 T cells, *C. difficile* induced cell death upon culture. Furthermore, this was only caused by *C. difficile* supernatant and was not observed when Vδ2 T cells were cultured with the BHIS media used for generating *C. difficile.* It would appear that a non-γδ population within the PBMC prevents the toxic effects of *C. difficile*

supernatant on Vδ2 T cells, perhaps by taking up the toxic agent. In order to test this hypothesis, individual cell populations were isolated from the same donor and cultured in various combinations to determine whether the presence of monocytes, B cells, αβ T cells or all 3 populations prevented the cell death induced by the inhibitory factor found in *C. difficile* supernatant. It was found that, individually, none of the cell populations were able to confer protection against *C. difficile*-induced toxicity, while the presence of all 3 populations, which simulates the conditions found in a PBMC sample, reduced the cell death and conserved the Vδ2 T cell percentages, which is in line with the lack of toxicity of *C. difficile* supernatant towards PBMC. Of note, the Vδ2 T cells used were not highly pure, and contained ~70% Vδ2 T cells, a requirement for Vδ2 T cell expansion presumably because they contain monocytes which are required to induce their proliferation (Tyler, et al., 2015).

Lastly, a fresh batch of *C. difficile* supernatant strain RT 001 was isolated with the aim of examining the cytokine profiles of *C. difficile*-expanded Vδ2 T cells, as opposed to those of HMB-PP-expanded cells which were examined earlier. First, we confirmed that these cells were able to induce Vδ2 T cell proliferation, and found that like HMB-PP they stimulated the expansion of both CD4 and CD8 subsets. Moreover, they induced production of the same cytokines by Vδ2 T cells as HMB-PP upon restimulation.

Thus, these results indicate that the stimulating agent produced by *C. difficile* has the same function as HMB-PP in Vδ2 T cell activation, and that this is significantly distinct to the effects of aminobisphosphonate stimulation, which fails to induce T_H1 cytokines by Vδ2 T cells (Fig. 3.22). Our group is currently in collaboration with collaborators in Maynooth University to identify using liquid chromatography/mass spectrometry, whether *C. difficile* supernatant contains HMB-PP or whether it contains a distinct Vδ2 T cell-stimulating agent, such as a pAg.

A confounding factor in this study was the great donor variability encountered upon stimulation with HMB-PP and *C. difficile*. Using a standardised cohort of healthy donors may help overcome this issue, as there was no information on age, gender or medical history on the donors used in this thesis.

The overall expansion of Vδ2 T cells seen with *C. difficile* supernatant may be due to a low concentration of pAg, although adding a higher concentration of *C. difficile* supernatant inhibited cell expansion due to an inhibitory factor found in the BHIS media, and thus we were not able to test this hypothesis. Along with the same collaborators, we hope to be able to purify separate fractions of *C. difficile* supernatant using a specialised column which could bind and ultimately elute HMB-PP or other compounds, and thus by using a large starting volume of *C. difficile* supernatant, we could isolate a high concentration of the stimulating agent and then test its bioactivity.

Overall, these results, which are summarised in Fig. 6.12, demonstrate a role for *C. difficile* in inducing an immune response through stimulation of Vδ2T cells. The evidence suggests the presence of a secreted factor of less than 3 kDa which has the same effects on Vδ2 T cells as the potent pAg HMB-PP, although the identity of this agent remains to be elucidated.

It is unclear how or why the MEP pathway evolved in several bacterial species in preference to the ancestral mevalonate pathway (Heuston, et al., 2012). Currently it is unknown whether the MEP pathway would be beneficial to the bacteria residing in the host. Evidence to support the role for this pathway during intracellular growth and survival of bacterial pathogens is provided by several studies (Begley, et al., 2008, Eskra, et al., 2001, Lai, et al., 2001, Shin, et al., 2006). Another possible explanation for favouring the MEP pathway is the energy efficiency of this pathway which synthesises a 5:1 ratio of IPP to its isomer, DMAPP. In contrast, this ratio is 3:7 in the mevalonate pathway (Ershov Iu, 2007). Furthermore, a study by Gill and colleagues found that MEP pathway genes were also abundant in the human indigenous microflora (Gill, et al., 2006). Nevertheless, it is advantageous for the host to have the ability to recognise these pathogens through Vγ9Vδ2 T cells, owing to the pAg production by these bacteria.

Future studies will examine the Vδ2 T cell numbers in patients with *C. difficile*. In addition, *C. difficile* extracts that do not contain BHIS, should be prepared and examined for the bioactivity.

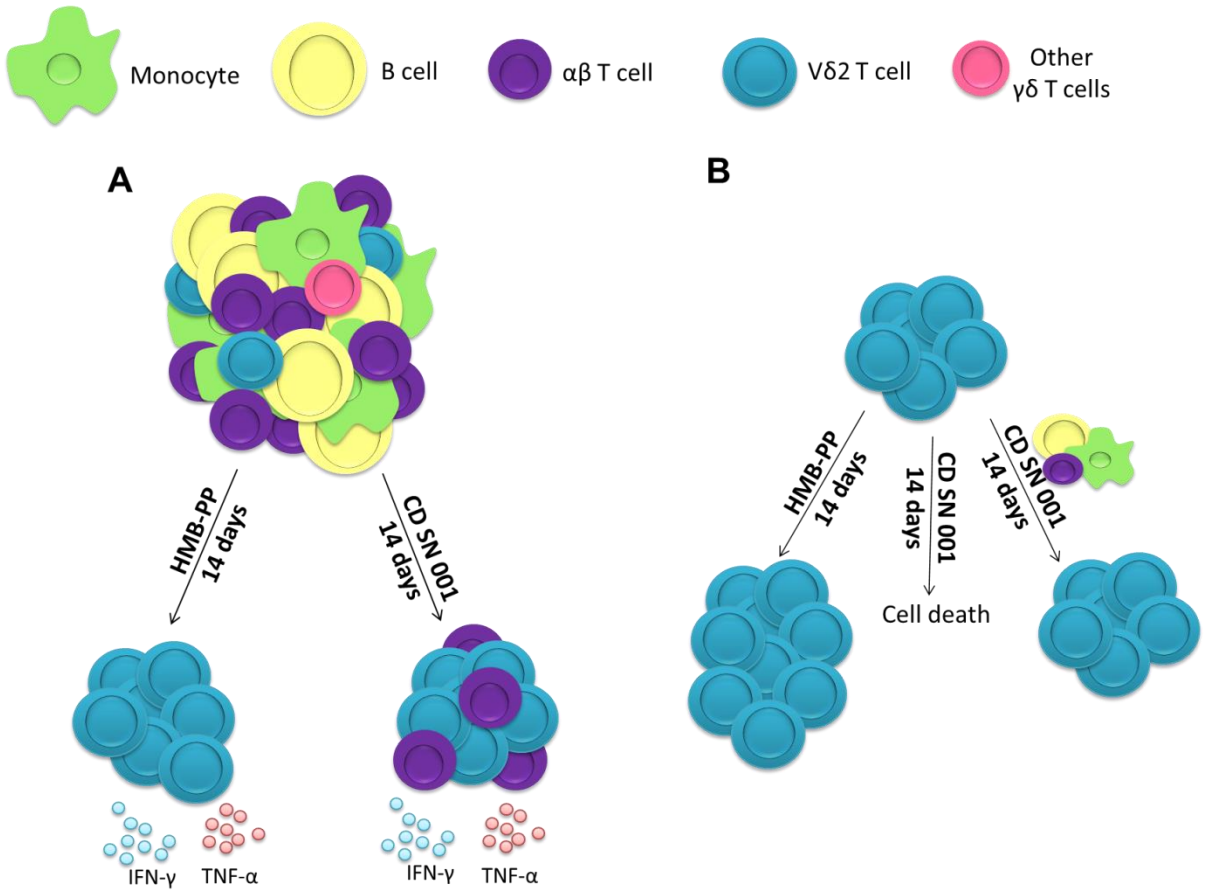

Figure 6.12. *Clostridium difficile* induces Vδ2 T cell expansion and cytokine production by Vδ2 T cells. A, *Clostridium difficile* supernatant (denoted CD SN 001) induces Vδ2 expansion from PBMC after 14 days. The resulting Vδ2 T cells exhibit the same cytokine profile as HMB-PP-expanded Vδ2 T cells. **B,** CD SN 001 is toxic to fresh γδ but not to PBMC or expanded Vδ2 T cells. The addition of αβ T cells, monocytes and B cells is protective against the toxic effect.

Chapter 7

Discussion

7.1 Discussion

Vγ9Vδ2 T cells show promise as candidates for cell-based immunotherapeutic modulation for cancer. They are frequently numerically and functionally impaired in cancer patients and can recognise tumour cells using cell-surface receptors. In addition, they can directly kill tumour cells and activate other immune effector cells directly and via the release of cytokines (Caccamo, et al., 2008, Dieli, et al., 2008). Two therapeutic strategies involving Vγ9Vδ2 T cells are under investigation in clinical trials in cancer patients. The first involves *in vivo* stimulation of Vγ9Vδ2 T cells using clinically approved aminobisphosphonates or BrHPP (Dieli, et al., 2007, Wilhelm, et al., 2003), which activate Vγ9Vδ2 T cells *in vivo,* inducing their expansion and effector functions. The second therapeutic approach involves *ex vivo* expansion of patients' Vγ9Vδ2 T cells and their adoptive transfer back into the patients (Bennouna, et al., 2008). Vδ1 T cells may also play important roles in fighting tumour cells (Siegers and Lamb, 2014) owing to their ability to recognise lipids presented by CD1 molecules and stress-induced self-ligands, and recent findings suggest that Vδ3 T cells share functions with Vδ1 T cells and thus may play similar roles. Thus, Vδ1 and Vδ3 T cells also have potential as therapeutic agents.

In the present study we characterised γδ T cell subsets frequencies, their phenotypes and functions and looked at methods of expanding γδ T cells to investigate their functional properties and to exploit their potential as potential immunotherapeutic agents. We confirmed that Vδ2 T cells comprised the majority of γδ T cells in peripheral blood, while Vδ1 and Vδ3 T cells, which are usually found in greater proportions in the peripheral tissues, were much less abundant (Halary, et al., 2005, Kalyan and Kabelitz, 2013). The human Vδ2 chain is known to specifically bind to Vγ9, and thus we confirmed that Vδ2 T cells were all Vγ9 positive (Dunne, et al., 2010), which allowed us to identify Vγ9Vδ2 T cells using solely the Vδ2 chain. In contrast, the Vδ1 and Vδ3 TCR chains did not preferentially bind to the Vγ9 chain, as also reported by Siegers and Lamb (2014).

We observed that, as previously described, both freshly isolated and expanded γδ T cells were mainly DN for CD4 and CD8 expression, which is thought to reflect the lack

of MHC requirement for γδ T cell activation (Hayday, 2000, Kabelitz, et al., 2000). However, while there were some $CD8^+$ T cells and virtually no $CD4^+$ T cells in magnetically enriched and expanded cells, these were found at higher frequencies in freshly isolated cells, thus suggesting that $CD4^+$ γδ T cells are not as likely to be selected by the magnetic particles. Furthermore, when we expanded Vδ2 T cells directly from PBMC using HMB-PP, we also found low numbers of $CD4^+$ T cells. In contrast, zoledronate appeared to promote expansion of higher numbers of $V\delta2^+$ CD4 T cells. Other studies have described methods that selectively expand particular CD4 and CD8 subsets of innate T cells (O'Reilly, et al., 2011, Zeng, et al., 2013, Dunne, et al., 2010b).

$V\delta1^+$, $V\delta2^+$ and $V\delta3^+$ γδ T cells showed predominantly naïve and central memory phenotypes, while upon expansion, these exhibited predominantly effector memory status (Dieli, et al., 2003, Dunne, et al., 2010, Caccamo, et al., 2005). They expressed TLR2 (Pietschmann, et al., 2009, Wesch, et al., 2006, Wesch, et al., 2011), NK and activation markers NKG2D, CD56, CD69 and CD161 (Zheng, et al., 2013, Biassoni, et al., 2001, Dunne, et al., 2010) thus allowing them to directly recognise PRR and tumour cells.

The potent pAg HMB-PP was used to expand Vδ2 T cells to generate cell lines for functional studies. We found high inter-individual variability in the proliferative responses of Vδ2 T cells to HMB-PP. However, we found that by carrying out a partial enrichment of γδ T cells, we obtained highly pure Vδ2 T cell lines. The reason for a partial rather than a complete enrichment was the requirement for monocytes in Vδ2 T cell activation (Eberl and Moser, 2009), which are required to present pAg to Vδ2 T cells. In contrast, we found the aminobisphosphonate zoledronate to consistently yield highly pure populations of Vδ2 T cells. These data show that HMB-PP-expanded Vδ2 T cells have the same functional properties as fresh Vδ2 T cells, while zoledronate-expanded Vδ2 T cells do not rapidly produce T_H1 cytokines upon restimulation but this can be restored using HMB-PP in conjunction with zoledronate. Therefore, we demonstrate a method of expanding Vγ9Vδ2 T cells *ex vivo* using HMB-PP or zoledronate which could then be adoptively transferred back into patients.

A major limitation with Vδ3 T cells is their scarcity in peripheral blood and thus the need to expand them to obtain satisfactory cell numbers for their study. Another challenge is the lack of a known ligand specific for Vδ3 T cells. We optimised a method for generating highly pure lines of Vδ3 T cells. In this method, $V\delta3^+$ T cells were isolated from PBMC by flow cytometric cell sorting and stimulated with the T cell mitogen PHA in the presence of irradiated feeder cells and expanded using IL-2. Using this method, high numbers (up to 1,000 fold increase from ~1,000 cells) of Vδ3 T cell populations were obtained within 3-4 weeks. .

Upon activation, γδ T cells are known to rapidly produce high levels of T_H cytokines which serve to activate cells of the innate and adaptive immune systems (Eberl, et al., 2009). Vδ2 T cells mainly secrete IFN-γ and TNF-α, while non-Vδ2 T cells can also produce T_H2 and T_H17 cytokines (Dechanet, et al., 1999, Fenoglio, et al., 2009, Maher, et al., 2015). T_H2 and T_H17 cytokines can also be produced by Vδ2 T cells under certain conditions (Spinozzi, et al., 1995, Wesch, et al., 2001, Ness-Schwickerath, et al., 2010, Caccamo, et al., 2011). We confirmed these findings and also found that some Vδ2 T cells secrete high amounts of IL-4 upon stimulation with HMB-PP, which may be important in B cell activation. In contrast, zoledronate stimulation abrogated the ability of Vδ2 T cells to produce T_H1 cytokines. Thus the difference in cytokine phenotypes induced reflects the distinct mode of activation between HMB-PP and zoledronate. Recently, Vδ3 T cells have been shown to produce T_H1, T_H2 and T_H17 cytokines upon activation (Mangan, et al., 2013). In the present investigation, we found that Vδ3 T cells were not able to produce IL-17 with PMA and ionomycin stimulation, but required the addition B cells alongside PMA and ionomycin.

Immunotherapeutic approaches involving γδ T cells have shown improved clinical outcomes in various cancers (Fournie, et al., 2013, Kobayashi, et al., 2011). Meraviglia and colleagues found disease stabilisation or partial remission in 3 patients with metastatic breast cancer which were correlated to sustained peripheral blood Vγ9Vδ2 T cell numbers (Meraviglia, et al., 2010). Dokouhaki and his colleagues found that lung cancer progression was remarkably inhibited in a xenograft model of human non-small lung cancer (Dokouhaki, et al., 2010). However, there are also limitations. Repeated pAg stimulation has been shown to yield a progressively weaker effect due to anergy

and exhaustion of Vγ9Vδ2 T cells. As Vγ9Vδ2 T cells are prone to activation-induced cell death, their effects are often transient (Gan, et al., 2001). Therefore the current therapies provide short-term responses for long term chronic diseases. Alternative strategies such as DC immunotherapies that use Vγ9Vδ2 T cells as adjuvants could be utilised to promote desired immune responses.

This prompted us to further examine interactions between Vδ2 T cells, DC and B cells. We found that they induced co-stimulatory marker expression by both DC and B cells, and while they promoted T_H1 responses by DC, they promoted differentiation of B cells into T_H2-promoting antibody-secreting plasma cells. Our findings confirm the ability of Vδ2 T cells to mature DC into APC (Dunne, et al., 2010, Conti, et al., 2005, Devilder, et al., 2006, Ismaili, et al., 2002, Martino, et al., 2005, Shrestha, et al., 2005). In addition we endorse the findings by other groups which also demonstrated the ability of Vδ2 T cells to induce differentiation of B cells into antibody-producing cells (Brandes, et al., 2003, Caccamo, et al., 2006, Bansal, et al., 2012, Caccamo, et al., 2012). We found that while co-stimulatory markers, T_H1 cytokines and cell contact were important in DC activation by Vδ2 T cells, they did not play a major role in B cell maturation. In comparison, others have reported that CD40L, ICOS, IL-4 and IL-10 were important for Vδ2 T cell-mediated antibody production by tonsillar B cells after 10 days (Caccamo, et al., 2006). However, those studies did not assess the role of those molecules in B cell maturation into APC. We found CD40L and cell contact to be important in B cell maturation into APC.

We also observed that DC can reciprocally activate Vδ2 T cells to express co-stimulatory markers and produce increased levels of IFN-γ, while B cells did not activate Vδ2 T cells. An increased frequency of Vδ2 T cells was found to result in increased cytokine production by DC, thus highlighting the importance of using *in vivo* expansion methods to increase numbers of circulating Vδ2 T cells to activate DC. In contrast, increasing the number of DC that come in contact with Vδ2 T cells, did not enhance the effector capabilities of Vδ2 T cells. While these studies were carried out under normal conditions of homeostasis, we also examined the interactions between Vδ2 T cells with DC or B cells under conditions of inflammation, by adding LPS to the co-cultures. We found that LPS stimulation induced the expression of costimulatory

markers by DC and B cells and induced the secretion of T_H1 and T_{reg} cytokines by DC and IL-12, IgG and IgA by B cells. Moreover, LPS and Vδ2 T cells had a synergistic effect on IL-12 production by DC, which might be explained by the ability of Vδ2 T cells to recognise TLR4 ligands (Devilder, et al., 2009). IFN-γ has previously been shown be important for IL-12 production by LPS-stimulated DC. Thus, DC and Vδ2 T cells reciprocally stimulate IFN-γ release, and under conditions of inflammation IFN-γ secretion by Vδ2 T cells stimulates high levels of IL-12 by DC.

Another important finding was the ability of Vδ2-matured DC to induce proliferation and T_H1 cytokines by resting alloreactive αβ T cells. Although we examined whether these mature DC could also present specific antigen to T cells, we found that the presence of tetanus toxoid or tuberculin did not augment αβ T cell activation compared to DC in the absence of added antigens, which may have been due to an insufficient number of antigen-reactive T cells in the cell population. B cells are known to be capable of presenting antigens to T cells (Rodriguez-Pinto, 2005). We saw that Vδ2-matured B cells also induced proliferation of resting T cells, but were not as efficient as Vδ2-matured DC in doing so. These findings suggest that Vδ2 T cells have the ability to control different arms of the immune system through this selective activation of distinct APC functions and thus could be used as adjuvants in cellular therapy to drive desired immune responses.

As Vδ2 T cells have proven to play a central role in orchestrating immune responses, we therefore wanted to investigate the roles of the less common Vδ3 T cell subset. A study from our group recently demonstrated the ability of Vδ3 T cells to induce maturation of DC into cytokine-secreting APC (Mangan, et al., 2013). Our study is the first to explore the reciprocal relationship between Vδ3 T cells and B cells. We found that like Vδ2 T cells, Vδ3 T cells induced an increase in co-stimulatory molecule expression by B cells, but did not induce cytokine secretion, nor antibody production, except for a possible increase in IgM. In turn, B cells induced co-stimulatory marker expression by Vδ3 T cells. Hence, while Vδ2 T cells activate B cells, B cells do not reciprocally activate Vδ2 T cells, whereas B cells reciprocally activate Vδ3 T cells. In addition, PMA and ionomycin-activated B cells induced IL-17 production by Vδ3 T cells, which were unable to produce IL-17 without B cell help, which is in contrast to the

findings by Mangan et al (2013), which found that Vδ3 T cell produced IL-17 upon PMA and ionomycin stimulation in the absence of B cells.

Several innate T cell subsets have gained focus due to their ability to recognise foreign and self-antigens in an MHC-independent manner. Some of these recognise antigens within the MHC-I-like CD1 molecules which are usually found on APC and include CD1a, CD1b, CD1c and CD1d (Russano, et al., 2007, Bai, et al., 2012, Spada, et al., 2000, de Jong, et al., 2010, Exley, et al., 2002, Uldrich, et al., 2011, de Lalla, et al., 2011). CD1 molecules were first described for γδ T cells, but are mainly studied in the context of iNKT cells which recognise glycolipid antigens presented by CD1d molecules (Bendelac, et al., 2007, Berzins, et al., 2011). Vδ1 T cells have also been reported to respond to CD1c and CD1d molecules presenting lipids and sulfatide (Russano, et al., 2007, Bai, et al., 2012, Spada, et al., 2000), and recently it was found that a proportion of Vδ3 T cells can recognise CD1d molecules independently of lipids. This prompted us to investigate whether certain lipids could enhance the reactivity of Vδ3 T cells to CD1 molecules. We investigated if Vδ3 T cells within freshly-isolated, γδ T cell-enriched PBMC and Vδ3 T cell lines could degranulate in response to HeLa cells transfected with CD1a, CD1b, CD1c or CD1d as APC. In contrast to a report by Mangan, et al., (2013), we found that our Vδ3 T cells lacked reactivity against each of the CD1 molecules examined. We then decided to test if Vδ3 T cells could respond to cardiolipin, sulfatide or ganglioside but found that they showed no reactivity against CD1a, CD1b, CD1c or CD1d, as they failed to degranulate and produce cytokines in response to these glyocolipids. It is possible that our failure to detect CD1d-restricted Vδ3 T cells is because they make up very low proportions of Vδ3T cells, as seen for CD1d-restricted Vδ1 T cells (Bai et al., 2012), or that they failed to expand significantly during the generation of Vδ3 T cell lines. Future work using antigen-loaded CD1 tetramers may ultimately confirm or refute the previous observation of CD1d restriction of Vδ3 T cells.

Isoprenoid biosynthesis is vital for cell survival and thus is employed by most cells including many bacterial species, parasites and eukaryotic cells. Eukaryotic cells and some bacterial species such as *Staphylococcus* and *Streptomyces* use the mevalonate pathway which results in IPP production (Heuston, et al., 2012). Cells with dysregulated metabolism, such as in tumour or microbe-infected cells can

overproduce IPP due to overexpression of rate-limiting enzymes in the pathway leading to IPP production. This can alert Vδ2 T cells, stimulating their proliferation. Thus IPP serves as a self-ligand for Vδ2 T cells to recognise cellular dysregulation due to altered metabolism. In contrast, most bacteria appear to utilise the MEP pathway of isoprenoid synthesis, which also results in the production of IPP but also the potent pAg HMB-PP. Evidence in support of this comes from genetic studies which revealed that these bacteria, including *C. difficile* express genes involved in the MEP pathway. Therefore, Vδ2 T cells can detect and respond to these bacterial-derived metabolites resulting in the initiation of immune responses against these pathogens. Vδ2 T cells can readily expand *in vivo* and *in vitro* in the presence of *Mycobacterium* and *Plasmodium* species (Kabelitz, et al., 2000, Chen, 2013, Behr, et al., 1996, Kabelitz, et al., 1990). Thus far, the ability of *C. difficile* to activate Vδ2 T cells has not been established. Therefore, we looked at *C. difficile* as a potential source of HMB-PP. *C. difficile* extract in the form of supernatant or lysate, from 3 distinct strains, were filtered to remove toxins and examined for their Vδ2-stimulating properties. As expected, *C. difficile,* from all strains and both sources was able to induce proliferation of Vδ2 T cells compared to controls. Furthermore, *C. difficile* induced expansion of both CD4 and CD8 subsets of Vδ2 T cells and the production of the same cytokines by Vδ2 T cells as were observed with HMB-PP stimulation. However, we found the medium used to culture *C. difficile* to be toxic towards human PBMC at high doses. This toxic effect was eliminated by reducing the amounts of *C. difficile*-containing medium. However, when monocytes, B cells and conventional T cells were partially removed from the cultures, the *C. difficile* extract appeared to induce cell death and this was prevented by the addition of monocytes, B cells and conventional T cells. Overall we found vast inter-donor variability in expansion using HMB-PP and *C. difficile*, but *C. difficile* and HMB-PP appeared to induce the same functions by Vδ2 T cells. We speculate that a factor of less than 3 kDa secreted by *C. difficile* is responsible for inducing expansion of Vδ2 T cells, but is either less potent, or is present at lower concentration than 10 nM HMB-PP. Future plans include optimising methods to reveal the identity of this stimulating factor by using LC-MS in collaboration with collaborators in the Biology Department at Maynooth University. Another aim is to design a column that could be used to isolate individual components of the *C. difficile*

extracts and investigate the bioactivity of these alongside HMB-PP. Finally, we wish to phenotype Vδ2 T cells in *C. difficile* patients to determine whether they show altered Vδ2 T cell frequencies compared to healthy individuals.

These studies highlight the role of γδ T cells in immunosurveillance, regulation of immune responses and acting as a bridge between the adaptive and innate immune systems. While they only represent a minority of circulating T cells, they have been proven to rapidly proliferate during microbial infections and accumulate around invasive pathogens and therefore they appear to partake in tissue homeostasis, anti-tumour and antimicrobial immunity due to their ability to recognise self-ligands and bacterial-derived metabolites and respond by directly killing target cells or by driving recruitment or activation of neighbouring cells (Zheng, et al., 2013, Moser and Eberl, 2007). Although the mechanism involved in differentiating between self and foreign ligands are not well understood, IPP levels produced by host cells are normally insufficient to trigger immune responses and thus avoid inducing autoimmune responses. Vδ2 T cells can only be activated by elevated IPP levels of micromolar concentrations which are found in tumour cells and infected cells. However, it appears that the role of Vγ9Vδ2 T cells in antimicrobial infection is of superior physiological importance than that of homeostasis, as they show an increased sensitivity to HMB-PP which is detected at nanomolar concentrations (Moser and Eberl, 2007). Thus, in the future, γδ T cells may be applied to treat bacterial infections, and some groups have already investigated the anti-bacterial properties of Vγ9Vδ2 T cells (Bonneville and Scotet, 2006, Huang, et al., 2009). Studies are needed to further address the distribution and function of γδ T cells following infusion into patients. Moreover, since pAg-responsive γδ T cells are unique to humans and primates, humanised mouse models are needed to further study their functions *in vivo*, and some are already under investigation (Santolaria, et al., 2013, Roth and Harui, 2015, Tu, et al., 2011, Tu, et al., 2014).

It is apparent that γδ T cells have evolved to recognise a unique set of antigens which are not recognised by conventional T cells, and thus, γδ T cells and other innate-like T cells have gained focus as major regulatory effector cells that orchestrate adaptive and innate immune responses.

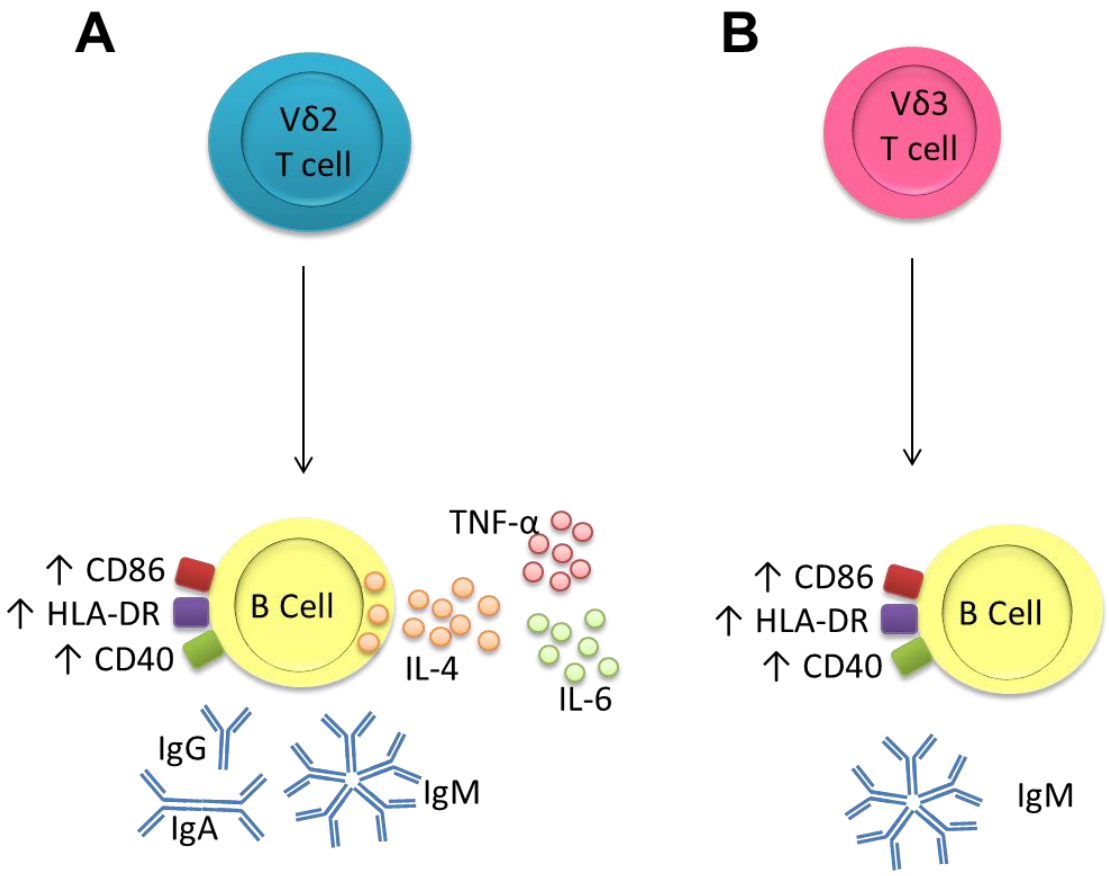

Figure 7.1 Vδ2 and Vδ3 T cells play distinct roles in B cell activation. A, Vδ2 T cells induce maturation of B cells into antibody secreting and cytokine producing cells. **B**, Vδ3 T cells induce maturation and IgM production by B cells.

7.2 Future directions

The results obtained in this thesis highlight potential for further research. In chapter 3 we analysed the expression of various markers by γδ T cell subsets. Although similar results have been reported by other groups with regards to Vδ1 and Vδ2 T cells, Vδ3 T cells and their functions are still poorly characterised, and should be examined in further detail. More importantly, there is a need for identification of a ligand specific for Vδ3 T cells.

In chapter 4, we demonstrated the ability of Vδ2 T cells to orchestrate different arms of the immune system through interaction with DC and B cells. Various factors, such as IFN-γ, IL-4, TNF-α, CD86, CD40 and cell contact, were investigated for their importance in B cell activation by Vδ2 T cells, but none of these proved to play a role. Therefore, future work should further examine the molecular mechanisms involved in the activation of B cells by Vδ2 T cells.

We also explored the interaction between Vδ3 T cells and B cells (chapter 5), and found that Vδ3 T cells induce maturation of B cells with distinct functions from those induced by Vδ2 T cells. The mechanisms involved in this activation, however, are yet to be examined. Furthermore, since we failed to observe CD1 restriction in our Vδ3 T cells lines using a cytotoxicity assay, we propose the use of distinct methods such as using antigen-loaded CD1 tetramers to further investigate the ability of Vδ3 T cells to recognise CD1 molecules. Lipids and other antigens should then be investigated for their potential to activate Vδ3 T cells.

In the final chapter, we showed that a factor secreted by *Clostridium difficile* is capable of inducing proliferation and cytokine production by Vδ2 T cells in a manner similar to that of phosphoantigens, but that factor has yet to be identified. The use of liquid chromatography/mass spectrometry or other similar techniques could reveal whether a phosphoantigen is contained within the *C. difficile* supernatant. A chemical signature for HMB-PP could be obtained and compared to that of factors secreted by *C. difficile*. Moreover, *C. difficile* patients should be phenotyped to determine whether Vδ2 T cell numbers or functions are affected by *C. difficile* infection.

8.0 References

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

Adams, E.J., Gu, S., Luoma, A.M. 2015. Human gamma delta T cells: Evolution and ligand recognition. Cell Immunol 296, 31.

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

Agrati, C., Cimini, E., Sacchi, A., Bordoni, V., Gioia, C., Casetti, R., Turchi, F., Tripodi, M., Martini, F. 2009. Activated V gamma 9V delta 2 T cells trigger granulocyte functions via MCP-2 release. J Immunol 182, 522.

Alexander, A.A., Maniar, A., Cummings, J.S., Hebbeler, A.M., Schulze, D.H., Gastman, B.R., Pauza, C.D., Strome, S.E., Chapoval, A.I. 2008. Isopentenyl pyrophosphate-activated CD56+ {gamma}{delta} T lymphocytes display potent antitumor activity toward human squamous cell carcinoma. Clin Cancer Res 14, 4232.

Altincicek, B., Moll, J., Campos, N., Foerster, G., Beck, E., Hoeffler, J.F., Grosdemange-Billiard, C., Rodriguez-Concepcion, M., Rohmer, M., Boronat, A., Eberl, M., Jomaa, H. 2001. Cutting edge: human gamma delta T cells are activated by intermediates of the 2-C-methyl-D-erythritol 4-phosphate pathway of isoprenoid biosynthesis. J Immunol 166, 3655.

Angelini, D.F., Zambello, R., Galandrini, R., Diamantini, A., Placido, R., Micucci, F., Poccia, F., Semenzato, G., Borsellino, G., Santoni, A., Battistini, L. 2011. NKG2A inhibits NKG2C effector functions of gammadelta T cells: implications in health and disease. J Leukoc Biol 89, 75.

Anguille, S., Smits, E.L., Bryant, C., Van Acker, H.H., Goossens, H., Lion, E., Fromm, P.D., Van Tendeloo, V.F., Berneman, Z.N. 2015. Dendritic Cells as Pharmacological Tools for Cancer Immunotherapy. Pharmacol Rev 67, 731.

Aoyagi, M., Shimojo, N., Sekine, K., Nishimuta, T., Kohno, Y. 2003. Respiratory syncytial virus infection suppresses IFN-gamma production of gammadelta T cells. Clin Exp Immunol 131, 312.

Appleman, L.J., Boussiotis, V.A. 2003. T cell anergy and costimulation. Immunol Rev 192, 161.

Arnett, H.A., Viney, J.L. 2014. Immune modulation by butyrophilins. Nat Rev Immunol 14, 559.

Arrenberg, P., Halder, R., Dai, Y., Maricic, I., Kumar, V. 2010. Oligoclonality and innate-like features in the TCR repertoire of type II NKT cells reactive to a beta-linked self-glycolipid. Proc Natl Acad Sci U S A 107, 10984.

Arrenberg, P., Maricic, I., Kumar, V. 2011. Sulfatide-mediated activation of type II natural killer T cells prevents hepatic ischemic reperfusion injury in mice. Gastroenterology 140, 646.

Arvand, M., Hauri, A.M., Zaiss, N.H., Witte, W., Bettge-Weller, G. 2009. Clostridium difficile ribotypes 001, 017, and 027 are associated with lethal C. difficile infection in Hesse, Germany. Euro Surveill 14.

Babcock, G.J., Broering, T.J., Hernandez, H.J., Mandell, R.B., Donahue, K., Boatright, N., Stack, A.M., Lowy, I., Graziano, R., Molrine, D., Ambrosino, D.M., Thomas, W.D., Jr. 2006. Human monoclonal antibodies directed against toxins A and B prevent Clostridium difficile-induced mortality in hamsters. Infect Immun 74, 6339.

Bai, L., Picard, D., Anderson, B., Chaudhary, V., Luoma, A., Jabri, B., Adams, E.J., Savage, P.B., Bendelac, A. 2012. The majority of CD1d-sulfatide-specific T cells in human blood use a semiinvariant Vdelta1 TCR. Eur J Immunol 42, 2505.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.J., Pulendran, B., Palucka, K. 2000. Immunobiology of dendritic cells. Annu Rev Immunol 18, 767.

Bansal, R.R., Mackay, C.R., Moser, B., Eberl, M. 2012. IL-21 enhances the potential of human gammadelta T cells to provide B-cell help. Eur J Immunol 42, 110.

Barbut, F., Richard, A., Hamadi, K., Chomette, V., Burghoffer, B., Petit, J.C. 2000. Epidemiology of recurrences or reinfections of Clostridium difficile-associated diarrhea. J Clin Microbiol 38, 2386.

Barnaba, V., Watts, C., de Boer, M., Lane, P., Lanzavecchia, A. 1994. Professional presentation of antigen by activated human T cells. Eur J Immunol 24, 71.

Barnes, P.F., Grisso, C.L., Abrams, J.S., Band, H., Rea, T.H., Modlin, R.L. 1992. Gamma delta T lymphocytes in human tuberculosis. J Infect Dis 165, 506.

Bartkowiak, J., Kulczyck-Wojdala, D., Blonski, J.Z., Robak, T. 2002. Molecular diversity of gammadelta T cells in peripheral blood from patients with B-cell chronic lymphocytic leukaemia. Neoplasma 49, 86.

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

Beetz, S., Wesch, D., Marischen, L., Welte, S., Oberg, H.H., Kabelitz, D. 2008. Innate immune functions of human gammadelta T cells. Immunobiology 213, 173.

Begley, M., Bron, P.A., Heuston, S., Casey, P.G., Englert, N., Wiesner, J., Jomaa, H., Gahan, C.G., Hill, C. 2008. Analysis of the isoprenoid biosynthesis pathways in Listeria monocytogenes reveals a role for the alternative 2-C-methyl-D-erythritol 4-phosphate pathway in murine infection. Infect Immun 76, 5392.

Behar, S.M., Podrebarac, T.A., Roy, C.J., Wang, C.R., Brenner, M.B. 1999. Diverse TCRs recognize murine CD1. J Immunol 162, 161.

Behr, C., Poupot, R., Peyrat, M.A., Poquet, Y., Constant, P., Dubois, P., Bonneville, M., Fournie, J.J. 1996. Plasmodium falciparum stimuli for human gammadelta T cells are related to phosphorylated antigens of mycobacteria. Infect Immun 64, 2892.

Bendelac, A., Matzinger, P., Seder, R.A., Paul, W.E., Schwartz, R.H. 1992. Activation events during thymic selection. J Exp Med 175, 731.

Bendelac, A., Savage, P.B., Teyton, L. 2007. The biology of NKT cells. Annu Rev Immunol 25, 297.

Bennouna, J., Bompas, E., Neidhardt, E.M., Rolland, F., Philip, I., Galea, C., Salot, S., Saiagh, S., Audrain, M., Rimbert, M., Lafaye-de Micheaux, S., Tiollier, J., Negrier, S. 2008. Phase-I study of Innacell gammadelta, an autologous cell-therapy product highly enriched in gamma9delta2 T lymphocytes, in combination with IL-2, in patients with metastatic renal cell carcinoma. Cancer Immunol Immunother 57, 1599.

Bennouna, J., Levy, V., Sicard, H., Senellart, H., Audrain, M., Hiret, S., Rolland, F., Bruzzoni-Giovanelli, H., Rimbert, M., Galea, C., Tiollier, J., Calvo, F. 2010. Phase I study of bromohydrin pyrophosphate (BrHPP, IPH 1101), a Vgamma9Vdelta2 T lymphocyte agonist in patients with solid tumors. Cancer Immunol Immunother 59, 1521.

Benzaid, I., Monkkonen, H., Stresing, V., Bonnelye, E., Green, J., Monkkonen, J., Touraine, J.L., Clezardin, P. 2011. High phosphoantigen levels in bisphosphonate-treated human breast tumors promote Vgamma9Vdelta2 T-cell chemotaxis and cytotoxicity in vivo. Cancer Res 71, 4562.

Berzins, S.P., Smyth, M.J., Baxter, A.G. 2011. Presumed guilty: natural killer T cell defects and human disease. Nat Rev Immunol 11, 131.

Biassoni, R., Cantoni, C., Pende, D., Sivori, S., Parolini, S., Vitale, M., Bottino, C., Moretta, A. 2001. Human natural killer cell receptors and co-receptors. Immunol Rev 181, 203.

Birkinshaw, R.W., Kjer-Nielsen, L., Eckle, S.B., McCluskey, J., Rossjohn, J. 2014. MAITs, MR1 and vitamin B metabolites. Curr Opin Immunol 26, 7.

Blumberg, R.S., Terhorst, C., Bleicher, P., McDermott, F.V., Allan, C.H., Landau, S.B., Trier, J.S., Balk, S.P. 1991. Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. J Immunol 147, 2518.

Bonneville, M., O'Brien, R.L., Born, W.K. 2010. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. Nat Rev Immunol 10, 467.

Bonneville, M., Scotet, E. 2006. Human Vgamma9Vdelta2 T cells: promising new leads for immunotherapy of infections and tumors. Curr Opin Immunol 18, 539.

Braakman, E., van de Winkel, J.G., van Krimpen, B.A., Jansze, M., Bolhuis, R.L. 1992. CD16 on human gamma delta T lymphocytes: expression, function, and specificity for mouse IgG isotypes. Cell Immunol 143, 97.

Brandes, M., Willimann, K., Bioley, G., Levy, N., Eberl, M., Luo, M., Tampe, R., Levy, F., Romero, P., Moser, B. 2009. Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses. Proc Natl Acad Sci U S A 106, 2307.

Brandes, M., Willimann, K., Lang, A.B., Nam, K.H., Jin, C., Brenner, M.B., Morita, C.T., Moser, B. 2003. Flexible migration program regulates gamma delta T-cell involvement in humoral immunity. Blood 102, 3693.

Brandes, M., Willimann, K., Moser, B. 2005. Professional antigen-presentation function by human gammadelta T Cells. Science 309, 264.

Brandt, L.J. 2013. American Journal of Gastroenterology Lecture: Intestinal microbiota and the role of fecal microbiota transplant (FMT) in treatment of C. difficile infection. Am J Gastroenterol 108, 177.

Braza, M.S., Klein, B. 2013. Anti-tumour immunotherapy with Vgamma9Vdelta2 T lymphocytes: from the bench to the bedside. Br J Haematol 160, 123.

Brazier, J.S., Raybould, R., Patel, B., Duckworth, G., Pearson, A., Charlett, A., Duerden, B.I. 2008. Distribution and antimicrobial susceptibility patterns of Clostridium difficile PCR ribotypes in English hospitals, 2007-08. Euro Surveill 13.

Breimer, M.E., Hansson, G.C., Karlsson, K.A., Larson, G., Leffler, H. 2012. Glycosphingolipid composition of epithelial cells isolated along the villus axis of small intestine of a single human individual. Glycobiology 22, 1721.

Brennan, P.J., Brigl, M., Brenner, M.B. 2013. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nat Rev Immunol 13, 101.

Brennan, P.J., Tatituri R.V., Brigl M., Kim E.Y., Tuli A., Sanderson J.P., Gadola S.D., Hsu F.F., Besra G.S., Brenner M.B. Invariant natural killer T cells recognise lipid self antigens induced by microbial danger signals. Nat Immunol 12, 1202.

Brigl, M., Brenner, M.B. 2004. CD1: antigen presentation and T cell function. Annu Rev Immunol 22, 817.

Bucy, R.P., Chen, C.L., Cihak, J., Losch, U., Cooper, M.D. 1988. Avian T cells expressing gamma delta receptors localize in the splenic sinusoids and the intestinal epithelium. J Immunol 141, 2200.

Bukowski, J.F., Morita, C.T., Brenner, M.B. 1999. Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. Immunity 11, 57.

Burk, M.R., Mori, L., De Libero, G. 1995. Human V gamma 9-V delta 2 cells are stimulated in a cross-reactive fashion by a variety of phosphorylated metabolites. Eur J Immunol 25, 2052.

Caccamo, N., Battistini, L., Bonneville, M., Poccia, F., Fournie, J.J., Meraviglia, S., Borsellino, G., Kroczek, R.A., La Mendola, C., Scotet, E., Dieli, F., Salerno, A. 2006a. CXCR5 identifies a subset of Vgamma9Vdelta2 T cells which secrete IL-4 and IL-10 and help B cells for antibody production. J Immunol 177, 5290.

Caccamo, N., Dieli, F., Wesch, D., Jomaa, H., Eberl, M. 2006b. Sex-specific phenotypical and functional differences in peripheral human Vgamma9/Vdelta2 T cells. J Leukoc Biol 79, 663.

Caccamo, N., La Mendola, C., Orlando, V., Meraviglia, S., Todaro, M., Stassi, G., Sireci, G., Fournie, J.J., Dieli, F. 2011. Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. Blood 118, 129.

Caccamo, N., Meraviglia, S., Ferlazzo, V., Angelini, D., Borsellino, G., Poccia, F., Battistini, L., Dieli, F., Salerno, A. 2005. Differential requirements for antigen or homeostatic cytokines for proliferation and differentiation of human Vgamma9Vdelta2 naive, memory and effector T cell subsets. Eur J Immunol 35, 1764.

Caccamo, N., Meraviglia, S., Scarpa, F., La Mendola, C., Santini, D., Bonanno, C.T., Misiano, G., Dieli, F., Salerno, A. 2008. Aminobisphosphonate-activated gammadelta T cells in immunotherapy of cancer: doubts no more. Expert Opin Biol Ther 8, 875.

Caccamo, N., Todaro, M., La Manna, M.P., Sireci, G., Stassi, G., Dieli, F. 2012. IL-21 regulates the differentiation of a human gammadelta T cell subset equipped with B cell helper activity. PLoS One 7, e41940.

Canchis, P.W., Bhan, A.K., Landau, S.B., Yang, L., Balk, S.P., Blumberg, R.S. 1993. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. Immunology 80, 561.

Cao, X., Sugita, M., Van Der Wel, N., Lai, J., Rogers, R.A., Peters, P.J., Brenner, M.B. 2002. CD1 molecules efficiently present antigen in immature dendritic cells and traffic independently of MHC class II during dendritic cell maturation. J Immunol 169, 4770.

Capietto, A.H., Martinet, L., Fournie, J.J. 2011. Stimulated gammadelta T cells increase the in vivo efficacy of trastuzumab in HER-2+ breast cancer. J Immunol 187, 1031.

Carnaud, C., Lee, D., Donnars, O., Park, S.H., Beavis, A., Koezuka, Y., Bendelac, A. 1999. Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. J Immunol 163, 4647.

Carroll, K.C., Bartlett, J.G. 2011. Biology of Clostridium difficile: implications for epidemiology and diagnosis. Annu Rev Microbiol 65, 501.

Catellani, S., Poggi, A., Bruzzone, A., Dadati, P., Ravetti, J.L., Gobbi, M., Zocchi, M.R. 2007. Expansion of Vdelta1 T lymphocytes producing IL-4 in low-grade non-Hodgkin lymphomas expressing UL-16-binding proteins. Blood 109, 2078.

Cavaillon, J.M. 2001. Pro- versus anti-inflammatory cytokines: myth or reality. Cell Mol Biol (Noisy-le-grand) 47, 695.

Chargui, J., Combaret, V., Scaglione, V., Iacono, I., Peri, V., Valteau-Couanet, D., Dubrel, M., Angevin, E., Puisieux, A., Romagne, F., Bergeron, C. 2010. Bromohydrin pyrophosphate-stimulated Vgamma9delta2 T cells expanded ex vivo from patients with poor-prognosis neuroblastoma lyse autologous primary tumor cells. J Immunother 33, 591.

Chatila, T., Silverman, L., Miller, R., Geha, R. 1989. Mechanisms of T cell activation by the calcium ionophore ionomycin. J Immunol 143, 1283.

Chen, K., Xu, W., Wilson, M., He, B., Miller, N.W., Bengten, E., Edholm, E.S., Santini, P.A., Rath, P., Chiu, A., Cattalini, M., Litzman, J., J, B.B., Huang, B., Meini, A., Riesbeck, K., Cunningham-Rundles, C., Plebani, A., Cerutti, A. 2009. Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. Nat Immunol 10, 889.

Chen, Z.W. 2013. Multifunctional immune responses of HMBPP-specific Vgamma2Vdelta2 T cells in M. tuberculosis and other infections. Cell Mol Immunol 10, 58.

Chen, Z.W., Letvin, N.L. 2003. Adaptive immune response of Vgamma2Vdelta2 T cells: a new paradigm. Trends Immunol 24, 213.

Cheroutre, H. 2005. IELs: enforcing law and order in the court of the intestinal epithelium. Immunol Rev 206, 114.

Chien, Y.H., Meyer, C., Bonneville, M. 2014. gammadelta T cells: first line of defense and beyond. Annu Rev Immunol 32, 121.

Chiplunkar, S., Dhar, S., Wesch, D., Kabelitz, D. 2009. gammadelta T cells in cancer immunotherapy: current status and future prospects. Immunotherapy 1, 663.

Cipriani, B., Borsellino, G., Poccia, F., Placido, R., Tramonti, D., Bach, S., Battistini, L., Brosnan, C.F. 2000. Activation of C-C beta-chemokines in human peripheral blood gammadelta T cells by isopentenyl pyrophosphate and regulation by cytokines. Blood 95, 39.

Collins, C., Wolfe, J., Roessner, K., Shi, C., Sigal, L.H., Budd, R.C. 2005. Lyme arthritis synovial gammadelta T cells instruct dendritic cells via fas ligand. J Immunol 175, 5656.

Conroy, M.J., Mac Nicholas, R., Taylor, M., O'Dea, S., Mulcahy, F., Norris, S., Doherty, D.G. 2015. Increased Frequencies of Circulating IFN-gamma-Producing Vdelta1(+) and Vdelta2(+) gammadelta T Cells in Patients with Asymptomatic Persistent Hepatitis B Virus Infection. Viral Immunol 28, 201.

Constant, P., Davodeau, F., Peyrat, M.A., Poquet, Y., Puzo, G., Bonneville, M., Fournie, J.J. 1994. Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. Science 264, 267.

Conti, L., Casetti, R., Cardone, M., Varano, B., Martino, A., Belardelli, F., Poccia, F., Gessani, S. 2005. Reciprocal activating interaction between dendritic cells and pamidronate-stimulated gammadelta T cells: role of CD86 and inflammatory cytokines. J Immunol 174, 252.

Coquet, J.M., Chakravarti, S., Kyparissoudis, K., McNab, F.W., Pitt, L.A., McKenzie, B.S., Berzins, S.P., Smyth, M.J., Godfrey, D.I. 2008. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. Proc Natl Acad Sci U S A 105, 11287.

Coscas, D., Chowers, M., Levite, M., Amariglio, N., Lang, A., Barshack, I., Bar-Meir, S., Chowers, Y. 2004. Unique TCRDV1-positive lymphocytes that infiltrate colonic neoplasia originate from a selected cell population present in the intestinal mucosa and the peripheral blood. Scand J Immunol 60, 529.

Cosman, D., Mullberg, J., Sutherland, C.L., Chin, W., Armitage, R., Fanslow, W., Kubin, M., 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.

Couzi, L., Levaillant, Y., Jamai, A., Pitard, V., Lassalle, R., Martin, K., Garrigue, I., Hawchar, O., Siberchicot, F., Moore, N., Moreau, J.F., Dechanet-Merville, J., Merville, P. 2010. Cytomegalovirus-induced gammadelta T cells associate with reduced cancer risk after kidney transplantation. J Am Soc Nephrol 21, 181.

Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S., et al. 1994. Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature 367, 455.

Das, H., Wang, L., Kamath, A., Bukowski, J.F. 2001. Vgamma2Vdelta2 T-cell receptor-mediated recognition of aminobisphosphonates. Blood 98, 1616.

D'Asaro, M., La Mendola, C., Di Liberto, D., Orlando, V., Todaro, M., Spina, M., Guggino, G., Meraviglia, S., Caccamo, N., Messina, A., Salerno, A., Di Raimondo, F., Vigneri, P., Stassi, G., Fournie, J.J., Dieli, F. 2010. V gamma 9V delta 2 T lymphocytes efficiently recognize and kill zoledronate-sensitized, imatinib-sensitive, and imatinib-resistant chronic myelogenous leukemia cells. J Immunol 184, 3260.

Davey, M.S., Lin, C.Y., Roberts, G.W., Heuston, S., Brown, A.C., Chess, J.A., Toleman, M.A., Gahan, C.G., Hill, C., Parish, T., Williams, J.D., Davies, S.J., Johnson, D.W., Topley, N., Moser, B., Eberl, M. 2011. Human neutrophil clearance of bacterial

pathogens triggers anti-microbial gammadelta T cell responses in early infection. PLoS Pathog 7, e1002040.

Davey, M.S., Morgan, M.P., Liuzzi, A.R., Tyler, C.J., Khan, M.W., Szakmany, T., Hall, J.E., Moser, B., Eberl, M. 2014. Microbe-specific unconventional T cells induce human neutrophil differentiation into antigen cross-presenting cells. J Immunol 193, 3704.

Davies, D. 2007. Cell Sorting by Flow Cytometry, Humana Press, 257 pp.

de Jong, A., Pena-Cruz, V., Cheng, T.Y., Clark, R.A., Van Rhijn, I., Moody, D.B. 2010. CD1a-autoreactive T cells are a normal component of the human alphabeta T cell repertoire. Nat Immunol 11, 1102.

de Koning, P.J., Tesselaar, K., Bovenschen, N., Colak, S., Quadir, R., Volman, T.J., Kummer, J.A. 2010. The cytotoxic protease granzyme M is expressed by lymphocytes of both the innate and adaptive immune system. Mol Immunol 47, 903.

de Lalla, C., Lepore, M., Piccolo, F.M., Rinaldi, A., Scelfo, A., Garavaglia, C., Mori, L., De Libero, G., Dellabona, P., Casorati, G. 2011. High-frequency and adaptive-like dynamics of human CD1 self-reactive T cells. Eur J Immunol 41, 602.

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

De Santo, C., Salio, M., Masri, S.H., Lee, L.Y., Dong, T., Speak, A.O., Porubsky, S., Booth, S., Veerapen, N., Besra, G.S., Grone, H.J., Platt, F.M., Zambon, M., Cerundolo, V. 2008. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. J Clin Invest 118, 4036.

DeBarros, A., Chaves-Ferreira, M., d'Orey, F., Ribot, J.C., Silva-Santos, B. 2011. CD70-CD27 interactions provide survival and proliferative signals that regulate T cell

receptor-driven activation of human gammadelta peripheral blood lymphocytes. Eur J Immunol 41, 195.

Dechanet, J., Merville, P., Lim, A., Retiere, C., Pitard, V., Lafarge, X., Michelson, S., Meric, C., Hallet, M.M., Kourilsky, P., Potaux, L., Bonneville, M., Moreau, J.F. 1999. Implication of gammadelta T cells in the human immune response to cytomegalovirus. J Clin Invest 103, 1437.

den Haan, J.M., Arens, R., van Zelm, M.C. 2014. The activation of the adaptive immune system: cross-talk between antigen-presenting cells, T cells and B cells. Immunol Lett 162, 103.

Deusch, K., Luling, F., Reich, K., Classen, M., Wagner, H., 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.

Devilder, M.C., Allain, S., Dousset, C., Bonneville, M., Scotet, E. 2009. Early triggering of exclusive IFN-gamma responses of human Vgamma9Vdelta2 T cells by TLR-activated myeloid and plasmacytoid dendritic cells. J Immunol 183, 3625.

Devilder, M.C., Maillet, S., Bouyge-Moreau, I., Donnadieu, E., Bonneville, M., Scotet, E. 2006. Potentiation of antigen-stimulated V gamma 9V delta 2 T cell cytokine production by immature dendritic cells (DC) and reciprocal effect on DC maturation. J Immunol 176, 1386.

Diefenbach, A., Raulet, D.H. 2002. The innate immune response to tumors and its role in the induction of T-cell immunity. Immunol Rev 188, 9.

Dieli, F., Caccamo, N., Meraviglia, S. 2008. Advances in immunotherapy of castration-resistant prostate cancer: bisphosphonates, phosphoantigens and more. Curr Opin Investig Drugs 9, 1089.

Dieli, F., Poccia, F., Lipp, M., Sireci, G., Caccamo, N., Di Sano, C., Salerno, A. 2003. Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. J Exp Med 198, 391.

Dieli, F., Troye-Blomberg, M., Ivanyi, J., Fournie, J.J., Krensky, A.M., Bonneville, M., Peyrat, M.A., Caccamo, N., Sireci, G., Salerno, A. 2001. Granulysin-dependent killing of intracellular and extracellular Mycobacterium tuberculosis by Vgamma9/Vdelta2 T lymphocytes. J Infect Dis 184, 1082.

Dieli, F., Vermijlen, D., Fulfaro, F., Caccamo, N., Meraviglia, S., Cicero, G., Roberts, A., Buccheri, S., D'Asaro, M., Gebbia, N., Salerno, A., Eberl, M., Hayday, A.C. 2007. Targeting human {gamma}delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer Res 67, 7450.

Dieude, M., Striegl, H., Tyznik, A.J., Wang, J., Behar, S.M., Piccirillo, C.A., Levine, J.S., Zajonc, D.M., Rauch, J. 2011. Cardiolipin binds to CD1d and stimulates CD1d-restricted gammadelta T cells in the normal murine repertoire. J Immunol 186, 4771.

Dimova, T., Brouwer, M., Gosselin, F., Tassignon, J., Leo, O., Donner, C., Marchant, A., Vermijlen, D. 2015. Effector Vgamma9Vdelta2 T cells dominate the human fetal gammadelta T-cell repertoire. Proc Natl Acad Sci U S A 112, E556.

Doherty, D.G. 2015. Immunity, tolerance and autoimmunity in the liver: A comprehensive review. J Autoimmun.

Dokouhaki, P., Han, M., Joe, B., Li, M., Johnston, M.R., Tsao, M.S., Zhang, L. 2010. Adoptive immunotherapy of cancer using ex vivo expanded human gammadelta T cells: A new approach. Cancer Lett 297, 126.

Donald, R.G., Flint, M., Kalyan, N., Johnson, E., Witko, S.E., Kotash, C., Zhao, P., Megati, S., Yurgelonis, I., Lee, P.K., Matsuka, Y.V., Severina, E., Deatly, A., Sidhu, M., Jansen, K.U., Minton, N.P., Anderson, A.S. 2013. A novel approach to generate a recombinant toxoid vaccine against Clostridium difficile. Microbiology 159, 1254.

Dougan, S.K., Kaser, A., Blumberg, R.S. 2007. CD1 expression on antigen-presenting cells. Curr Top Microbiol Immunol 314, 113.

Dudal, S., Turriere, C., Bessoles, S., Fontes, P., Sanchez, F., Liautard, J., Liautard, J.P., Lafont, V. 2006. Release of LL-37 by activated human Vgamma9Vdelta2 T cells: a microbicidal weapon against Brucella suis. J Immunol 177, 5533.

Dunne, M.R., Elliott, L., Hussey, S., Mahmud, N., Kelly, J., Doherty, D.G., Feighery, C.F. 2013. Persistent changes in circulating and intestinal gammadelta T cell subsets, invariant natural killer T cells and mucosal-associated invariant T cells in children and adults with coeliac disease. PLoS One 8, e76008.

Dunne, M.R., Madrigal-Estebas, L., Tobin, L.M., Doherty, D.G. 2010a. (E)-4-hydroxy-3-methyl-but-2 enyl pyrophosphate-stimulated Vgamma9Vdelta2 T cells possess T helper type 1-promoting adjuvant activity for human monocyte-derived dendritic cells. Cancer Immunol Immunother 59, 1109.

Dunne, M.R., Mangan, B.A., Madrigal-Estebas, L., Doherty, D.G. 2010b. Preferential Th1 cytokine profile of phosphoantigen-stimulated human Vgamma9Vdelta2 T cells. Mediators Inflamm 2010, 704941.

Dusseaux, M., Martin, E., Serriari, N., Peguillet, I., Premel, V., Louis, D., Milder, M., Le Bourhis, L., Soudais, C., Treiner, E., Lantz, O. 2011. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. Blood 117, 1250.

Eberl, M., Hintz, M., Reichenberg, A., Kollas, A.K., Wiesner, J., Jomaa, H. 2003. Microbial isoprenoid biosynthesis and human gammadelta T cell activation. FEBS Lett 544, 4.

Eberl, M., Moser, B. 2009. Monocytes and gammadelta T cells: close encounters in microbial infection. Trends Immunol 30, 562.

Eberl, M., Roberts, G.W., Meuter, S., Williams, J.D., Topley, N., Moser, B. 2009. A rapid crosstalk of human gammadelta T cells and monocytes drives the acute inflammation in bacterial infections. PLoS Pathog 5, e1000308.

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

Elliott, B., Chang, B.J., Golledge, C.L., Riley, T.V. 2007. Clostridium difficile-associated diarrhoea. Intern Med J 37, 561.

Elliott, J.F., Rock, E.P., Patten, P.A., Davis, M.M., Chien, Y.H. 1988. The adult T-cell receptor delta-chain is diverse and distinct from that of fetal thymocytes. Nature 331, 627.

Ershov Iu, V. 2007. [2-C-Methylerythritol phosphate pathway of isoprenoid biosynthesis as a target in identifying of new antibiotics, herbicides, and immunomodulators (Review)]. Prikl Biokhim Mikrobiol 43, 133.

Eskra, L., Canavessi, A., Carey, M., Splitter, G. 2001. Brucella abortus genes identified following constitutive growth and macrophage infection. Infect Immun 69, 7736.

Exley, M.A., He, Q., Cheng, O., Wang, R.J., Cheney, C.P., Balk, S.P., Koziel, M.J. 2002. Cutting edge: Compartmentalization of Th1-like noninvariant CD1d-reactive T cells in hepatitis C virus-infected liver. J Immunol 168, 1519.

Eynon, E.E., Parker, D.C. 1992. Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. J Exp Med 175, 131.

Fais, F., Tenca, C., Cimino, G., Coletti, V., Zanardi, S., Bagnara, D., Saverino, D., Zarcone, D., De Rossi, G., Ciccone, E., Grossi, C.E. 2005. CD1d expression on B-precursor acute lymphoblastic leukemia subsets with poor prognosis. Leukemia 19, 551.

Fang, H., Welte, T., Zheng, X., Chang, G.J., Holbrook, M.R., Soong, L., Wang, T. 2010. gammadelta T cells promote the maturation of dendritic cells during West Nile virus infection. FEMS Immunol Med Microbiol 59, 71.

Fenoglio, D., Poggi, A., Catellani, S., Battaglia, F., Ferrera, A., Setti, M., Murdaca, G., Zocchi, M.R. 2009. Vdelta1 T lymphocytes producing IFN-gamma and IL-17 are expanded in HIV-1-infected patients and respond to Candida albicans. Blood 113, 6611.

Fisch, P., Malkovsky, M., Kovats, S., Sturm, E., Braakman, E., Klein, B.S., Voss, S.D., Morrissey, L.W., DeMars, R., Welch, W.J., et al. 1990. Recognition by human V gamma 9/V delta 2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. Science 250, 1269.

Fisher, J.P., Heuijerjans, J., Yan, M., Gustafsson, K., Anderson, J. 2014. gammadelta T cells for cancer immunotherapy: A systematic review of clinical trials. Oncoimmunology 3, e27572.

Fournie, J.J., Sicard, H., Poupot, M., Bezombes, C., Blanc, A., Romagne, F., Ysebaert, L., Laurent, G. 2013. What lessons can be learned from gammadelta T cell-based cancer immunotherapy trials? Cell Mol Immunol 10, 35.

Fournie, J.J., Sicard, H., Poupot, M., Bezombes, C., Blanc, A., Romagne, F., Ysebaert, L., Laurent, G. 2013. What lessons can be learned from gammadelta T cell-based cancer immunotherapy trials? Cell Mol Immunol 10, 35.

Fowell, D.J., Magram, J., Turck, C.W., Killeen, N., Locksley, R.M. 1997. Impaired Th2 subset development in the absence of CD4. Immunity 6, 559.

Fuchs, E.J., Matzinger, P. 1992. B cells turn off virgin but not memory T cells. Science 258, 1156.

Fujii, S., Shimizu, K., Hemmi, H., Steinman, R.M. 2007. Innate Valpha14(+) natural killer T cells mature dendritic cells, leading to strong adaptive immunity. Immunol Rev 220, 183.

Fujii, S., Shimizu, K., Smith, C., Bonifaz, L., Steinman, R.M. 2003. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. J Exp Med 198, 267.

Galli, G., Nuti, S., Tavarini, S., Galli-Stampino, L., De Lalla, C., Casorati, G., Dellabona, P., Abrignani, S. 2003. CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. J Exp Med 197, 1051.

Gan, Y.H., Wallace, M., Malkovsky, M. 2001. Fas-dependent, activation-induced cell death of gammadelta cells. J Biol Regul Homeost Agents 15, 277.

Garcia, V.E., Sieling, P.A., Gong, J., Barnes, P.F., Uyemura, K., Tanaka, Y., Bloom, B.R., Morita, C.T., Modlin, R.L. 1997. Single-cell cytokine analysis of gamma delta T cell responses to nonpeptide mycobacterial antigens. J Immunol 159, 1328.

Geisberger, R., Lamers, M., Achatz, G. 2006. The riddle of the dual expression of IgM and IgD. Immunology 118, 429.

Gill, S.R., Pop, M., Deboy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., Gordon, J.I., Relman, D.A., Fraser-Liggett, C.M., Nelson, K.E. 2006. Metagenomic analysis of the human distal gut microbiome. Science 312, 1355.

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

Glatman Zaretsky, A., Taylor, J.J., King, I.L., Marshall, F.A., Mohrs, M., Pearce, E.J. 2009. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. J Exp Med 206, 991.

Glatzel, A., Wesch, D., Schiemann, F., Brandt, E., Janssen, O., Kabelitz, D. 2002. Patterns of chemokine receptor expression on peripheral blood gamma delta T lymphocytes: strong expression of CCR5 is a selective feature of V delta 2/V gamma 9 gamma delta T cells. J Immunol 168, 4920.

Gleimer, M., Parham, P. 2003. Stress management: MHC class I and class I-like molecules as reporters of cellular stress. Immunity 19, 469.

Gober, H.J., Kistowska, M., Angman, L., Jeno, P., Mori, L., De Libero, G. 2003. Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med 197, 163.

Godder, K.T., Henslee-Downey, P.J., Mehta, J., Park, B.S., Chiang, K.Y., Abhyankar, S., Lamb, L.S. 2007. Long term disease-free survival in acute leukemia patients recovering with increased gammadelta T cells after partially mismatched related donor bone marrow transplantation. Bone Marrow Transplant 39, 751.

Godfrey, D.I., Hammond, K.J., Poulton, L.D., Smyth, M.J., Baxter, A.G. 2000. NKT cells: facts, functions and fallacies. Immunol Today 21, 573.

Goding, J.W. 1978. Allotypes of IgM and IgD receptors in the mouse: a probe for lymphocyte differentiation. Contemp Top Immunobiol 8, 203.

Gold, M.C., Eid, T., Smyk-Pearson, S., Eberling, Y., Swarbrick, G.M., Langley, S.M., Streeter, P.R., Lewinsohn, D.A., Lewinsohn, D.M. 2013. Human thymic MR1-restricted MAIT cells are innate pathogen-reactive effectors that adapt following thymic egress. Mucosal Immunol 6, 35.

Gonzalez, S., Groh, V., Spies, T. 2006. Immunobiology of human NKG2D and its ligands. Curr Top Microbiol Immunol 298, 121.

Goodman, T., Lefrancois, L. 1988. Expression of the gamma-delta T-cell receptor on intestinal CD8+ intraepithelial lymphocytes. Nature 333, 855.

Groh, V., Rhinehart, R., Secrist, H., Bauer, S., Grabstein, K.H., 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.

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

Gumperz, J.E., Miyake, S., Yamamura, T., Brenner, M.B. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. J Exp Med 195, 625.

Gumperz, J.E., Roy, C., Makowska, A., Lum, D., Sugita, M., Podrebarac, T., Koezuka, Y., Porcelli, S.A., Cardell, S., Brenner, M.B., Behar, S.M. 2000. Murine CD1d-restricted T cell recognition of cellular lipids. Immunity 12, 211.

Guo, H., Kumar, P., Malarkannan, S. 2011. Evasion of natural killer cells by influenza virus. J Leukoc Biol 89, 189.

Guo, T., Chamoto, K., Hirano, N. 2015. Adoptive T Cell Therapy Targeting CD1 and MR1. Front Immunol 6, 247.

Hacker, G., Kromer, S., Falk, M., Heeg, K., Wagner, H., Pfeffer, K. 1992. V delta 1+ subset of human gamma delta T cells responds to ligands expressed by EBV-infected Burkitt lymphoma cells and transformed B lymphocytes. J Immunol 149, 3984.

Halary, F., Peyrat, M.A., Champagne, E., Lopez-Botet, M., Moretta, A., Moretta, L., Vie, H., Fournie, J.J., Bonneville, M. 1997. Control of self-reactive cytotoxic T lymphocytes expressing gamma delta T cell receptors by natural killer inhibitory receptors. Eur J Immunol 27, 2812.

Halary, F., Pitard, V., Dlubek, D., Krzysiek, R., de la Salle, H., Merville, P., Dromer, C., Emilie, D., Moreau, J.F., Dechanet-Merville, J. 2005. Shared reactivity of V{delta}2(neg) {gamma}{delta} T cells against cytomegalovirus-infected cells and tumor intestinal epithelial cells. J Exp Med 201, 1567.

Halder, R.C., Aguilera, C., Maricic, I., Kumar, V. 2007. Type II NKT cell-mediated anergy induction in type I NKT cells prevents inflammatory liver disease. J Clin Invest 117, 2302.

Hannani, D., Ma, Y., Yamazaki, T., Dechanet-Merville, J., Kroemer, G., Zitvogel, L. 2012. Harnessing gammadelta T cells in anticancer immunotherapy. Trends Immunol 33, 199.

Hara, T., Mizuno, Y., Takaki, K., Takada, H., Akeda, H., Aoki, T., Nagata, M., Ueda, K., Matsuzaki, G., Yoshikai, Y., et al. 1992. Predominant activation and expansion of V gamma 9-bearing gamma delta T cells in vivo as well as in vitro in Salmonella infection. J Clin Invest 90, 204.

Harizi, H. 2013. Reciprocal crosstalk between dendritic cells and natural killer cells under the effects of PGE2 in immunity and immunopathology. Cell Mol Immunol 10, 213.

Harly, C., Guillaume, Y., Nedellec, S., Peigne, C.M., Monkkonen, H., Monkkonen, J., Li, J., Kuball, J., Adams, E.J., Netzer, S., Dechanet-Merville, J., Leger, A., Herrmann, T., Breathnach, R., Olive, D., Bonneville, M., Scotet, E. 2012. Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human gammadelta T-cell subset. Blood 120, 2269.

Harly, C., Peigne, C.M., Scotet, E. 2014. Molecules and Mechanisms Implicated in the Peculiar Antigenic Activation Process of Human Vgamma9Vdelta2 T Cells. Front Immunol 5, 657.

Harris, D.P., Haynes, L., Sayles, P.C., Duso, D.K., Eaton, S.M., Lepak, N.M., Johnson, L.L., Swain, S.L., Lund, F.E. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol 1, 475.

Hata, S., Satyanarayana, K., Devlin, P., Band, H., McLean, J., Strominger, J.L., Brenner, M.B., Krangel, M.S. 1988. Extensive junctional diversity of rearranged human T cell receptor delta genes. Science 240, 1541.

Hayday, A., Theodoridis, E., Ramsburg, E., Shires, J. 2001. Intraepithelial lymphocytes: exploring the Third Way in immunology. Nat Immunol 2, 997.

Hayday, A., Vantourout, P. 2013. A long-playing CD about the gammadelta TCR repertoire. Immunity 39, 994.

Hayday, A.C. 2000. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. Annu Rev Immunol 18, 975.

Hayday, A.C. 2009. Gammadelta T cells and the lymphoid stress-surveillance response. Immunity 31, 184.

Hein, W.R., Mackay, C.R. 1991. Prominence of gamma delta T cells in the ruminant immune system. Immunol Today 12, 30.

Heuston, S., Begley, M., Gahan, C.G., Hill, C. 2012. Isoprenoid biosynthesis in bacterial pathogens. Microbiology 158, 1389.

Hintz, M., Reichenberg, A., Altincicek, B., Bahr, U., Gschwind, R.M., Kollas, A.K., Beck, E., Wiesner, J., Eberl, M., Jomaa, H. 2001. Identification of (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human gammadelta T cells in Escherichia coli. FEBS Lett 509, 317.

Hinz, T., Wesch, D., Halary, F., Marx, S., Choudhary, A., Arden, B., Janssen, O., Bonneville, M., Kabelitz, D. 1997. Identification of the complete expressed human TCR V gamma repertoire by flow cytometry. Int Immunol 9, 1065.

Holtmeier, W., Kabelitz, D. 2005. gammadelta T cells link innate and adaptive immune responses. Chem Immunol Allergy 86, 151.

Holtmeier, W., Pfander, M., Hennemann, A., Zollner, T.M., Kaufmann, R., 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.

Hoq, M.M., Suzutani, T., Toyoda, T., Horiike, G., Yoshida, I., Azuma, M. 1997. Role of gamma delta TCR+ lymphocytes in the augmented resistance of trehalose 6,6'-dimycolate-treated mice to influenza virus infection. J Gen Virol 78 (Pt 7), 1597.

Hsiao, C.H., Lin, X., Barney, R.J., Shippy, R.R., Li, J., Vinogradova, O., Wiemer, D.F., Wiemer, A.J. 2014. Synthesis of a phosphoantigen prodrug that potently activates Vgamma9Vdelta2 T-lymphocytes. Chem Biol 21, 945.

Huang, D., Chen, C.Y., Ali, Z., Shao, L., Shen, L., Lockman, H.A., Barnewall, R.E., Sabourin, C., Eestep, J., Reichenberg, A., Hintz, M., Jomaa, H., Wang, R., Chen, Z.W. 2009. Antigen-specific Vgamma2Vdelta2 T effector cells confer homeostatic protection against pneumonic plaque lesions. Proc Natl Acad Sci U S A 106, 7553.

Hwang, S.J., Kim, S., Park, W.S., Chung, D.H. 2006. IL-4-secreting NKT cells prevent hypersensitivity pneumonitis by suppressing IFN-gamma-producing neutrophils. J Immunol 177, 5258.

Idrees, A.S., Sugie, T., Inoue, C., Murata-Hirai, K., Okamura, H., Morita, C.T., Minato, N., Toi, M., Tanaka, Y. 2013. Comparison of gammadelta T cell responses and farnesyl diphosphate synthase inhibition in tumor cells pretreated with zoledronic acid. Cancer Sci 104, 536.

Indra, A., Huhulescu, S., Fiedler, A., Kernbichler, S., Blaschitz, M., Allerberger, F. 2009. Outbreak of Clostridium difficile 027 infection in Vienna, Austria 2008-2009. Euro Surveill 14.

Indra, A., Huhulescu, S., Hasenberger, P., Schmid, D., Alfery, C., Wuerzner, R., Fille, M., Gattringer, K., Kuijper, E., Allerberger, F. 2006. First isolation of Clostridium difficile PCR ribotype 027 in Austria. Euro Surveill 11, E060914.3.

Ismaili, J., Olislagers, V., Poupot, R., Fournie, J.J., Goldman, M. 2002. Human gamma delta T cells induce dendritic cell maturation. Clin Immunol 103, 296.

Iwasaki, M., Tanaka, Y., Kobayashi, H., Murata-Hirai, K., Miyabe, H., Sugie, T., Toi, M., Minato, N. 2011. Expression and function of PD-1 in human gammadelta T cells that recognize phosphoantigens. Eur J Immunol 41, 345.

Izumi, T., Kondo, M., Takahashi, T., Fujieda, N., Kondo, A., Tamura, N., Murakawa, T., Nakajima, J., Matsushita, H., Kakimi, K. 2013. Ex vivo characterization of gammadelta T-cell repertoire in patients after adoptive transfer of Vgamma9Vdelta2 T cells expressing the interleukin-2 receptor beta-chain and the common gamma-chain. Cytotherapy 15, 481.

Jafari, N.V., Kuehne, S.A., Bryant, C.E., Elawad, M., Wren, B.W., Minton, N.P., Allan, E., Bajaj-Elliott, M. 2013. Clostridium difficile modulates host innate immunity via toxin-independent and dependent mechanism(s). PLoS One 8, e69846.

Janeway, C.A., Jr. 2001. How the immune system works to protect the host from infection: a personal view. Proc Natl Acad Sci U S A 98, 7461.

Janeway, C.A., Jr. 2010. Janeway's Immunobiology. Taylor & Francis, Inc, New York.

Janeway, C.A., Jr., Jones, B., Hayday, A. 1988. Specificity and function of T cells bearing gamma delta receptors. Immunol Today 9, 73.

Jomaa, H., Feurle, J., Luhs, K., Kunzmann, V., Tony, H.P., Herderich, M., Wilhelm, M. 1999. Vgamma9/Vdelta2 T cell activation induced by bacterial low molecular mass compounds depends on the 1-deoxy-D-xylulose 5-phosphate pathway of isoprenoid biosynthesis. FEMS Immunol Med Microbiol 25, 371.

Kabelitz, D., Bender, A., Schondelmaier, S., Schoel, B., Kaufmann, S.H. 1990. A large fraction of human peripheral blood gamma/delta + T cells is activated by Mycobacterium tuberculosis but not by its 65-kD heat shock protein. J Exp Med 171, 667.

Kabelitz, D., Glatzel, A., Wesch, D. 2000. Antigen recognition by human gammadelta T lymphocytes. Int Arch Allergy Immunol 122, 1.

Kabelitz, D., He, W. 2012. The multifunctionality of human Vgamma9Vdelta2 gammadelta T cells: clonal plasticity or distinct subsets? Scand J Immunol 76, 213.

Kabelitz, D., Hinz, T., Dobmeyer, T., Mentzel, U., Marx, S., Bohme, A., Arden, B., Rossol, R., Hoelzer, D. 1997. Clonal expansion of Vgamma3/Vdelta3-expressing gammadelta T cells in an HIV-1/2-negative patient with CD4 T-cell deficiency. Br J Haematol 96, 266.

Kabelitz, D., Wesch, D., He, W. 2007. Perspectives of gammadelta T cells in tumor immunology. Cancer Res 67, 5.

Kabelitz, D., Wesch, D., Hinz, T. 1999. gamma delta T cells, their T cell receptor usage and role in human diseases. Springer Semin Immunopathol 21, 55.

Kabelitz, D., Wesch, D., Pitters, E., Zoller, M. 2004. Characterization of tumor reactivity of human V gamma 9V delta 2 gamma delta T cells in vitro and in SCID mice in vivo. J Immunol 173, 6767.

Kalyan, S., Chow, A.W. 2009. Linking innate and adaptive immunity: human Vgamma9Vdelta2 T cells enhance CD40 expression and HMGB-1 secretion. Mediators Inflamm 2009, 819408.

Kalyan, S., Kabelitz, D. 2013. Defining the nature of human gammadelta T cells: a biographical sketch of the highly empathetic. Cell Mol Immunol 10, 21.

Kelly, C.P., Pothoulakis, C., LaMont, J.T. 1994. Clostridium difficile colitis. N Engl J Med 330, 257.

Kenna, T., Golden-Mason, L., Norris, S., Hegarty, J.E., O'Farrelly, C., Doherty, D.G. 2004. Distinct subpopulations of gamma delta T cells are present in normal and tumor-bearing human liver. Clin Immunol 113, 56.

Kim, S., Iizuka, K., Aguila, H.L., Weissman, I.L., Yokoyama, W.M. 2000. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. Proc Natl Acad Sci U S A 97, 2731.

Kitamura, H., Iwakabe, K., Yahata, T., Nishimura, S., Ohta, A., Ohmi, Y., Sato, M., Takeda, K., Okumura, K., Van Kaer, L., Kawano, T., Taniguchi, M., Nishimura, T. 1999. The natural killer T (NKT) cell ligand alpha-galactosylceramide

demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. J Exp Med 189, 1121.

Kjer-Nielsen, L., Patel, O., Corbett, A.J., Le Nours, J., Meehan, B., Liu, L., Bhati, M., Chen, Z., Kostenko, L., Reantragoon, R., Williamson, N.A., Purcell, A.W., Dudek, N.L., McConville, M.J., O'Hair, R.A., Khairallah, G.N., Godfrey, D.I., Fairlie, D.P., Rossjohn, J., McCluskey, J. 2012. MR1 presents microbial vitamin B metabolites to MAIT cells. Nature 491, 717.

Knight, A., Madrigal, A.J., Grace, S., Sivakumaran, J., Kottaridis, P., Mackinnon, S., Travers, P.J., Lowdell, M.W. 2010. The role of Vdelta2-negative gammadelta T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. Blood 116, 2164.

Kobayashi, H., Tanaka, Y., Yagi, J., Minato, N., Tanabe, K. 2011. Phase I/II study of adoptive transfer of gammadelta T cells in combination with zoledronic acid and IL-2 to patients with advanced renal cell carcinoma. Cancer Immunol Immunother 60, 1075.

Kobayashi, H., Tanaka, Y., Yagi, J., Osaka, Y., Nakazawa, H., Uchiyama, T., Minato, N., Toma, H. 2007. Safety profile and anti-tumor effects of adoptive immunotherapy using gamma-delta T cells against advanced renal cell carcinoma: a pilot study. Cancer Immunol Immunother 56, 469.

Koizumi, H., Liu, C.C., Zheng, L.M., Joag, S.V., Bayne, N.K., Holoshitz, J., Young, J.D. 1991. Expression of perforin and serine esterases by human gamma/delta T cells. J Exp Med 173, 499.

Koo, H.L., Garey, K.W., Dupont, H.L. 2010. Future novel therapeutic agents for Clostridium difficile infection. Expert Opin Investig Drugs 19, 825.

Kozbor, D., Trinchieri, G., Monos, D.S., Isobe, M., Russo, G., Haney, J.A., Zmijewski, C., Croce, C.M. 1989. Human TCR-gamma+/delta+, CD8+ T lymphocytes recognize tetanus toxoid in an MHC-restricted fashion. J Exp Med 169, 1847.

Kuijper, E.J., Coignard, B., Brazier, J.S., Suetens, C., Drudy, D., Wiuff, C., Pituch, H., Reichert, P., Schneider, F., Widmer, A.F., Olsen, K.E., Allerberger, F., Notermans, D.W., Barbut, F., Delmee, M., Wilcox, M., Pearson, A., Patel, B.C., Brown, D.J., Frei, R., Akerlund, T., Poxton, I.R., Tull, P. 2007. Update of Clostridium difficile-associated disease due to PCR ribotype 027 in Europe. Euro Surveill 12, E1.

Kunzmann, V., Bauer, E., Wilhelm, M. 1999. Gamma/delta T-cell stimulation by pamidronate. N Engl J Med 340, 737.

Kurosaki, T., Kometani, K., Ise, W. 2015. Memory B cells. Nat Rev Immunol 15, 149.

Laggner, U., Di Meglio, P., Perera, G.K., Hundhausen, C., Lacy, K.E., Ali, N., Smith, C.H., Hayday, A.C., Nickoloff, B.J., Nestle, F.O. 2011. Identification of a novel proinflammatory human skin-homing Vgamma9Vdelta2 T cell subset with a potential role in psoriasis. J Immunol 187, 2783.

Lai, Y.C., Peng, H.L., Chang, H.Y. 2001. Identification of genes induced in vivo during Klebsiella pneumoniae CG43 infection. Infect Immun 69, 7140.

Lamb, L.S., Jr., Henslee-Downey, P.J., Parrish, R.S., Godder, K., Thompson, J., Lee, C., Gee, A.P. 1996. Increased frequency of TCR gamma delta + T cells in disease-free survivors following T cell-depleted, partially mismatched, related donor bone marrow transplantation for leukemia. J Hematother 5, 503.

Lanier, L.L., Chang, C., Azuma, M., Ruitenberg, J.J., Hemperly, J.J., Phillips, J.H. 1991. Molecular and functional analysis of human natural killer cell-associated neural cell adhesion molecule (N-CAM/CD56). J Immunol 146, 4421.

Lanzavecchia, A., Bernasconi, N., Traggiai, E., Ruprecht, C.R., Corti, D., Sallusto, F. 2006. Understanding and making use of human memory B cells. Immunol Rev 211, 303.

Lanzavecchia, A., Roosnek, E., Gregory, T., Berman, P., Abrignani, S. 1988. T cells can present antigens such as HIV gp120 targeted to their own surface molecules. Nature 334, 530.

Lanzavecchia, A., Sallusto, F. 2001. The instructive role of dendritic cells on T cell responses: lineages, plasticity and kinetics. Curr Opin Immunol 13, 291.

Lara-Tejero, M., Galan, J.E. 2001. CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. Infect Immun 69, 4358.

Le Bourhis, L., Martin, E., Peguillet, I., Guihot, A., Froux, N., Core, M., Levy, E., Dusseaux, M., Meyssonnier, V., Premel, V., Ngo, C., Riteau, B., Duban, L., Robert, D., Huang, S., Rottman, M., Soudais, C., Lantz, O. 2010. Antimicrobial activity of mucosal-associated invariant T cells. Nat Immunol 11, 701.

Lee, P.T., Benlagha, K., Teyton, L., Bendelac, A. 2002. Distinct functional lineages of human V(alpha)24 natural killer T cells. J Exp Med 195, 637.

Lehner, T., Mitchell, E., Bergmeier, L., Singh, M., Spallek, R., Cranage, M., Hall, G., Dennis, M., Villinger, F., Wang, Y. 2000. The role of gammadelta T cells in generating antiviral factors and beta-chemokines in protection against mucosal simian immunodeficiency virus infection. Eur J Immunol 30, 2245.

Leon, B., Lopez-Bravo, M., Ardavin, C. 2005. Monocyte-derived dendritic cells. Semin Immunol 17, 313.

Lepore, M., de Lalla, C., Gundimeda, S.R., Gsellinger, H., Consonni, M., Garavaglia, C., Sansano, S., Piccolo, F., Scelfo, A., Haussinger, D., Montagna, D., Locatelli, F., Bonini, C., Bondanza, A., Forcina, A., Li, Z., Ni, G., Ciceri, F., Jeno, P., Xia, C., Mori, L., Dellabona, P., Casorati, G., De Libero, G. 2014. A novel self-lipid antigen targets human T cells against CD1c(+) leukemias. J Exp Med 211, 1363.

Lepore, M., Kalinichenko, A., Colone, A., Paleja, B., Singhal, A., Tschumi, A., Lee, B., Poidinger, M., Zolezzi, F., Quagliata, L., Sander, P., Newell, E., Bertoletti, A., Terracciano, L., De Libero, G., Mori, L. 2014. Parallel T-cell cloning and deep

sequencing of human MAIT cells reveal stable oligoclonal TCRbeta repertoire. Nat Commun 5, 3866.

Leslie, D.S., Vincent, M.S., Spada, F.M., Das, H., Sugita, M., Morita, C.T., Brenner, M.B. 2002. CD1-mediated gamma/delta T cell maturation of dendritic cells. J Exp Med 196, 1575.

Li, B., Rossman, M.D., Imir, T., Oner-Eyuboglu, A.F., Lee, C.W., Biancaniello, R., Carding, S.R. 1996. Disease-specific changes in gammadelta T cell repertoire and function in patients with pulmonary tuberculosis. J Immunol 157, 4222.

Lin, H., Nieda, M., Rozenkov, V., Nicol, A.J. 2006. Analysis of the effect of different NKT cell subpopulations on the activation of CD4 and CD8 T cells, NK cells, and B cells. Exp Hematol 34, 289.

Liu, D., Song, L., Brawley, V.S., Robison, N., Wei, J., Gao, X., Tian, G., Margol, A., Ahmed, N., Asgharzadeh, S., Metelitsa, L.S. 2013. Medulloblastoma expresses CD1d and can be targeted for immunotherapy with NKT cells. Clin Immunol 149, 55.

Liu, Z., Eltoum, I.E., Guo, B., Beck, B.H., Cloud, G.A., Lopez, R.D. 2008. Protective immunosurveillance and therapeutic antitumor activity of gammadelta T cells demonstrated in a mouse model of prostate cancer. J Immunol 180, 6044.

Longhi, M.S., Hussain, M.J., Mitry, R.R., Arora, S.K., Mieli-Vergani, G., Vergani, D., Ma, Y. 2006. Functional study of CD4+CD25+ regulatory T cells in health and autoimmune hepatitis. J Immunol 176, 4484.

Loo, V.G., Poirier, L., Miller, M.A., Oughton, M., Libman, M.D., Michaud, S., Bourgault, A.M., Nguyen, T., Frenette, C., Kelly, M., Vibien, A., Brassard, P., Fenn, S., Dewar, K., Hudson, T.J., Horn, R., Rene, P., Monczak, Y., Dascal, A. 2005. A predominantly clonal multi-institutional outbreak of Clostridium difficile-associated diarrhea with high morbidity and mortality. N Engl J Med 353, 2442.

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., Savage, P.B., Jabri, B., Bendelac, A., Adams, E.J. 2013. Crystal structure of Vdelta1 T cell receptor in complex with CD1d-sulfatide shows MHC-like recognition of a self-lipid by human gammadelta T cells. Immunity 39, 1032.

Lutz, M.B., Schuler, G. 2002. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol 23, 445.

Lyras, D., O'Connor, J.R., Howarth, P.M., Sambol, S.P., Carter, G.P., Phumoonna, T., Poon, R., Adams, V., Vedantam, G., Johnson, S., Gerding, D.N., Rood, J.I. 2009. Toxin B is essential for virulence of Clostridium difficile. Nature 458, 1176.

Macho-Fernandez, E., Brigl, M. 2015. The Extended Family of CD1d-Restricted NKT Cells: Sifting through a Mixed Bag of TCRs, Antigens, and Functions. Front Immunol 6, 362.

MacLennan, I.C., Toellner, K.M., Cunningham, A.F., Serre, K., Sze, D.M., Zuniga, E., Cook, M.C., Vinuesa, C.G. 2003. Extrafollicular antibody responses. Immunol Rev 194, 8.

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

Maher, C.O., Dunne, K., Comerford, R., O'Dea, S., Loy, A., Woo, J., Rogers, T.R., Mulcahy, F., Dunne, P.J., Doherty, D.G. 2015. Candida albicans stimulates IL-23 release by human dendritic cells and downstream IL-17 secretion by Vdelta1 T cells. J Immunol 194, 5953.

Mangan, B.A., Dunne, M.R., O'Reilly, V.P., Dunne, P.J., Exley, M.A., O'Shea, D., Scotet, E., Hogan, A.E., Doherty, D.G. 2013. Cutting edge: CD1d restriction and Th1/Th2/Th17 cytokine secretion by human Vdelta3 T cells. J Immunol 191, 30.

Marcu-Malina, V., Balbir-Gurman, A., Dardik, R., Braun-Moscovici, Y., Segel, M.J., Bank, I. 2014. A Novel Prothrombotic Pathway in Systemic Sclerosis Patients: Possible Role of Bisphosphonate-Activated gammadelta T Cells. Front Immunol 5, 414.

Martino, A., Casetti, R., D'Alessandri, A., Sacchi, A., Poccia, F. 2005. Complementary function of gamma delta T-lymphocytes and dendritic cells in the response to isopentenyl-pyrophosphate and lipopolysaccharide antigens. J Clin Immunol 25, 230.

Martino, A., Casetti, R., Sacchi, A., Poccia, F. 2007. Central memory Vgamma9Vdelta2 T lymphocytes primed and expanded by bacillus Calmette-Guerin-infected dendritic cells kill mycobacterial-infected monocytes. J Immunol 179, 3057.

Matsuda, J.L., Naidenko, O.V., Gapin, L., Nakayama, T., Taniguchi, M., Wang, C.R., Koezuka, Y., Kronenberg, M. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J Exp Med 192, 741.

Mattarollo, S.R., Kenna, T., Nieda, M., Nicol, A.J. 2007. Chemotherapy and zoledronate sensitize solid tumour cells to Vgamma9Vdelta2 T cell cytotoxicity. Cancer Immunol Immunother 56, 1285.

McCarthy, N.E., Bashir, Z., Vossenkamper, A., Hedin, C.R., Giles, E.M., Bhattacharjee, S., Brown, S.G., Sanders, T.J., Whelan, K., MacDonald, T.T., Lindsay, J.O., Stagg, A.J. 2013. Proinflammatory Vdelta2+ T cells populate the human intestinal mucosa and enhance IFN-gamma production by colonic alphabeta T cells. J Immunol 191, 2752.

McDonald, L.C., Killgore, G.E., Thompson, A., Owens, R.C., Jr., Kazakova, S.V., Sambol, S.P., Johnson, S., Gerding, D.N. 2005. An epidemic, toxin gene-variant strain of Clostridium difficile. N Engl J Med 353, 2433.

McFarland, L.V. 2009. Evidence-based review of probiotics for antibiotic-associated diarrhea and Clostridium difficile infections. Anaerobe 15, 274.

Mellman, I., Steinman, R.M. 2001. Dendritic cells: specialized and regulated antigen processing machines. Cell 106, 255.

Meraviglia, S., Eberl, M., Vermijlen, D., Todaro, M., Buccheri, S., Cicero, G., La Mendola, C., Guggino, G., D'Asaro, M., Orlando, V., Scarpa, F., Roberts, A., Caccamo, N., Stassi, G., Dieli, F., Hayday, A.C. 2010. In vivo manipulation of Vgamma9Vdelta2 T cells with zoledronate and low-dose interleukin-2 for immunotherapy of advanced breast cancer patients. Clin Exp Immunol 161, 290.

Meraviglia, S., Eberl, M., Vermijlen, D., Todaro, M., Buccheri, S., Cicero, G., La Mendola, C., Guggino, G., D'Asaro, M., Orlando, V., Scarpa, F., Roberts, A., Caccamo, N., Stassi, G., Dieli, F., Hayday, A.C. 2010. In vivo manipulation of Vgamma9Vdelta2 T cells with zoledronate and low-dose interleukin-2 for immunotherapy of advanced breast cancer patients. Clin Exp Immunol 161, 290.

Metelitsa, L.S., Weinberg, K.I., Emanuel, P.D., Seeger, R.C. 2003. Expression of CD1d by myelomonocytic leukemias provides a target for cytotoxic NKT cells. Leukemia 17, 1068.

Meuter, S., Eberl, M., Moser, B. 2010. Prolonged antigen survival and cytosolic export in cross-presenting human gammadelta T cells. Proc Natl Acad Sci U S A 107, 8730.

Mills, K.H. 2004. Regulatory T cells: friend or foe in immunity to infection? Nat Rev Immunol 4, 841.

Miyagawa, F., Tanaka, Y., Yamashita, S., Minato, N. 2001. Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human gamma delta T cells by aminobisphosphonate antigen. J Immunol 166, 5508.

Moens, E., Brouwer, M., Dimova, T., Goldman, M., Willems, F., Vermijlen, D. 2011. IL-23R and TCR signaling drives the generation of neonatal Vgamma9Vdelta2 T

cells expressing high levels of cytotoxic mediators and producing IFN-gamma and IL-17. J Leukoc Biol 89, 743.

Mollenhauer, H.H., Morre, D.J., Rowe, L.D. 1990. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. Biochim Biophys Acta 1031, 225.

Morgan, D.A., Ruscetti, F.W., Gallo, R. 1976. Selective in vitro growth of T lymphocytes from normal human bone marrows. Science 193, 1007.

Morita, C.T., Jin, C., Sarikonda, G., Wang, H. 2007. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. Immunol Rev 215, 59.

Morita, C.T., Mariuzza, R.A., Brenner, M.B. 2000. Antigen recognition by human gamma delta T cells: pattern recognition by the adaptive immune system. Springer Semin Immunopathol 22, 191.

Moser, B., Brandes, M. 2006. Gammadelta T cells: an alternative type of professional APC. Trends Immunol 27, 112.

Moser, B., Eberl, M. 2007. gammadelta T cells: novel initiators of adaptive immunity. Immunol Rev 215, 89.

Motohashi, S., Okamoto, Y., Yoshino, I., Nakayama, T. 2011. Anti-tumor immune responses induced by iNKT cell-based immunotherapy for lung cancer and head and neck cancer. Clin Immunol 140, 167.

Nakajima, J., Murakawa, T., Fukami, T., Goto, S., Kaneko, T., Yoshida, Y., Takamoto, S., Kakimi, K. 2010. A phase I study of adoptive immunotherapy for recurrent non-small-cell lung cancer patients with autologous gammadelta T cells. Eur J Cardiothorac Surg 37, 1191.

Nedellec, S., Sabourin, C., Bonneville, M., Scotet, E. 2010. NKG2D costimulates human V gamma 9V delta 2 T cell antitumor cytotoxicity through protein kinase C

theta-dependent modulation of early TCR-induced calcium and transduction signals. J Immunol 185, 55.

Ness-Schwickerath, K.J., Jin, C., Morita, C.T. 2010. Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells. J Immunol 184, 7268.

Ni Eidhin, D.B., O'Brien, J.B., McCabe, M.S., Athie-Morales, V., Kelleher, D.P. 2008. Active immunization of hamsters against Clostridium difficile infection using surface-layer protein. FEMS Immunol Med Microbiol 52, 207.

O'Brien, J.B., McCabe, M.S., Athie-Morales, V., McDonald, G.S., Ni Eidhin, D.B., Kelleher, D.P. 2005. Passive immunisation of hamsters against Clostridium difficile infection using antibodies to surface layer proteins. FEMS Microbiol Lett 246, 199.

Okada, R., Kondo, T., Matsuki, F., Takata, H., Takiguchi, M. 2008. Phenotypic classification of human CD4+ T cell subsets and their differentiation. Int Immunol 20, 1189.

Oliaro, J., Dudal, S., Liautard, J., Andrault, J.B., Liautard, J.P., Lafont, V. 2005. Vgamma9Vdelta2 T cells use a combination of mechanisms to limit the spread of the pathogenic bacteria Brucella. J Leukoc Biol 77, 652.

Olweus, J., BitMansour, A., Warnke, R., Thompson, P.A., Carballido, J., Picker, L.J., Lund-Johansen, F. 1997. Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. Proc Natl Acad Sci U S A 94, 12551.

O'Reilly, V., Zeng, S.G., Bricard, G., Atzberger, A., Hogan, A.E., Jackson, J., Feighery, C., Porcelli, S.A., Doherty, D.G. 2011. Distinct and overlapping effector functions of expanded human CD4+, CD8alpha+ and CD4-CD8alpha- invariant natural killer T cells. PLoS One 6, e28648.

O'Sullivan, B., Thomas, R. 2003. CD40 and dendritic cell function. Crit Rev Immunol 23, 83.

Parker, C.M., Groh, V., Band, H., Porcelli, S.A., Morita, C., Fabbi, M., Glass, D., Strominger, J.L., Brenner, M.B. 1990. Evidence for extrathymic changes in the T cell receptor gamma/delta repertoire. J Exp Med 171, 1597.

Pechhold, K., Wesch, D., Schondelmaier, S., Kabelitz, D. 1994. Primary activation of V gamma 9-expressing gamma delta T cells by Mycobacterium tuberculosis. Requirement for Th1-type CD4 T cell help and inhibition by IL-10. J Immunol 152, 4984.

Penack, O., Gentilini, C., Fischer, L., Asemissen, A.M., Scheibenbogen, C., Thiel, E., Uharek, L. 2005. CD56dimCD16neg cells are responsible for natural cytotoxicity against tumor targets. Leukemia 19, 835.

Pena-Cruz, V., Ito, S., Dascher, C.C., Brenner, M.B., Sugita, M. 2003. Epidermal Langerhans cells efficiently mediate CD1a-dependent presentation of microbial lipid antigens to T cells. J Invest Dermatol 121, 517.

Pepin, J., Valiquette, L., Alary, M.E., Villemure, P., Pelletier, A., Forget, K., Pepin, K., Chouinard, D. 2004. Clostridium difficile-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. Cmaj 171, 466.

Perera, M.K., Carter, R., Goonewardene, R., Mendis, K.N. 1994. Transient increase in circulating gamma/delta T cells during Plasmodium vivax malarial paroxysms. J Exp Med 179, 311.

Petrasca, A., Doherty, D.G. 2014. Human Vdelta2(+) gammadelta T Cells Differentially Induce Maturation, Cytokine Production, and Alloreactive T Cell Stimulation by Dendritic Cells and B Cells. Front Immunol 5, 650.

Pietschmann, K., Beetz, S., Welte, S., Martens, I., Gruen, J., Oberg, H.H., Wesch, D., Kabelitz, D. 2009. Toll-like receptor expression and function in subsets of human gammadelta T lymphocytes. Scand J Immunol 70, 245.

Poles, M.A., Barsoum, S., Yu, W., Yu, J., Sun, P., Daly, J., He, T., Mehandru, S., Talal, A., Markowitz, M., Hurley, A., Ho, D., Zhang, L. 2003. Human immunodeficiency virus type 1 induces persistent changes in mucosal and blood gammadelta T cells despite suppressive therapy. J Virol 77, 10456.

Poli, A., Michel, T., Theresine, M., Andres, E., Hentges, F., Zimmer, J. 2009. CD56bright natural killer (NK) cells: an important NK cell subset. Immunology 126, 458.

Porcelli, S., Brenner, M.B., Band, H. 1991. Biology of the human gamma delta T-cell receptor. Immunol Rev 120, 137.

Porcelli, S., Yockey, C.E., Brenner, M.B., Balk, S.P. 1993. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. J Exp Med 178, 1.

Poxton, I.R., McCoubrey, J., Blair, G. 2001. The pathogenicity of Clostridium difficile. Clin Microbiol Infect 7, 421.

Puan, K.J., Jin, C., Wang, H., Sarikonda, G., Raker, A.M., Lee, H.K., Samuelson, M.I., Marker-Hermann, E., Pasa-Tolic, L., Nieves, E., Giner, J.L., Kuzuyama, T., Morita, C.T. 2007. Preferential recognition of a microbial metabolite by human Vgamma2Vdelta2 T cells. Int Immunol 19, 657.

Puttur, F.K., Fernandez, M.A., White, R., Roediger, B., Cunningham, A.L., Weninger, W., Jones, C.A. 2010. Herpes simplex virus infects skin gamma delta T cells before Langerhans cells and impedes migration of infected Langerhans cells by inducing apoptosis and blocking E-cadherin downregulation. J Immunol 185, 477.

Qin, G., Mao, H., Zheng, J., Sia, S.F., Liu, Y., Chan, P.L., Lam, K.T., Peiris, J.S., Lau, Y.L., Tu, W. 2009. Phosphoantigen-expanded human gammadelta T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses. J Infect Dis 200, 858.

Rajoriya, N., Fergusson, J.R., Leithead, J.A., Klenerman, P. 2014. Gamma Delta T-lymphocytes in Hepatitis C and Chronic Liver Disease. Front Immunol 5, 400.

Raulet, D.H. 2003. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol 3, 781.

Reil, M., Erhard, M., Kuijper, E.J., Kist, M., Zaiss, H., Witte, W., Gruber, H., Borgmann, S. 2011. Recognition of Clostridium difficile PCR-ribotypes 001, 027 and 126/078 using an extended MALDI-TOF MS system. Eur J Clin Microbiol Infect Dis 30, 1431.

Reis e Sousa, C. 2006. Dendritic cells in a mature age. Nat Rev Immunol 6, 476.

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

Rhodes, D.A., Stammers, M., Malcherek, G., Beck, S., Trowsdale, J. 2001. The cluster of BTN genes in the extended major histocompatibility complex. Genomics 71, 351.

Ribot, J.C., debarros, A., Silva-Santos, B. 2011. Searching for "signal 2": costimulation requirements of gammadelta T cells. Cell Mol Life Sci 68, 2345.

Riganti, C., Massaia, M., Davey, M.S., Eberl, M. 2012. Human gammadelta T-cell responses in infection and immunotherapy: common mechanisms, common mediators? Eur J Immunol 42, 1668.

Rodriguez-Pinto, D. 2005. B cells as antigen presenting cells. Cell Immunol 238, 67.

Rossi, D., Zlotnik, A. 2000. The biology of chemokines and their receptors. Annu Rev Immunol 18, 217.

Rossol, R., Dobmeyer, J.M., Dobmeyer, T.S., Klein, S.A., Rossol, S., Wesch, D., Hoelzer, D., Kabelitz, D., Helm, E.B. 1998. Increase in Vdelta1+ gammadelta 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.

Roth, M.D., Harui, A. 2015. Human tumor infiltrating lymphocytes cooperatively regulate prostate tumor growth in a humanized mouse model. J Immunother Cancer 3, 12.

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

Russano, A.M., Bassotti, G., Agea, E., Bistoni, O., Mazzocchi, A., Morelli, A., Porcelli, S.A., Spinozzi, F. 2007. CD1-restricted recognition of exogenous and self-lipid antigens by duodenal gammadelta+ T lymphocytes. J Immunol 178, 3620.

Rust, C.J., Verreck, F., Vietor, H., Koning, F. 1990. Specific recognition of staphylococcal enterotoxin A by human T cells bearing receptors with the V gamma 9 region. Nature 346, 572.

Ryan, A., Lynch, M., Smith, S.M., Amu, S., Nel, H.J., McCoy, C.E., Dowling, J.K., Draper, E., O'Reilly, V., McCarthy, C., O'Brien, J., Ni Eidhin, D., O'Connell, M.J., Keogh, B., Morton, C.O., Rogers, T.R., Fallon, P.G., O'Neill, L.A., Kelleher, D., Loscher, C.E. 2011. A role for TLR4 in Clostridium difficile infection and the recognition of surface layer proteins. PLoS Pathog 7, e1002076.

Sallusto, F., Lanzavecchia, A. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med 179, 1109.

Sallusto, F., Mackay, C.R. 2004. Chemoattractants and their receptors in homeostasis and inflammation. Curr Opin Immunol 16, 724.

Sandstrom, A., Peigne, C.M., Leger, A., Crooks, J.E., Konczak, F., Gesnel, M.C., Breathnach, R., Bonneville, M., Scotet, E., Adams, E.J. 2014. The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vgamma9Vdelta2 T cells. Immunity 40, 490.

Santolaria, T., Robard, M., Leger, A., Catros, V., Bonneville, M., Scotet, E. 2013. Repeated systemic administrations of both aminobisphosphonates and human Vgamma9Vdelta2 T cells efficiently control tumor development in vivo. J Immunol 191, 1993.

Schwartz, R.H. 2003. T cell anergy. Annu Rev Immunol 21, 305.

Sciammas, R., Bluestone, J.A. 1999. TCRgammadelta cells and viruses. Microbes Infect 1, 203.

Seo, G.Y., Youn, J., Kim, P.H. 2009. IL-21 ensures TGF-beta 1-induced IgA isotype expression in mouse Peyer's patches. J Leukoc Biol 85, 744.

Shin, S.J., Wu, C.W., Steinberg, H., Talaat, A.M. 2006. Identification of novel virulence determinants in Mycobacterium paratuberculosis by screening a library of insertional mutants. Infect Immun 74, 3825.

Shrestha, N., Ida, J.A., Lubinski, A.S., Pallin, M., Kaplan, G., Haslett, P.A. 2005. Regulation of acquired immunity by gamma delta T-cell/dendritic-cell interactions. Ann N Y Acad Sci 1062, 79.

Sicard, H., Ingoure, S., Luciani, B., Serraz, C., Fournie, J.J., Bonneville, M., Tiollier, J., Romagne, F. 2005. In vivo immunomanipulation of V gamma 9V delta 2 T cells with a synthetic phosphoantigen in a preclinical nonhuman primate model. J Immunol 175, 5471.

Siegers, G.M., Lamb, L.S., Jr. 2014. Cytotoxic and regulatory properties of circulating Vdelta1+ gammadelta T cells: a new player on the cell therapy field? Mol Ther 22, 1416.

Smith, K.A. 1988. Interleukin-2: inception, impact, and implications. Science 240, 1169.

Soriano, M.M., Liao, S., Danziger, L.H. 2013. Fidaxomicin: a minimally absorbed macrocyclic antibiotic for the treatment of Clostridium difficile infections. Expert Rev Anti Infect Ther 11, 767.

Sougioultzis, S., Kyne, L., Drudy, D., Keates, S., Maroo, S., Pothoulakis, C., Giannasca, P.J., Lee, C.K., Warny, M., Monath, T.P., Kelly, C.P. 2005. Clostridium difficile toxoid vaccine in recurrent C. difficile-associated diarrhea. Gastroenterology 128, 764.

Spada, F.M., Grant, E.P., Peters, P.J., Sugita, M., Melian, A., Leslie, D.S., Lee, H.K., van Donselaar, E., Hanson, D.A., Krensky, A.M., Majdic, O., Porcelli, S.A., Morita, C.T., Brenner, M.B. 2000. Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. J Exp Med 191, 937.

Spanoudakis, E., Hu, M., Naresh, K., Terpos, E., Melo, V., Reid, A., Kotsianidis, I., Abdalla, S., Rahemtulla, A., Karadimitris, A. 2009. Regulation of multiple myeloma survival and progression by CD1d. Blood 113, 2498.

Spinozzi, F., Agea, E., Bistoni, O., Forenza, N., Monaco, A., Falini, B., Bassotti, G., De Benedictis, F., Grignani, F., Bertotto, A. 1995. Local expansion of allergen-specific CD30+Th2-type gamma delta T cells in bronchial asthma. Mol Med 1, 821.

Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 9, 271.

Steinman, R.M. 2003. Some interfaces of dendritic cell biology. Apmis 111, 675.

Steinman, R.M. 2007. Dendritic cells: understanding immunogenicity. Eur J Immunol 37 Suppl 1, S53.

Steinman, R.M., Hawiger, D., Liu, K., Bonifaz, L., Bonnyay, D., Mahnke, K., Iyoda, T., Ravetch, J., Dhodapkar, M., Inaba, K., Nussenzweig, M. 2003a. Dendritic cell function in vivo during the steady state: a role in peripheral tolerance. Ann N Y Acad Sci 987, 15.

Steinman, R.M., Hawiger, D., Nussenzweig, M.C. 2003b. Tolerogenic dendritic cells. Annu Rev Immunol 21, 685.

Stetson, D.B., Mohrs, M., Reinhardt, R.L., Baron, J.L., Wang, Z.E., Gapin, L., Kronenberg, M., Locksley, R.M. 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. J Exp Med 198, 1069.

Subramanian, L., Blumenfeld, H., Tohn, R., Ly, D., Aguilera, C., Maricic, I., Mansson, J.E., Buschard, K., Kumar, V., Delovitch, T.L. 2012. NKT cells stimulated by long fatty acyl chain sulfatides significantly reduce the incidence of type 1 diabetes in nonobese diabetic mice [corrected]. PLoS One 7, e37771.

Takahashi, T., Suzuki, T. 2012. Role of sulfatide in normal and pathological cells and tissues. J Lipid Res 53, 1437.

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

Taylor, P.R., Martinez-Pomares, L., Stacey, M., Lin, H.H., Brown, G.D., Gordon, S. 2005. Macrophage receptors and immune recognition. Annu Rev Immunol 23, 901.

Thedrez, A., Sabourin, C., Gertner, J., Devilder, M.C., Allain-Maillet, S., Fournie, J.J., Scotet, E., Bonneville, M. 2007. Self/non-self discrimination by human gammadelta T cells: simple solutions for a complex issue? Immunol Rev 215, 123.

Thompson, K., Rogers, M.J. 2004. Statins prevent bisphosphonate-induced gamma,delta-T-cell proliferation and activation in vitro. J Bone Miner Res 19, 278.

Thompson, K., Rojas-Navea, J., Rogers, M.J. 2006. Alkylamines cause Vgamma9Vdelta2 T-cell activation and proliferation by inhibiting the mevalonate pathway. Blood 107, 651.

Tian, J.H., Fuhrmann, S.R., Kluepfel-Stahl, S., Carman, R.J., Ellingsworth, L., Flyer, D.C. 2012. A novel fusion protein containing the receptor binding domains of C. difficile toxin A and toxin B elicits protective immunity against lethal toxin and spore challenge in preclinical efficacy models. Vaccine 30, 4249.

Tilloy, F., Treiner, E., Park, S.H., Garcia, C., Lemonnier, F., de la Salle, H., Bendelac, A., Bonneville, M., Lantz, O. 1999. An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. J Exp Med 189, 1907.

Tisch, R. 2010. Immunogenic versus tolerogenic dendritic cells: a matter of maturation. Int Rev Immunol 29, 111.

Todaro, M., D'Asaro, M., Caccamo, N., Iovino, F., Francipane, M.G., Meraviglia, S., Orlando, V., La Mendola, C., Gulotta, G., Salerno, A., Dieli, F., Stassi, G. 2009. Efficient killing of human colon cancer stem cells by gammadelta T lymphocytes. J Immunol 182, 7287.

Tokuyama, H., Hagi, T., Mattarollo, S.R., Morley, J., Wang, Q., So, H.F., Moriyasu, F., Nieda, M., Nicol, A.J. 2008. V gamma 9 V delta 2 T cell cytotoxicity against tumor cells is enhanced by monoclonal antibody drugs--rituximab and trastuzumab. Int J Cancer 122, 2526.

Tonegawa, S. 1983. Somatic generation of antibody diversity. Nature 302, 575.

Treiner, E., Duban, L., Bahram, S., Radosavljevic, M., Wanner, V., Tilloy, F., Affaticati, P., Gilfillan, S., Lantz, O. 2003. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. Nature 422, 164.

Tseng, C.T., Miskovsky, E., Houghton, M., Klimpel, G.R. 2001. Characterization of liver T-cell receptor gammadelta T cells obtained from individuals chronically infected with hepatitis C virus (HCV): evidence for these T cells playing a role in the liver pathology associated with HCV infections. Hepatology 33, 1312.

Tu, W., Zheng, J., Liu, Y., Sia, S.F., Liu, M., Qin, G., Ng, I.H., Xiang, Z., Lam, K.T., Peiris, J.S., Lau, Y.L. 2011. The aminobisphosphonate pamidronate controls influenza pathogenesis by expanding a gammadelta T cell population in humanized mice. J Exp Med 208, 1511.

Tu, W.W., Lau, Y.L., Peiris, J.S. 2014. Use of humanised mice to study antiviral activity of human gammadelta-T cells against influenza A viruses. Hong Kong Med J 20 Suppl 6, 4.

Tyler, C.J., Doherty, D.G., Moser, B., Eberl, M. 2015. Human Vgamma9/Vdelta2 T cells: Innate adaptors of the immune system. Cell Immunol 296, 10.

Uchida, R., Ashihara, E., Sato, K., Kimura, S., Kuroda, J., Takeuchi, M., Kawata, E., Taniguchi, K., Okamoto, M., Shimura, K., Kiyono, Y., Shimazaki, C., Taniwaki, M., Maekawa, T. 2007. Gamma delta T cells kill myeloma cells by sensing mevalonate metabolites and ICAM-1 molecules on cell surface. Biochem Biophys Res Commun 354, 613.

Uldrich, A.P., Patel, O., Cameron, G., Pellicci, D.G., Day, E.B., Sullivan, L.C., Kyparissoudis, K., Kjer-Nielsen, L., Vivian, J.P., Cao, B., Brooks, A.G., Williams, S.J., Illarionov, P., Besra, G.S., Turner, S.J., Porcelli, S.A., McCluskey, J., Smyth, M.J., Rossjohn, J., Godfrey, D.I. 2011. A semi-invariant Valpha10+ T cell antigen receptor defines a population of natural killer T cells with distinct glycolipid antigen-recognition properties. Nat Immunol 12, 616.

Underdown, B.J., Schiff, J.M. 1986. Immunoglobulin A: strategic defense initiative at the mucosal surface. Annu Rev Immunol 4, 389.

Vantourout, P., Hayday, A. 2013. Six-of-the-best: unique contributions of gammadelta T cells to immunology. Nat Rev Immunol 13, 88.

Vavassori, S., Kumar, A., Wan, G.S., Ramanjaneyulu, G.S., Cavallari, M., El Daker, S., Beddoe, T., Theodossis, A., Williams, N.K., Gostick, E., Price, D.A., Soudamini, D.U., Voon, K.K., Olivo, M., Rossjohn, J., Mori, L., De Libero, G. 2013.

Butyrophilin 3A1 binds phosphorylated antigens and stimulates human gammadelta T cells. Nat Immunol 14, 908.

Viey, E., Fromont, G., Escudier, B., Morel, Y., Da Rocha, S., Chouaib, S., Caignard, A. 2005. Phosphostim-activated gamma delta T cells kill autologous metastatic renal cell carcinoma. J Immunol 174, 1338.

Vincent, M.S., Leslie, D.S., Gumperz, J.E., Xiong, X., Grant, E.P., Brenner, M.B. 2002. CD1-dependent dendritic cell instruction. Nat Immunol 3, 1163.

Voth, D.E., Ballard, J.D. 2005. Clostridium difficile toxins: mechanism of action and role in disease. Clin Microbiol Rev 18, 247.

Walzer, T., Jaeger, S., Chaix, J., Vivier, E. 2007. Natural killer cells: from CD3(-)NKp46(+) to post-genomics meta-analyses. Curr Opin Immunol 19, 365.

Wang, H., Henry, O., Distefano, M.D., Wang, Y.C., Raikkonen, J., Monkkonen, J., Tanaka, Y., Morita, C.T. 2013. Butyrophilin 3A1 plays an essential role in prenyl pyrophosphate stimulation of human Vgamma2Vdelta2 T cells. J Immunol 191, 1029.

Wang, H., Sarikonda, G., Puan, K.J., Tanaka, Y., Feng, J., Giner, J.L., Cao, R., Monkkonen, J., Oldfield, E., Morita, C.T. 2011. Indirect stimulation of human Vgamma2Vdelta2 T cells through alterations in isoprenoid metabolism. J Immunol 187, 5099.

Wang, L., Das, H., Kamath, A., Bukowski, J.F. 2001. Human V gamma 2V delta 2 T cells produce IFN-gamma and TNF-alpha with an on/off/on cycling pattern in response to live bacterial products. J Immunol 167, 6195.

Wen, L., Barber, D.F., Pao, W., Wong, F.S., Owen, M.J., Hayday, A. 1998. Primary gamma delta cell clones can be defined phenotypically and functionally as Th1/Th2 cells and illustrate the association of CD4 with Th2 differentiation. J Immunol 160, 1965.

Weng, X., Liao, C.M., Bagchi, S., Cardell, S.L., Stein, P.L., Wang, C.R. 2014. The adaptor protein SAP regulates type II NKT-cell development, cytokine production, and cytotoxicity against lymphoma. Eur J Immunol 44, 3646.

Wesch, D., Beetz, S., Oberg, H.H., Marget, M., Krengel, K., Kabelitz, D. 2006. Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes. J Immunol 176, 1348.

Wesch, D., Glatzel, A., Kabelitz, D. 2001. Differentiation of resting human peripheral blood gamma delta T cells toward Th1- or Th2-phenotype. Cell Immunol 212, 110.

Wesch, D., Kabelitz, D. 2003. Differential expression of natural killer receptors on Vdelta1 gammadelta T cells in HIV-1-infected individuals. J Acquir Immune Defic Syndr 33, 420.

Wesch, D., Peters, C., Oberg, H.H., Pietschmann, K., Kabelitz, D. 2011. Modulation of gammadelta T cell responses by TLR ligands. Cell Mol Life Sci 68, 2357.

Wilhelm, M., Kunzmann, V., Eckstein, S., Reimer, P., Weissinger, F., Ruediger, T., Tony, H.P. 2003. Gammadelta T cells for immune therapy of patients with lymphoid malignancies. Blood 102, 200.

Worku, S., Gorse, G.J., Belshe, R.B., Hoft, D.F. 2001. Canarypox vaccines induce antigen-specific human gammadelta T cells capable of interferon-gamma production. J Infect Dis 184, 525.

Wren, B.W. 2006. Clostridium difficile comes of age. Future Microbiol 1, 243.

Wrobel, P., Shojaei, H., Schittek, B., Gieseler, F., Wollenberg, B., Kalthoff, H., Kabelitz, D., Wesch, D. 2007. Lysis of a broad range of epithelial tumour cells by human gamma delta T cells: involvement of NKG2D ligands and T-cell receptor- versus NKG2D-dependent recognition. Scand J Immunol 66, 320.

Yao, L., Sgadari, C., Furuke, K., Bloom, E.T., Teruya-Feldstein, J., Tosato, G. 1999. Contribution of natural killer cells to inhibition of angiogenesis by interleukin-12. Blood 93, 1612.

Zar, F.A., Bakkanagari, S.R., Moorthi, K.M., Davis, M.B. 2007. A comparison of vancomycin and metronidazole for the treatment of Clostridium difficile-associated diarrhea, stratified by disease severity. Clin Infect Dis 45, 302.

Zeng, M.Y., Pham, D., Bagaitkar, J., Liu, J., Otero, K., Shan, M., Wynn, T.A., Brombacher, F., Brutkiewicz, R.R., Kaplan, M.H., Dinauer, M.C. 2013. An efferocytosis-induced, IL-4-dependent macrophage-iNKT cell circuit suppresses sterile inflammation and is defective in murine CGD. Blood 121, 3473.

Zeng, S.G., Ghnewa, Y.G., O'Reilly, V.P., Lyons, V.G., Atzberger, A., Hogan, A.E., Exley, M.A., Doherty, D.G. 2013. Human invariant NKT cell subsets differentially promote differentiation, antibody production, and T cell stimulation by B cells in vitro. J Immunol 191, 1666.

Zhao, J., Weng, X., Bagchi, S., Wang, C.R. 2014. Polyclonal type II natural killer T cells require PLZF and SAP for their development and contribute to CpG-mediated antitumor response. Proc Natl Acad Sci U S A 111, 2674.

Zheng, J., Liu, Y., Lau, Y.L., Tu, W. 2013. gammadelta-T cells: an unpolished sword in human anti-infection immunity. Cell Mol Immunol 10, 50.

Ziegler-Heitbrock, L. 2014. Monocyte subsets in man and other species. Cell Immunol 289, 135.

Zocchi, M.R., Poggi, A. 2004. Role of gammadelta T lymphocytes in tumor defense. Front Biosci 9, 2588.