Human peripheral γδT cell phenotypic and functional shift in response to stimulation with *E.coli*

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ABSTRACT

It is known that γδT cells provide immune-surveillance and host defense against infection and cancer. Our understanding of γδT cell functionality and antimicrobial immunity, however, remains poor. Limited data suggests that γδT cells can phagocytose particles and act as professional antigen-presenting cells (pAPCs). In order to examine γδT cell bacterial interactions, an *ex vivo* co-culture model of human peripheral blood mononuclear cell (PBMC) responses to *Escherichia coli* was employed. Following PBMC stimulation with *E.coli,* Vγ9Vδ2 cells underwent rapid T cell receptor (TCR)-dependent proliferation and functional transition from cytotoxic, inflammatory cytokine immunity, to cell expansion with diminished cytokine but increased costimulatory molecule expression, and capacity for professional phagocytosis. Phagocytosis was augmented by IgG opsonization, and inhibited by TCR-blockade, suggesting a licensing interaction involving the TCR and FcγR. Vγ9Vδ2 cells displayed potent cytotoxicity through TCR-dependent and independent mechanisms. Vγ9Vδ2 cell cytokine responses and cytotoxicity further presented with variable sensitivity to blocking of host butyrophilin 3A (BTN3A), in response to both, self and non-self, stimuli. We conclude that i) Vγ9Vδ2 T cells transition from early inflammatory cytotoxic killers to myeloid-like pAPCs in response to infectious stimuli, and that ii) Vγ9Vδ2 T cell recognition of targets is predominantly governed by TCR recognition of self stress markers in a BTN3A-dependent manner.

DECLARATION

I, Marta Barisa, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated as such in the thesis.

ACKNOWLEDGEMENTS

Thank you, Kenth, John and Mona, for your support and bottomless patience. Under your watchful eye and the safety of your wing, my pursuit of a career in research commences.

Paldies arī maniem mīļajiem vecākiem!
Paldies par ticību maniem spēkiem, par pacietību, par padomu, par plecu un smaidu. Šis ceļš, ko eju, ir bijis iespējams dēļ jūsu mīlestības un atbalsta. Varu tikai cerēt, ka došu jums ar ko lepoties.

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ABBREVIATIONS

APC – antigen presenting cell

APC (in the context of FACS staining only, in the Materials and Methods sections) – Allophycocyanin

pAPC – professional antigen presenting cell

α- - anti

αβT cell – alpha beta T cell

BTN - butyrophilin

BTN3A – butyrophilin 3A

CCR – chemokine receptor

CD – cluster of differentiation

CDR – complimentarity-determining region

CFU – colony forming unit

Ctrl – control

CyD – Cytochalasin D

Cy5.5 – Cyanine 5.5

Cy7 – Cyanine 7

C. albicans – *Candida albicans*

C. jejuni – Campylobacter jejuni

DAPI - 4',6-diamidino-2-phenylindole

DMSO - Dimethyl sulfoxide

D1-D14 – day 1 to day 14

E.coli – Eschericia coli

FITC - Fluorescein isothiocyanate

FcR – Fc receptor

FcγR – Fc gamma receptor

GFP – Green Fluorescent Protein

GM-CSF – granulocyte macrophage colony stimulating factor

γδT cell – gamma delta T cell

HMBPP - (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate

HCMV – human cytomegalovirus

HLA – human leukocyte antigen

IEL – intraepithelial lymphocyte

IFN – Interferon

Ig - immunoglobulin

IgG – immunoglobulin G

IL – Interleukin

iNKT – invariant natural killer T cell

IPP – isopentenyl pyrophosphate

IPTG - Isopropyl β-D-1-thiogalactopyranoside

Iso – isotype control

ITAM - Immunoreceptor tyrosine-based activation motif

LCK – lymphocyte-specific protein tyrosine kinase

L. monocytogenes – Lysteria monocytogenes

MFI – median fluorescence intensity

MHC – major histocompatibility complex

MICA - MHC class I chain-related A

M. morganii - Morganella morganii

M. tuberculosis – Mycobacterium tuberculosis

neg – negative

NK – natural killer cell

NKG2D - Natural Killer Group 2D receptor

NKT – natural killer T cell

Ops. – opsonized (usually IgG-opsonized)

O/N – overnight

OD – optical density

pAg – phosphoantigen

PBMC – peripheral blood mononuclear cells

PE – Phycoerythrin

PMN – polymorphonuclear cells

pos – positive

PRR – pattern recognition receptor

P. falciparum – Plasmodium falciparum

S. typhimurium - *Salmonella typhimurium*

S. aureus – *Staphylococcus aureus*

TB – Trypan Blue

TCR – T cell receptor

Th1- Th1 T helper cell

Th2 – Th2 T helper cell

Th17 – Th17 T helper cell

TLR – toll-like receptor

TM – transmembrane region

TNF – tumour necrosis factor

Treg – regulatory T cell

T. gondii – *Toxoplasma gondii*

UV - ultraviolet radiation

w/ - with

Zol. - zoledronate

1. INTRODUCTION

Gamma delta T (γδT) cells are an atypical subset of $CD3^{pos}$ lymphocytes with heterogeneous phenotypic patterns, effector functions and anatomical distribution that differ significantly from canonical alpha beta T (αβT) cells. Recent immunotherapeutic insights have highlighted γδT cells as an attractive putative tool against malignancy and infectious disease. Particular attention in this context has been given to the unusual capacity of γδT cells to act as both cytotoxic effectors against target cells and professional antigen presenting cells (pAPCs) to αβT cells. The following thesis presents an *ex vivo* experimental investigation of peripheral human γδT cell cytokine, cytotoxic and pAPC effector phenotype in response to various antigenic challenges, and the molecular factors that regulate it, therein.

A significant portion of the data contained within this thesis has been published in a June 2017 issue of *Scientific Reports* journal. The article, titled "*E. coli* promotes human Vγ9Vδ2 T cell transition from cytokine producing bactericidal effectors to professional phagocytic killers in a TCR-dependent manner", can be viewed in the Appendix section.

1.1. Introduction to γδT cells

Expressing a T cell receptor (TCR) composed of γ and δ chains, γδT cells comprise between 1% and 20% of the human peripheral T cell compartment, and up to 50% of epithelial lymphocytes. Often referred to in the literature as "innately-adaptive" lymphocytes (1), γδT cells are characterized by potent and rapid pro-inflammatory cytokine release and cytotoxicity upon stimulation, which is nonetheless paired with a lymphoid-like capacity for extensive clonal expansion (2–4). Some 70% of γδT cells are $CD4^{neg}CD8^{neg}$; the remainder are largely $CD4^{neg}CD8^{pos}$, with a $CD4^{pos}CD8^{neg}$ phenotype

a rarity. Whilst their baseline clonality remains controversial, it is thought that γδT cells have a predominantly oligoclonal rather than polyclonal TCR repertoire, which may underlie their swift and potent responses to primary antigenic stimulation (5,6).

γδT cells undergo a distinct developmental process. Unlike their classic, well-characterized counterparts – peripheral αβT cells - γδT cells appear to develop in several waves, the first of which is already evident in the prenatal thymus (reviewed by Vantourout and Hayday(7)). "Innate-like" $CD27^{pos}$ and $CD27^{neg}$ γδ T cells, possibly derived from foetal liver progenitors and characterized by secretion of interferon-γ (IFN-γ) and interleukin 17 (IL-17), respectively, have been detected in the prenatal thymus of both, mouse and man (7). A second wave of bone marrow-derived "lymphoid-like" γδT cells appears to emerge postnatally with a proposed default potential for the production of IFN-γ. In a significant deviation from αβ T cell development, some γδ T cells then migrate to distinct anatomical locations, rather than entering lymphatic re-circulation; human and mouse "innate-like" γδT cells have a particular propensity for epithelial sites, such as the gastrointestinal mucosa and skin, whilst "lymphoid-like" γδ T cells reside mostly within systemic circulation (7).

A summary of the basic features, shared and divergent, between the main human adaptive lymphocyte cell types - γδT cells, αβT cells and B cells – can be viewed in Table 1.1. Anatomical distribution, surface marker expression, cytokine secretion and proliferative behaviour have been used as a basis for differential γδT cell investigation and discussion in the literature. Perhaps the most basic and frequently used of these divisions relies on anatomical localization, with relatively scarce "lymphoid-like" γδT cells residing in suspension within the circulatory system, and highly abundant "innate-like" γδT cells residing at mucosal epithelial sites, largely as intraepithelial lymphocytes (IELs) (7).

Table 1.1 | **Features of human γδT, αβT and B cells**

Characteristic	**αβT cells**	**γδT cells**	**B cells**
Antigen-receptor configuration	CD3 complex + αβTCR	CD3 complex + γδTCR	Imunnoglobulin (Ig)
Theoretical receptor number	~10^{15}	~10^{20}	~10^{11}
Antigen recognition	Peptide + MHC	Protein and non-protein	Protein and non-protein
Antigen restriction	Yes	Rare	No
Phenotype	$CD4^{pos}$ or $CD8^{pos}$	Mostly $CD4^{neg}CD8^{neg}$; some IEL are $CD8(\alpha\alpha)^{pos}$	$CD19^{pos}CD20^{pos}$
Frequency in blood	65-75%	1-10% (25-60% in gut)	5-10%
Distribution	Blood and lymphoid tissue	Blood, lymphoid and epithelial/mucosal tissue	Blood and lymphoid tissue
Effector capability	Cytotoxicity ($CD8^{pos}$), Cytokine release (Th1/Th2/Th17)	Cytotoxicity, Cytokine release (Th1>Th17>Th2)	Ig production
Function	Immune protection and pathogen eradication	Immunoregulation and immunosurveillance	Humoral immunity

Adapted from Carding and Egan (2002) (8))

Human γδT cells are further often denoted based on their respective use of Vδ TCR chains. In most healthy human adults the peripheral γδT cell compartment is dominated by Vδ2 cells, whereas the predominant γδTCR^{pos} IEL population are Vδ1 cells. There are a number of further Vδ chains employed by both peripheral and tissue-resident, γδT cells, but these rare populations are poorly understood and are often referred to simply as Vδ1^{-} Vδ2^{-} γδT cells (for a basic subset summary refer to Table 1.2). Meaningful discussion of γδT cell physiological functions and therapeutic potential must note that the subdivision of γδT cells into "innate-like" and "lymphoid-like" subsets has been disputed. Significant further investigation is required for the establishment of well-defined, reproducible physiological differences between the multiple γδT cell compartments, regardless of subset definition.

Table 1.2 | **Features of human γδT cell subsets**

Subset	Most common VγVδ pairs	Tissue distribution	Key cytokines	Cytotoxicity
Vδ1^{pos}	Not defined	Epithelia, dermis, spleen and liver	IFN-γ and TNF; can produce IL-17	Potent
Vδ2^{pos}	Vγ9Vδ2	Blood	IFN-γ and TNF; can produce IL-17	Potent
Vδ1^{neg} Vδ2^{neg}	Not defined	Liver and gut epithelium	Not defined	Not defined

Adapted from Silva-Santos, et al. (2015) (9)

A majority of our insight into human γδT cell physiology derives from the study of the peripheral blood mononuclear cell (PBMC) compartment. The peripherally dominant Vδ2 γδT cells – with a Vγ9Vδ2 TCR - are the most-studied and best understood of the many, apparently heterogeneous γδT cell subsets. While data from peripheral Vδ2 γδT cell investigations in the context of immunotherapy remain promising (reviewed recently by Casetti, Martino and others (10,11)), it is crucial to achieve a broader understanding of all γδT cell subsets, and to establish whether inferences of γδT cell physiology from the human peripheral circulation are relevant to the more enigmatic tissue-resident lymphocyte compartments. It is, moreover, crucial to note that it remains, as of yet, unclear

to what extent human and murine γδT cell physiology is related. A significant portion of γδT cell literature examines murine γδT cells, which lack a homologous population to the human Vγ9Vδ2 cells, and possess oligoclonal, highly tissue-specific γδT cell populations such as dendritic epidermal T cells. While the overall differences between mouse and human γδT cell functionality and significance remain inconveniently obscure (reviewed recently by Pang, Pennington, et al. (12)), I make an effort in this thesis to focus my attention on human γδT cells and clinical scenarios.

1.2. γδT cells in health and disease

Current thought prevails that the predominant Vγ9Vδ2 human peripheral γδT cell subset mediates immuno-surveillance of stress signals emanating from endogenous and microbial pyrophosphates, sourced from *e.g.* tumour cells and infected cells, respectively (13). A significant increase in systemic and mucosal γδT cell numbers is seen in acute systemic bacterial and parasitic (as well as viral) infections. These include *Brucella, Streptococci, Coxiella, Listeria, Francisella, Escherichia, Campylobacter, Shigella, Leptospira, Plasmodium* and *Mycobacterium*, among others (14–24) . While further exploration into the functionality of these expanded γδT cells is necessary, data indicate an activated phenotype, as measured by high cell surface CD69, and significantly increased expression of MHC class II (*e.g.* HLA-DR) and CD86 (21,22,24–26). The presence of such $CD69^{pos}HLA\text{-}DR^{pos}$ γδT cells during sepsis and systemic inflammatory response syndrome appears to correlate negatively with mortality (25,27). The natural or experimental elevation of functional, activated γδT cell numbers has, moreover, been shown to correlate with improved prognosis and even protection from such chronic infections as *M. tuberculosis,* human cytomegalovirus (HCMV), *Toxoplasma gondii, Listeria monocytogenes* and a number of intestinal infections, among others, in murine, non-human primate and human models (28–36). Particularly important in a clinical setting – and when considering γδT cells as an immunotherapeutic tool - γδT cells are routinely shown to infiltrate and play a role in such common human tumour types as breast, colon, ovarian and lung cancers, as well as melanoma (37–41). Strikingly, the presence of γδT

cells was recently identified as the single most favourable lymphocytic predictive factor in human malignancy in a meta-analysis of 18,000 human tumours of 25 different types; γδT cells were shown to correlate with positive outcomes in both pan-cancer analysis, which included multiple myeloma and various leukaemias, as well as solid tumour analysis (42).

Explanations of the protective properties of γδT cells are extremely varied, and illustrate the expansive immune arsenal of these "innately-adaptive" lymphocytes in limiting or preventing the onset of disease. The most commonly discussed mechanisms of γδT cell effector function include potent, en-masse release of Interferon-γ (IFN-γ) and other Th1 cytokines for the resolution of intracellular infections, secretion of IL-17 for rapid mobilization of innate immunity at epithelial sites during bacterial invasion, upregulation of tumor necrosis factor-α (TNF-α) during systemic infection, effective cytotoxicity against transformed or infected self-cells and pathogens, mobilization and maturation of dendritic cells (DCs) as well as the antibody response in systemic and intestinal disease, priming and regulation of the αβT cell response in infectious or malignant scenarios through professional antigen presentation, maintenance of the integrity of epithelial barriers and even direct suppression of αβT cell-mediated inflammation in immunopathological settings (30,37,40,41,43–54). A simplified summary of the six main ways in which γδT cells contribute to the human immune response can be viewed in Figure 1.1, adapted from Vantourout and Hayday (7).

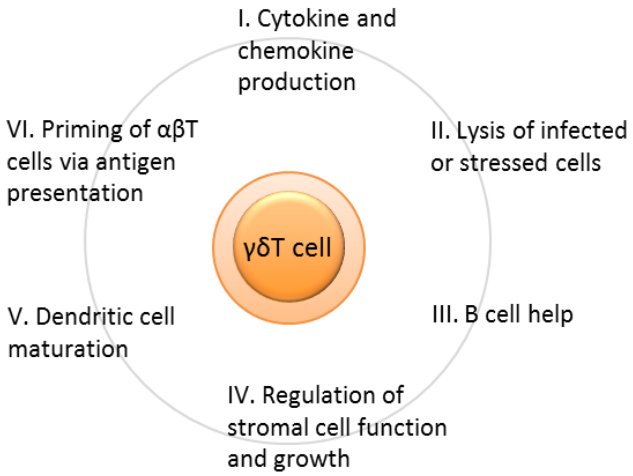

Fig. 1.1. γδT cell contribution to the human immune response. Literature documents that γδT cells have a central role in defending the host against a range of infectious and sterile stresses through six main mechanisms: I) Production of a diverse set of cytokines and chemokines to regulate other immune and non-immune cells. II) Direct lysis and, consequently, elimination of infected or stressed cells through the production of, e.g. granzymes. III) Help for B cells and promotion of IgE production. IV) Regulation stromal cell function through the production of growth factors. V) Triggering of dendritic cell maturation. VI) Antigen presentation to αβ T cells, resulting in priming.

1.3. γδT cells as professional antigen presenting cells

While a T cell pAPC is by no means an accepted concept in mainstream immunology, there are multiple lines of evidence suggesting that γδT cells may indeed act as pAPCs to classic αβT cells, which range from functional studies to expression of antigen presenting and co-stimulatory molecules, to apparent phagocytic capacity - aspects of which have been explored in humans, mice and other mammals (49,50,55–59). While the seminal publication on the pAPC capacity of human γδT cells may be attributed to Brandes and Moser, *et al.* (60), our own laboratory has previously published work documenting the phagocytic capacity of human γδT cells and their ability to act as pAPC even in the context of cross-presentation to cytotoxic CD8 αβT cells (49,50).

Further to their documented oncolytic properties, γδT cells also have recorded pro-tumorigenic roles. It appears that the very effector functions which confer them with the ability to recruit and shape myeloid, as well as lymphoid immunity, and affect the growth and development of epithelial/stromal tissue, enable γδT cells to aid and even protect tumour development and survival (37,39–41). IL-17-producing γδT cells have, for example, been shown to aid development of breast cancer metastases (61). If we consider, howerver, the central aetiological role of epithelial site integrity in infectious and malignant disease, the restoration or, more artificially, boosting of appropriate γδT cell function emerges as a key tool in the maintenance and restoration of human health. As outlined above, overall solid and non-solid tumour meta-analysis identifies γδT cells as a factor strongly favorable to tumour clearance and recovery (42). The plethora of functional outcomes upon γδT cell stimulation illuminates the potential of their use in therapy, and highlights our lack of understanding regarding their functionality. Basic investigation of factors determining the development of γδT cell phenotypes is crucial if we hope to harvest and manipulate their vast array of effector functions in a reproducible and clinically viable manner.

Vδ2 γδT cell *in vitro* responses to different bacterial stimuli have been explored by various groups and laboratories. IFN-γ production and expansion in response to exposure have been described for *M. tuberculosis, L. monocytogenes, S. aureus* and *E.coli,* as well as products from *Morganella morganii* and *Salmonella typhimurium,* among others (35,62–66). Potent Vδ2 γδT cell responses have, thus, been described to Gram-negative, Gram-positive bacteria as well as intracellular bacteria. The significance of γδT cells for *in vivo* resolution of infectious disease has remained ambiguous, largely due to the highly complex nature of events that occur during bacterial infection at epithelial surfaces. This complexity emanates from the multitude of intricate relationships between the highly populous, poorly understood and often intra-redundant mucosal immune compartment, epithelia and commensal as well as invading microorganisms.

Recent studies have finally provided unambiguous experimental evidence for the protective antibacterial properties of human γδT cells *in vivo.* Wang *et al.* were able to

provide effective infectious control and resolution of both Gram-positive (*S. aureus*) and Gram-negative (*M. morganii*) infections in severe combined immuno-deficiency mice by adoptive transfer of human Vδ2 cells (14). Curiously, however, these were not Vγ9Vδ2 cells, but rather Vγ2Vδ2 cells. Successful *in vivo* experimental control of intracellular infection, such as *M.tuberculosis*, has meanwhile been achieved by adoptive transfer of Vγ9Vδ2 cells in non-human primate models (35). IFN-γ is of central importance in these anti-microbial responses. It does, however, remain unclear whether IFN-γ production is the only significant effector response of bacteria-exposed γδT cells, and whether the anti-microbial effects observed are the result of direct Vδ2 cell killing of free bacteria or infected cells, or an indirect activation of other immune components / bystander effects.

An area of ongoing debate in the fields of basic γδT cell biology and immunotherapy concerns the capacity of γδT cells – often Vδ2 cells, specifically – to act as pAPCs to αβT cells. As described, the controversy stems largely from the significant unorthodoxy of a T cell pAPC, and the well-founded concern of myeloid contamination of the experimental setups. Evidence for the role and significance of *in vivo* human Vγ9Vδ2 cell pAPC function is, as of yet, lacking. Compelling, if not direct, evidence can be sourced from numerous studies on mouse *TCRδ*$^{-/-}$ knockout models. The observations on the immune outcome in the absence of γδT cells in these models vary with disease model and experimental question being asked. A common thread emerges, however, of increased sensitivity to infection and dysregulated, aberrant lymphoid responses – including αβT cell responses - particularly at epithelial sites (45–48,67). Significantly, induction of oral tolerance could not be achieved in *TCRδ*$^{-/-}$ knockout mice (46). Re-introduction of γδT cells restored suppression of both CD4+ and CD8+ αβT cell responses. Further models examining αβT cell knockout mice and γδT cell knockout mice in parallel found that *TCRα*$^{-/-}$ mice present with defects in protective immunity, while *TCRδ*$^{-/-}$ mice present with exaggerated intestinal damage as an apparent consequence of a lack of immune regulation at the site of infection (45). γδT cell-deficient mice have further been found to present with substantially increased sensitivity to chemically-induced malignancy (68).

Any discussion of the *in vivo* pAPC role of γδT cells would be incomplete sans mention of mouse dendritic epidermal T cells (DETC). Described for the first time decades ago,

DETC were, in fact, found to express a distinctly oligoclonal, tissue-specific γδTCR (69). Multiple lines of compelling evidence highlight the antigen presenting and tissue homoeostatic roles of DETC (56,70–72). It is important to note, however, that a human homologue to mouse DETC has not thus far been identified, nor one for Skint1 -the ligand that the DETC TCR appears to recognize (69). Nor, as mentioned earlier - a human pAg-activated Vγ9Vδ2 cell homologue in mice. I, therefore, refrain from in-depth discussion of the plethora of available γδT cell APC data generated in mouse models. The differences between mouse and human immune responses are a fascinating field of study and warrant significantly more attention from medical researchers, given how reliant we have grown on the extrapolation of mouse-generated data to human physiology and pathophysiology. A recent *Nature* editorial, based on work by Moss and group, outlines various reasons as to why murine models don't always translate easily to relevant human settings, and how these issues may be addressed, including the use of humanized mouse models (73). Reviews on the specific physiological differences between the mouse and human immune response, including γδT cells, have been compiled by Zschaler *et al.* and Mestas, *et al.* (74,75).

1.4. Phagocytosis

An important aspect of antigen presentation that necessitates consideration in the context of a γδT cell pAPC is the uptake of antigenic material destined for presentation. Cell internalization of material can take many forms and serves multiple physiologically important roles. Recent reviews by Flannagan *et al.* (76) and Underhill *et al.* (77) summarize what is known about the complex networks of information processing that occur during cell uptake of extracellular particles. While some forms of uptake serve a crucial immunological function, others are a part of routine cell homeostasis – and others still can be both, depending on the physiological context and cell type.

Endocytosis, the process by which cells internalize small molecules of *e.g.* nutritional value, is actin dependent, and occurs via multiple mechanisms. These include

internalization via clathrin-coated pits or cholesterol-rich caveolae. Macropinocytosis - sometimes referred to as "cell drinking" - involves extensive utilization of the actin cytoskeleton. It occurs via an eruption of membrane ruffles at the cell surface, which collapse back and fuse back with the cell membrane engulfing extracellular liquid in the process. The resulting vacuole, or macropinosome, is then shuttled down the normal endocytic pathway (78). Macropinocytosis is a normal part of all cell physiology. Via autophagy a cell may engulf organelles, soluble factors or microorganisms within its own cytosol. Trogocytosis describes the intracellular transfer of membrane patches from an APC to a lymphocyte (79). Cells can further engage in 'cannibalism', consuming whole, healthy self-cells, which are then degraded; such cannibalism has been seen with, for example, tumour cell 'eating' of lymphocytes (80). The unusual phenomenon of cell 'burrowing' is called entosis; the cell burrowing into another cell can die or re-emerge from its temporary host (80).

Phagocytosis – perhaps the most studied mode of material internalization – is actin and receptor-dependent uptake of specifically particulate targets >0.5μm in size (81). Potential targets range from dead or dying cells to microorganisms, to environmental debris. The ability to phagocytose material is restricted to specialized cells, which are predominantly immune effectors. There are multiple known mechanisms of phagocytosis, and these include i) reaching phagocytosis, mediated by *e.g.* Fc receptors or dectin 1, ii) triggered phagocytosis, which may occur if large particulate antigens are taken up during macropinocytosis, iii) sinking phagocytosis that is mediated by complement, and iv) macroautophagy, which involves cell 'eating' of large cytosolic material. All of these uptake routes result in the formation of a mature phagosome, and consequential killing and degradation of phagocytosed particles (77).

A wide range of receptors has been implicated in mediating phagocytosis, categorized broadly into opsonic, non-opsonic and triggered (non-specific) receptors and phagocytosis, therein. A brief summary, adapted from Underhill and Goodridge (77) can be seen in Table 1.3.

Table 1.3 | **Types of phagocytic receptors**

Type of receptor	Receptor	Ligand	Refs
Opsonic	Fc gamma family (FcγRI, FcγRIIA and FcγRIIA)	Antibody-opsonized targets	(85)
	Complement receptors (C1, C3, C4)	Complement-opsonized targets	(86)
	α5β1 integrin	Fibronectin	(87)
Non-opsonic	Dectin 1	β-glucan	(88)
	Macrophage receptor MARCO	Bacteria (undefined)	(89)
	Scavenger receptor A	Bacteria (diverse charged molecules)	(90)
	αVβ5 integrin	Apoptotic cells	(87)
Triggered	Toll like receptors	Various, including lipopolysccharides and lipopeptides	(91)

Phagocytosis proceeds through a number of stages, all of which involve tight control and multiple possible signaling events. Initial contact of cell and target is known as *tasting*, and involves receptor-mediated identification of target chemical identity by the cell. *Feeling*, the next step, occurs through dynamic membrane interactions between particle and cell; this permits the cell to identify the physical shape of the target. The specifics of subsequent *swallowing* are determined by signaling through the particular phagocytic receptor that was engaged. The phagocytic receptor(s) engaged further form a part of the phagosome membrane and inform phagosome handling and maturation, which result in *digesting* of the phagocytosed particle. The phagocytic process concludes with triggering of the *inflammatory response* within the cell. The inflammatory response involves secretion of specific inflammatory mediators from the cell, recruitment and activation of other cells, as well as professional antigen presentation of digested target peptides on MHC to αβT cells if the phagocyte is a pAPC.

Definitions of what exactly constitutes a *professional* APC may vary, but there is general agreement that these cells are efficient phagocytes (or in the case of B cells – efficient at

B cell receptor-mediated endocytosis), efficient at processing internalized material for presentation on MHC and express both, MHC class II and co-stimulatory molecules such as CD80 and CD86 (89). Occasionally, the ability to cross-present exogenous antigen on MHC class I is also included as a pre-requisite for a cell to be deemed a pAPC. In brief, pAPCs are cells which specialize in presenting antigen to T cells.

Some distinction as to 'professional' and 'non-professional' has also been made in reference to phagocytosis. Fibroblasts and epithelial cells have been shown to take up, for example, latex beads >0.5μm in size or red blood cells in a receptor-mediated manner, in a process which was enhanced by opsonization of the target. What prevents their categorization as professional phagocytes by those who venture to define the difference (90) is the limited range of particles that fibroblasts and epithelial cells appear to be capable of phagocytosing. The limited range of targets is attributable to the limited range of phagocytic receptors on the cell surface. Compared to professional phagocytes, non-professional phagocytes further lack the ability to produce a range of microbicidal molecules or mount a diverse cytokine response.

1.5. γδT cell recall and memory

An important aspect of γδT cell physiology in the context of their use as immunotherapeutics that has remained impervious to conclusive debate is "memory". In order to avoid semantic misunderstanding, the intended meaning of "memory" further in the text implies the phenomenon described by Janeway, *et al.* as: "… the ability of the immune system to respond more rapidly and effectively to pathogens that have been encountered previously, and reflects the preexistence of a clonally expanded population of antigen-specific lymphocytes", adding that "a long-standing debate about whether specific memory is maintained by distinct populations of long-lived memory cells that can

persist without residual antigen, or by lymphocytes that are under perpetual stimulation by residual antigen, appears to have been settled in favour of the former hypothesis." (91)

While a number of publications, disease models and research groups have suggested that γδT cells can confer residual protection from recurring disease, whether this protection is antigen-specific has been heavily contested. Publications have reported antigen-specific γδT cell memory responses to *M.tuberculosis* in humans, macaques, mice and cattle, as well as murine intestinal *L. monocytogenes* or imiquimod-induced inflammation-specific skin-resident γδT cells with indications of the hallmarks of canonical αβT cell memory: i) trafficking to draining lymph nodes, ii) migration through blood to distant tissue sites and peripheral lymph nodes, iii) persistence in these locations and iv) increased proliferative, and cytokine-producing capacity upon re-challenge (31,62,63,92). These findings are, however, inconsistent across various labs and have, importantly, struggled to find support in the patchy γδTCR sequencing data available in the literature. The number of known γδTCR antigen specificities is limited, and traditional T cell vaccination approaches have largely failed to induce reproducible, meaningful γδT cell memory to infectious re-challenge. It is, moreover, unclear to what extent the γδTCR plays a role in γδT cell activation, how it is modulated by a range of agonistic and antagonistic co-receptors, how this may differ between various γδT cell subsets or across species, such as mouse and human, and what resemblance exactly this bears to the central, tightly-regulated and non-redundant role of the TCR in canonical αβT cells.

A detailed understanding of the mode of γδTCR-antigen interaction remains elusive. As alluded to earlier in the text, it is known that γδT cells are capable of responding to antigen in the absence of major histocompatibility complex (MHC) and classic APC antigen processing and presentation. Instead, in what has been described as an "antibody-like breadth in antigen recognition" (7), γδT cells are capable of recognizing antigen presented on atypical MHC-like molecules (*e.g.* the CD1 family), altered or absent antigen presenting molecules themselves, and even free antigen (54,93–95). The notion that the γδTCR may be capable of binding free antigen is supported by the exceedingly high

γδTCR CDR3 diversity – found largely in the *TCRD* locus - which suggests a lack of restriction by the γδTCR for any particular antigen delivery structure (reviewed by Chien and Konigshofer (96)). The classes of antigen engaged by the γδTCR are consequently varied, and range from phosphoantigens (pAg) to peptide and lipid antigens. Some have suggested that γδT cells are further capable of responding to antigen in the absence of direct TCR stimulation, depending instead on the aforementioned pattern recognition receptors (PRRs) (3,44,97–99). A number of studies argue that γδT cells may be capable of engaging a single antigen through several different receptors, as is the case - in a foreign-antigen context - with bacterial lipopolysaccharide (LPS)-derived Lipid A, which triggers both the γδTCR and TLR-4 or, in a self-antigen context, with MHC class I-homologue MICA that is recognized by the γδTCR and NK-like receptor, NKG2D (93,95).

The question of γδT cell memory is hugely complicated by the popular hypothesis that γδT cells may, in fact, be largely auto-reactive, whence their responses are triggered primarily by recognition of self-stress markers, such as self-phospholipid cargos or aberrantly expressed antigen presenting molecules themselves, rather than foreign antigens (for a limited list of known γδT cell activating ligands refer to Table 1.4). These self-stress markers can be specific and induced by infection, transformation or other injury to tissue; γδT cells may thus mount a potent pro-inflammatory response to, for example, *E.coli* invasion, and yet remain entirely self-reactive. Such an explanation to the divergence between the rapid, potent, en-masse γδT cell responses and the delayed, highly antigen-specific, canonical αβT cell responses is intriguing. It may shed light on the ability of γδT cells to react so effectively to seemingly heterogeneous stimuli with what has been stipulated to be a relatively oligoclonal TCR repertoire.

Table 1.4 | **Activating ligands for human γδT cells**

Subset	Antigen
Vδ1^{pos}	MICA, CD1c, CD1d tetramers loaded with sulphatide, lipohexapeptides, phycoerythrin
Vδ2^{pos}	ULBP4, phosphoantigens, F1-ATPase, histidyl-tRNA synthetase, phycoerythrin, HMBPP
Vδ1^{neg}Vδ2^{neg}	Endothelial protein C receptor, phycoerythrin

Adapted from Hayday, et al. (2013) (7))

Here again, however, γδT cells elude simplicity and categorization; some of the most potent documented γδT cell stimuli, such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (or HMBPP), are of microbial origin (31). Some have proposed that the mechanism of HMBPP-induced activation of Vδ2 γδT cells may, in fact, be mediated via HMBPP induction of self-stress responses through dysregulation of the host mevalonate pathway, manifested as conformational changes in host cell surface butyrophilin (BTN) - BTN3A specifically - molecules (100). BTN changes through a similar mechanism have further been implicated as activatory to γδT cells, upon exposure to *E.coli* and other bacterial pathogens (101,102). Curiously, BTN3A conformational alterations further appear to be the mode of Vδ2 γδT cell activation in the context of stimulation with zoledronate. Zoledronate, a clinically-relevant bisphosphonate drug, which efficiently expands Vδ2 γδT cells *in vitro*, disrupts the host mevalonate pathway and causes the accumulation of endogenous pyrophosphates (IPP), which consequently induce conformational changes in BTN3A molecules on "stressed" self-cells (103). It is, perhaps, unsurprising that IPP and HMBPP appear to exert similar effects on Vδ2 γδT cells, given their high structural homology (Fig.1.2). The striking structural similarity between IPP and HMBPP does, however, present a hurdle in discerning the differential mechanisms by which these compounds may lead to γδT cell activation, and complicates our understanding of how γδT cells may distinguish between self and non-self danger signals. It is known, for example, that HMBPP, in the absence of any further immunogenic signals, is a far more potent activator of Vδ2 cells than IPP (104).

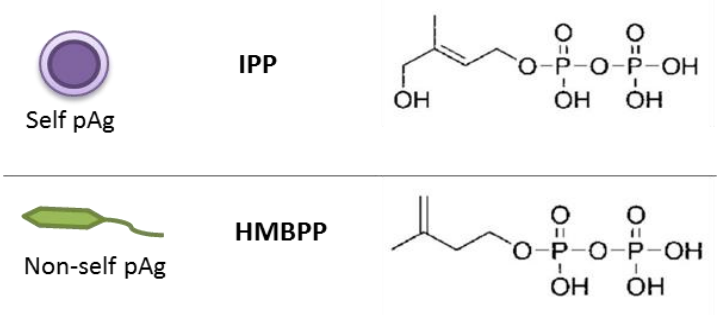

Fig. 1.2. Chemical structure of IPP and HMBPP phosphoantigens. Sourced from entirely different organisms, isopentenyl pyrophosphate (IPP), of vertebrate origin, and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), of microbial origin, present with highly homologous chemical structures.

A simplified diagram listing the various ways in which γδT cells may recognize pAg can be viewed in figure 1.3. There are four potential modes by which γδT cells detect self and non-self pAg danger signals: i) detection of the accumulation of self pAgs within self cells, induced by self-stress, *e.g.* transformation or the application of drugs such as zoledronate (Fig.1.3A), ii) detection of non-self pAgs directly, sourced from invading pAg-expressing microorganisms, *e.g. E.coli* (Fig.1.3B), iii) detection of non-self pAgs directly, but in a manner that is dependent on self cells manifesting these non-self pAgs, *e.g.* via HMBPP-induced butyrophilin changes (Fig.1.3C), or iv) detection of non-self pAgs indirectly, wherein non-self stressors such as *E.coli* induce self-stress and consequent accumulation of self pAgs that are then detected by γδT cells on self cells (Fig.1.3D). It is possible that all four mechanisms play a role in γδT cell detection of immune stress signals. γδT cell recognition of pAgs is explored in significant detail in subsequent chapters of the thesis.

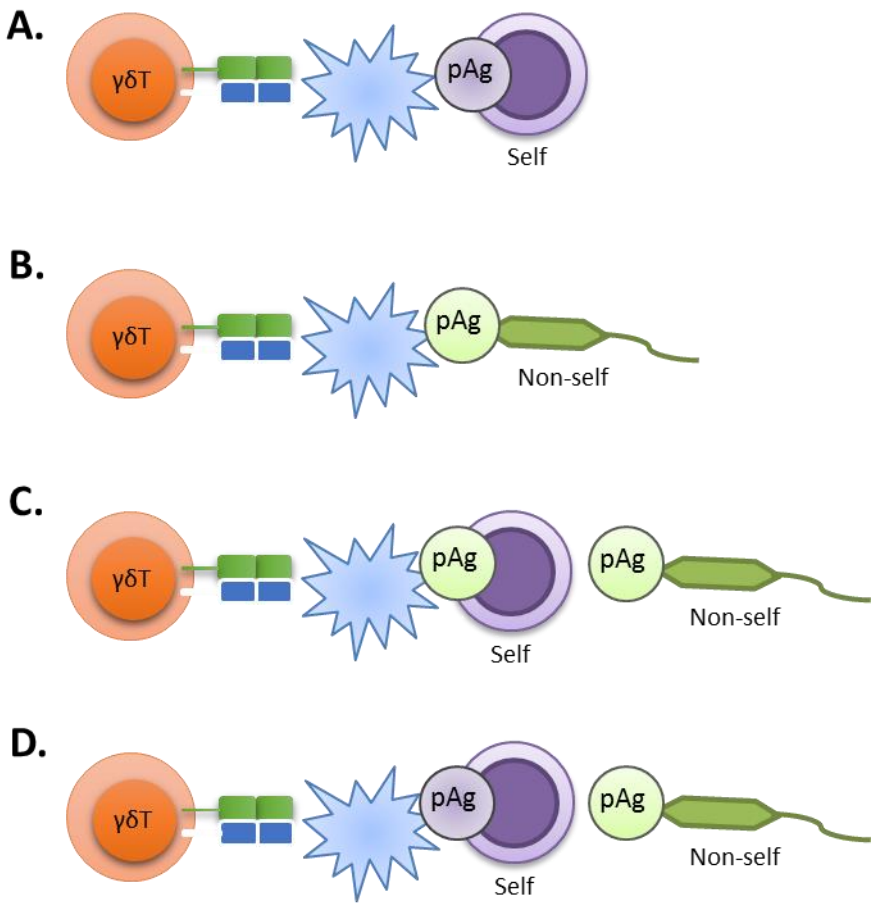

Fig. 1.3. γδT cell recognition of self and non-self phosphoantigen danger signals. It is known that γδT cells can recognise efficiently both endogenous cellular stress and invading microorganisms. While this can occur in a phosphoantigen (pAg)-dependent manner, exact mechanisms of recognition are lacking. Four different mechanisms of pAg detection in a self and non-self context are possible: (**A**) Direct detection of self pAgs, induced by self-stress, *e.g.* transformation or drugs such as zoledronate, (**B**) Direct detection of non-self pAgs, sourced from invading pAg-expressing microorganisms, *e.g. E.coli* (**C**) Direct detection of non-self pAgs, but in a manner that is dependent on self cells manifesting these non-self pAgs, *e.g.* via HMBPP-induced butyrophilin changes, or (**D**) Indirect detection of non-self pAgs, wherein non-self stressors such as *E.coli* induce self-stress and consequent accumulation of self pAgs that are then detected by γδT cells on self cells.

It remains important to consider in this context the extreme complexity of the immune environments that γδT cells predominantly operate in. It is conceivable that the gastrointestinal mucosa, an epithelial landscape densely populated by various immune cells and trillions of commensal microorganisms, requires effector cells sensitive to signals of self-stress as well as foreign pathogens. It is likely, thus, that γδT cells employ avenues in addition to the TCR and its machinery to fine-tune antigen recognition and determine appropriate effector function. Work to clarify the role in antigen specificity of the γδTCR *versus* the wide variety of other classically lymphoid and myeloid-associated pathogen recognition receptors (PRRs) that have been found on γδT cells, including NK-like receptors, Fc receptors (FcRs) and Toll-like receptors (TLRs) (reviewed by Hayday and Vantrout (7)), is crucial if we hope to manipulate antigen-specific γδT cell responses in a clinical setting.

The concept of a predominating γδT cell autoreactivity complicates their categorisation into traditional αβT cell memory phenotypes, such as "effector memory" or "naïve", given that these are traditionally prescribed as resulting from various degrees of antigen exposure. As memory phenotype remains a traditionally important parameter in predicting T cell behaviour in response to antigen, numerous cell surface markers, including CD27, CD45RA, CD45RO, CD62L, CCR7, and combinations thereof, have been used to attempt γδT cell classification into memory phenotypes with homologues in the αβT cell compartment. General consensus is lacking, and, importantly, the relevance of these markers in the context of classical αβT cell memory subsets for γδT cells is unclear at best. Similar lack of clarity persists with other classic and important parameters of T cell physiology. Markers, such as CD69 for early activation or PD-1 for exhaustion, for example, are thought to represent γδT cell parallels to αβT cell behaviour, but whether these are the definitive readouts of γδT cell activation or exhaustion states remains to be established. I proceed, nonetheless, with their use in investigation for want of obviously better current alternatives.

1.6. Categorisation of γδT cell subsets

Significant efforts have been poured into establishing reproducible and clear human γδT cell functional subsets, providing more detail than merely a reference to the expression of a Vδ1 or Vδ2 TCR chain. Particularly important in the pursuit of γδT cells as immunotherapeutic tools is the classification of their effector subsets prior to and post stimulation. Expansion *ex vivo* has been a significant component of the γδT cell field since its inception. The relative scarcity of γδT cells in human peripheral blood has necessitated expansion as a means of amplifying cell number for use in functional assays, protein analyses and other experimental probes. Additionally, the promise that γδT cells have demonstrated in the clinic has created a new urgency in establishing effective, reproducible γδT cell expansion protocols, with a detailed understanding of the effector subsets of expanded cells therein. Novel approaches for γδT cell amplification are being developed as technological advances permit deepening examination of the poorly understood epithelial immune compartment, where γδT cells can constitute up to 60% of total intraepithelial lymphocytes (IEL) (7). While expansion with stimuli such as IPP, zoledronate and concanavalin A (ConA) have become a routine in basic science as well as clinical γδT cell manipulation, our understanding of specific stimuli that drive and regulate γδT cell expansion remains limited (4,103,105). Moreover, most established protocols appear only effective in expanding the peripherally dominant Vγ9Vδ2 γδT cell subset. Significant donor variation in terms of rate-expansion remains an issue in optimizing γδT cell use in the clinic; the factors, which impact Vγ9Vδ2 cell expansion rate, remain uncertain.

Fascinating insight into the functional subgroups of human Vδ2 cells has been provided by a recent publication from the group of Pennington *et. al.* (106), which describes the surprisingly stable yet heterogeneous populations of peripheral Vδ2 cells in 63 healthy individuals, which were found to be independent of donor age, sex, ethnicity or, apparently, previous pAg exposure. They were able to identify two predominant Vδ2 cell populations, which were deemed $\gamma\delta^{(28+)}$ and $\gamma\delta^{(16+)}$ based on their respective expression of either CD28 or CD16. It was observed that $\gamma\delta^{(28+)}$ cells exhibited a $CCR6^{pos}$ phenotype,

which futher correlated with increased expression of cytokine receptors (*e.g.* IL-18R) and chemokine receptors (*e.g.* CCR2 and CCR5), as well as granzyme K. $\gamma\delta^{(16+)}$ cells, meanwhile, favoured expression of CXCR1, and presented with an increased perforin/granzyme B cytotoxic potential, paired with lowered proliferative capacity. Such observations are likely to carry significant implications for the design of personalized, cell-based anti-tumour therapies, whence a match between tumour cell type (which may be *e.g.* granzyme B inhibitor, PI-9, positive) and immune effector cell killing mechanism is important.

Meanwhile, in my characterization of unstimulated or differentially-expanded Vδ2 cells, I placed a particular emphasis on their expression patterns of APC molecules, MHC class II (HLA-DR, specifically) and co-stimulatory CD86, as well as homing receptors, chemokine receptor 7 (CCR7) and CD62L (also known as L-selectin). HLA-DR and CD86 expression has been documented on human γδT cells in various contexts, ranging from *in vitro* stimulation to *in vivo* infectious disease. Both endogenous pAgs and Gram-negative bacteria-stimulated γδT cells have been reported to upregulate expression of HLA-DR and CD86 (102,105). The dynamics, time scale and stability of this upregulation has, however, not been reported in detail.

CCR7, a homing receptor expressed on naïve T cells and activated APC, plays a central role in cell migration to lymphoid tissue. Its corresponding ligands, CCL19 and CCL21, are expressed strongly in the thymus and lymph nodes. Naïve $CCR7^{pos}$ T cells continue re-circulation through blood and the lymphatic system until encountering an activating APC, at which point CCR7 is downregulated and chemokine receptors for tissue-specific migration are upregulated. Central memory T cells (Tcm) engage in a similar blood-to-secondary lymphoid organ re-circulation and, as naïve T cells, downregulate CCR7 and upregulate tissue-specific homing receptors upon activation (107). CD62L is a lymphoid homing receptor functionally closely related to CCR7, and mediates blood-borne T cell rolling on high endothelial venules, allowing their extravasation into lymphatic circulation. CCR7 and CD62L are both used as classic markers of T cell migration to secondary lymphoid tissue.

CCR7 upregulation on APC, such as dendritic cells (DC), is, meanwhile, a marker of cell activation. Upon encounter with appropriate stimulation (*e.g.* invading bacteria) tissue-resident DC are activated, upregulate CCR7 and migrate to secondary lymphoid tissue. Once there, DC engage in presentation of phagocytosed and processed antigen to re-circulating T cells (108,109). A similar role, although less well understood, has been ascribed to CD62L. The two main sources of DCs to lymph nodes are thought to be peripheral DCs entering via afferent lymph and monocyte-derived DCs entering via high endothelial venules. Luster *et al.* (110) have produced a comprehensive review on the plethora of chemokines and chemokine receptors involved in shaping lymphoid as well as myeloid migration during the immune response.

While CCR7 and CD62L represent but a fraction of potentially significant γδT cell homing receptors, I chose to focus on them set against the curious background that these receptors appear to follow different patterns post-activation, depending on whether expression is examined in T cells or myeloid APC. If peripheral γδT cells do indeed perform an APC function during systemic infection - such as modeled in my experimental setup - does this APC function occur in blood or in secondary lymphoid tissue? Are APC and homing marker expression patterns on γδT cells reminiscent of those on classic αβT cells or myeloid APC? Moreover, does PBMC stimulation with the highly differential stimuli of zoledronate (representing 'self') or *E.coli* (representing 'non-self') induce similar upregulation of APC molecules, MHC class II and CD86, and lymphoid homing markers, CCR7 and CD62L? Finally, are these similarities or differences in differentially-expanded γδT cell APC and homing phenotype reflected in the TCR repertoire?

1.7. Role of the TCR in γδT cell physiology

Meaningful discussion of the nature and manipulation of γδT cell effector phenotypes would be incomplete without examination of the factors that influence the development of said phenotypes. Stimulation via the TCR is central to thymocyte development and effector T cell function. It determines the deletion or maintenance of a nascent T cell, its

activation status and subsequently regulates its 'fate decisions'. It is striking that decades after its discovery and establishment of its importance in the functioning of a healthy immune system, the exact mode of TCR engagement – even in canonical αβT cells – remains unclear. A number of models have been proposed, and their popularity in the field ebbs and flows with the income of new experimental evidence. Van der Merwe and Dushek have recently compiled a comprehensive review of various models of TCR triggering (111), accompanied by helpful diagrammatic material. In the interest of accuracy and clarity, they have defined 'TCR triggering' as: "[t]he process by which TCR binding to peptide-MHC molecules leads to biochemical changes in the cytoplasmic regions of the CD3 complex […]". These models are based on the central dogma that all TCR CD3 complex subunits contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains, which – via biochemical modification - are the central mediators of T cell activation resulting from TCR triggering. Phosphorylation by the Src family kinases, Lck and FYN, has been proposed as one of these essential biochemical ITAM changes. The models described have been organized into three main groups, depending on the primary mechanism proposed: i) aggregation, ii) conformational change and iii) segregation or re-distrubtion.

Models within the 'aggregation' group include: a) co-receptor heterodimerization and b) pseudodimerisation. Briefly, the co-receptor heterodimerisation model (arguably, the most popular and widely accepted model) proposes that co-receptors bind to the same peptide-MHC complex as the TCR, which then brings co-receptor-associated Lck into proximity with CD3 complex ITAMs, resulting in phosphorylation. A basic illustration of the heterodimerization model can be viewed in Figure 1.4. The pseudodimer model postulates that two TCRs are brought together by one of the two binding low-affinity self, and the other – binding high affinity self or non-self peptide-MHC complexes. The co-receptor associated with the low affinity TCR then engages the agonist (high affinity) peptide-MHC complex that has been bound by the high affinity TCR, thereby forming a dimer between the two TCRs. This then permits co-receptor-associated Lck phosphorylation of both TCR ITAMs.

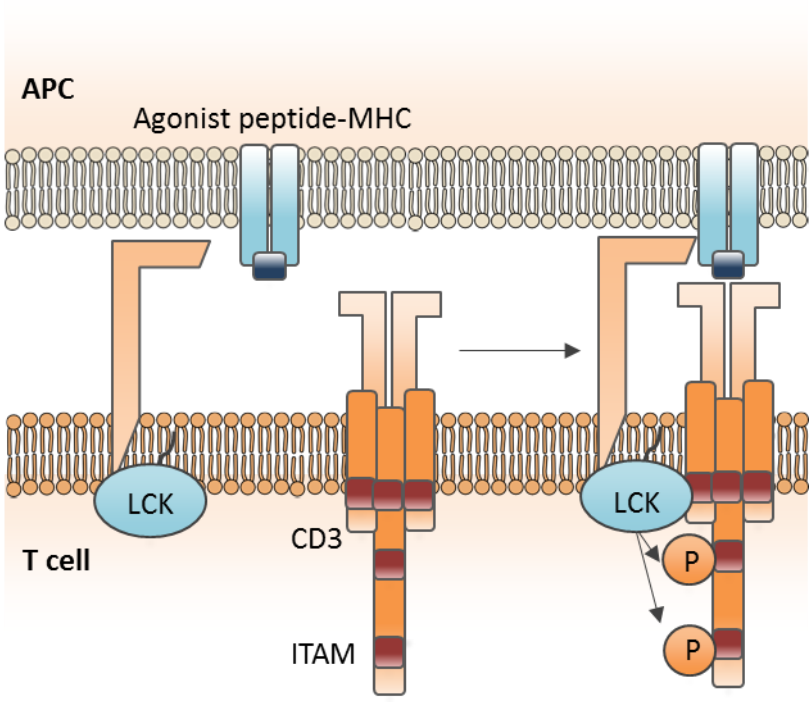

Fig. 1.4. Co-receptor heterodimerisation aggregation model of TCR triggering. The model proposes that co-receptor binding to the same peptide-MHC complex as the TCR brings co-receptor-associated Lck into proximity with TCR-CD3 ITAMs, thereby enhancing phosphorylation. Elements of this diagram have been adapted from van der Merwe and Dushek.

Models of the 'conformational change' group include: a) piston-like movement and b) induced clustering. The piston-like movement model describes mechanical 'pulling' of the TCR by cognate peptide-MHC complex, which then drives phosphorylation of the CD3 ITAMs. The induced clustering model argues that TCR clustering is induced by a conformational change in the peptide-MHC-engaged TCR-CD3 complex, which then enhances kinase activity and leads to the phosphorylation of CD3 ITAMS.

'Segregation or re-distribution' models include: a) kinetic segregation and b) lipid rafts. Briefly, the kinetic segregation model proposes that TCR binding to peptide-MHC ligand traps the TCR-CD3 complex in close-contact zones, thereby segregating it from inhibitory signals (*e.g.* inhibitory tyrosine phosphatase CD45) leading to stable phosphorylation of ITAMs by LCK. Lipid raft models postulate that peptide-MHC engagement results in partitioning of the TCR-CD3 complex into regions of membrane enriched in Lck and deficient in CD45.

It is conceivable that elements of all of the above-described models play a role in TCR triggering; each model applicable in a particular context, depending on, for example, the type of co-receptors a T cell expresses or the type of antigen presenting cell that is engaging the T cell at hand. It is important to note that the mechanistic aspects of TCR engagement by target do not end with triggering. Questions regarding subsequent events, such as "How is productive TCR signaling different from non-productive signaling? " and "How is productive signaling achieved?" are yet to be answered definitively. A multitude of further models that consider the mechanisms of post-triggering TCR signaling, including kinetic 'proofreading', segregation, sequestration of ITAMs, allosteric malleability and serial triggering, are evaluated by Chakraborty and Weiss (112). As with models of TCR triggering, their discussion of various proposed mechanisms makes several assumptions about the processes that occur. They too place importance on Lck, as well as a tyrosine kinase, Zap70. An emphasis is placed on the interactions between Lck, Zap70 and subunits of the invariant signaling protein CD3 complex, [αβTCR] co-receptors CD4 and CD8, and modulatory molecules, therein. Specifically, Chakraborty and Weiss describe a new model of TCR signaling, whereupon TCR engagement by peptide-MHC invokes basal phosphorylation of CD3, which then poises Zap70 for activation by Lck

largely via stabilization of 'active' Lck. A significant role is ascribed to the formation of TCR microclusters in permitting signal amplification by a triggered TCR.

Navarro and Cantrell have compiled current thought on what takes place after a TCR has been triggered and engaged productively (113). Once again, several different models persist, but most include signal transduction by serine threonine kinases. Membrane-proximal signals from TCR activation are transduced to elicit cytosolic and nuclear responses. TCR signaling triggers increased levels of second messengers (Ca^{2+}, diacylglycerol, PI3K, etc.), which facilitates activation of downstream kinases (protein kinase C, PDK1, Akt, etc.), each of which play a role in the unfolding signaling cascades. The signaling outcome of productive TCR engagement is the influencing of transcription factor activity, and, ultimately, altered gene expression and metabolic activity. Malissen *et al.* have described that Zap70 activation, downstream of productive TCR engagement, exerts an influence even on such T cell functions as rearrangements of the actin cytoskeleton and integrin affinity (114).

Canonical αβT cell TCR signaling, thus, remains an expanding, but, as of yet, contested intellectual landscape. Controversy persists regarding even basic conceptual models. A whole further level of complexity and controversy is introduced when contemplating signaling of the atypical, poorly understood γδT cell population. Rudimental understanding on γδTCR engagement of targets is lacking. It is unclear, for example, if γδTCR engagement is even necessary for the cells to exert many of their effector functions. It is unclear whether a TCR-MHC-peptide triad homologue exists for the γδTCR; some have even suggested that γδT cells are capable of recognizing free antigen, in the same way that B cells do (7). Data published by various groups, in various *in vitro* scenarios, of various donor species is frequently contradictory. As outlined in previous chapters, there are indeed significant species differences, as have been described for mouse *versus* human γδT cells. Subset and anatomical localization differences are likely additional factors explaining the heterogeneity of data generated. The recognition of 'stressed self' – if that is indeed what some (or all) γδT cells recognize – is particularly difficult to study. This is especially so, when considering that most γδT cells in the human body reside at epithelial surfaces, which are, as of yet, nearly impossible to replicate fully

in vitro. γδT cells are, moreover, known to express a plethora of non-TCR receptors implicated in the recognition of stressed or transformed self (*e.g.* NK cell receptors, such as NKG2D), making dissection of the unique role of the TCR exacting.

A list of documented human γδT cell clones can be viewed in Table 1.5, elements of which have been adapted from a review by Carding and Egan (8). While representing a mixture of suspected self and non-self TCR specificities, definitive evidence as to the exact mode of antigen engagement (*e.g.* crystllized TCR-antigen-antigen presenting molecule complex) is limited. It is possible that most (if not all) documented non-self ligand triggering of γδT cells in a TCR-dependent manner is merely a reflection of non-self induction of self-stress molecules, which are then ultimately what is recognized by the γδTCR. It is possible that the answer to this question is γδT cell subset-specific.

Table 1.5 | Characteristics of human γδT cell clones

γδTCR composition (origin)	Antigen Specificity	Functional phenotype	Biological function	Ref.
VγX-Vδ1 (IEL lines and clones)	MICA, MICB	Cytotoxicity	Elimination of stressed/transformed cells	(154)
VγX-Vδ1 (synovial fluid)	Lipohexapeptides + DC + IL-2	?	Fas-mediated modulation of immune responses to infection	(155,156)
VγX-Vδ1 (peripheral blood)	HLA-A2, HLA-A24	Cytotoxicity, Th1 cytokine release	?	(157,158)
VγX-Vδ1 (peripheral blood)	CD1c	Cytotoxicity	?	(159,160)
Vγ9-Vδ2 (peripheral blood)	Organic phosphoesters, alkylamines, nucleotide-conjugates and cell-cell contact	Cytotoxicity, Th1 cytokine release	Unique role in immunity to infection, homeostatic control of immune and non-immune processes?	(117,161, 162)
Vγ9-Vδ2 (peripheral blood)	Pamidronate + monocytic APCs	Cytotoxicity, IFN-γ release	Elimination of myeloid-cell targets?	(163)
Vγ9-Vδ2/1 (peripheral blood)	Staphylococcal enterotoxin	Cytotoxicity	Anti-microbial immunity?	(164,165)
Vγ9-Vδ2 (synovial fluid)	Mycobacterial Hsp65 / tetanus toxoid + HLA-DR	Cytotoxicity	Elimination of autoreactive cells?	(166,167)
Vγ9-Vδ2 (peripheral blood)	GroEL heat shock protein	Cytotoxicity, cytokine release	Tumour surveillance?	(168)

The recognition of 'self' by the TCR has been discussed by Hogquist and Jameson (131). Their focus, in particular, was the functional consequences of TCR signaling in CD4+CD8+ double-positive thymocytes and mature T cells. They argue that all T cells (canonical αβT cells in this instance) are at least partially self-reactive, as they recognize a diverse repertoire of peptides, but only in combination with self MHC molecules. They further highlight the classic model of thymic T cell selection, whereupon thymocytes undergoing positive *versus* negative selection present with significantly different Ca^{2+} flux patterns. Strong TCR signaling in the thymus generally leads to negative selection, whereas a weaker or 'intermediate' signal leads to positive selection. Importantly, however, they note that multiple T cell subsets, including IELs (which constitute the majority of human γδT cells), invariant NKT cells and regulatory T cells, require strong TCR signaling for selection. It is, therefore, clear that factors other than TCR signal strength alone are involved in shaping of the TCR repertoire. What these factors may be remains poorly understood, although anatomical location of the selection process or origin of the T cell may play a role.

The sensitivity of a TCR to high- or low-affinity peptide interactions evolves as cells develop from CD4+CD8+ double positive thymocytes to mature T cells (131). Over the developmental process, T cells appear to dampen their sensitivity to self peptide-MHC complexes. This process of 'tuning' is parallel to alternations in the gene expression profile of the cell, as well as changes to basal intracellular Ca^{2+} concentration. Nonetheless, basal TCR signaling in response to self peptide-MHC takes place throughout the lifetime of a naïve T cell, and is likely necessary for homeostasis.

Fascinating insight into the possible TCR dependency of human γδT cells has been provided by recent investigations of the mouse γδT cell compartment. Sutton *et al.* (132) found that γδT cells rapidly produce the potent pro-inflammatory cytokine IL-17 in response solely to stimulation with IL-1 and IL-23. This stands in stark contrast to the dogma that governs our understanding of canonical T cells, which are thought to engage potent effector function only after receiving a stimulus via the TCR. The cells producing IL-17 in response to cytokine stimulation alone were negative for the co-stimulatory receptor CD27, and were subsequently deemed 'innate-like' γδT cells (133). Such 'innate-

like' IL17^{pos} γδT cells are particularly abundant in murine and human neonates; their role has, thus, been postulated as one of rapid anti-microbial (anti-fungal and anti-bacterial, specifically) responders in vulnerable young animals. The same cells have further been implicated in a number of immunopathologies of both the young and adults in human and animal models, including psoriasis, rheumatoid arthritis and experimental allergic encephalomyelitis (134–139).

Mouse IL-17A-producing CD27^{neg} 'innate' γδT cells were considered to emerge by default from thymic progenitor cells that receive only weak TCR signals during development, in contrast to the strong signals received by CD27^{pos} 'lymphoid' γδT cells, delineating clearly the TCR as less important for the development and functioning of 'innate' γδT cells (133,140,141). It is known, however, that CD27^{neg} mouse γδT cells constitutively display markers associated with TCR activation (133,139,142). A recent investigation by Wencker *et al.* (143) showed that, in total contrast to previous assumption, CD27^{neg} mouse γδT cells selectively depend on strong TCR signaling, which thereafter alters and significantly mutes their TCR responsiveness. The group, thus, proposes that 'innate-like' lymphocytes reconcile their lymphoid and rapidly-responsive myeloid-like nature as follows: "During the development of innate-like T cells, a requisite activation of TCR signaling components markedly alters the antigen receptor response mode, removing it as the primary checkpoint for peripheral responsiveness. Thus freed, the cells can respond rapidly to innate signals." Wencker *et al.* further propose that this novel, atypical relationship to the TCR may permit innate T cells to utilize their TCR in novel ways. These include steady-state TCR-mediated engagement of epithelial cells by IELs or particular responses to large amounts of antigen in the context of particular cytokines (139,144). As such, TCR-expressing, 'innate' cells offer a vast expansion of the innate immune response repertoire. Indeed, γδT cells are known to express TCR repertoires that range from oligoclonal to polyclonal, depending on the subset and species of origin at hand (7). An 'innate' cell with a TCR may, moreover, provide an additional layer of quality control for lymphocytes entering the innate compartment.

While providing a fascinating insight into the workings of 'innate' mouse γδT cells, I emphasize that it is unclear what bearing this may have on human γδT cell counterparts. I stipulate that similar dependence on TCR may be found with human γδT IELs (largely of the Vδ1 subset). Importantly, not all mouse γδT cell subsets display TCR independence for the engagement of effector function, nor are all subsets stimulated by exposure to cytokines alone. It is possible that human γδT cells display a similar subset-specific dependence on the TCR.

1.8. Role of the BTN3A in γδT cell physiology

Detailed examination of human γδT cells function, particularly that of the peripherally dominant Vγ9Vδ2 subset, would be amiss without consideration of the role of the B7-like butyrophilins (BTN). Study of the B7 co-stimulatory immunoglobulin superfamily has expanded our understanding of the factors that regulate and fine-tune T cell activation. Classic examples of well-studied B7 member interactions include: I) activatory pairing of B7.1 (CD80) and B7.2 (CD86) on APCs to CD28 on T cells, and II) inhibitory pairing of B7.1 and B7.2 on APCs to CTLA4 on T cells. Further well-known and important members of the B7 family include PD1, PDL1 and B7RP1 (also known as ICOSL). Recent discoveries reveal that another immunoglobulin superfamily, closely related to the B7 family, may play a significant role in regulating the immune response.

The BTN immunoglobulin superfamily was first described in the 1980's with the discovery of BTN1 (145). BTN1 (BTN1A1, specifically) was found to play a role in milk fat globule secretion and stabilization. The immune significance of the BTN family took decades to establish and is being unraveled still. With the number of known human BTN family members currently at 13, their roles appear almost entirely geared toward immune regulation. Even BTN1A1 has been ascribed a role in thymic stromal and B cell interactions with activated T cells and macrophages (146). While the exact immunomodulatory roles of BTN family members remain unclear, two main lines of evidence speak to their putative importance: i) the high phylogenetic homology of BTN

and B7 proteins, and ii) the extremely broad BTN expression on immune cells or cells that interact closely with immune cells (*e.g.* thymic stromal cells and intestinal epithelial cells). The multiple similarities between the BTN and B7 immunoglobulin superfamilies was explored recently by Arnett and Viney (147). Perhaps most obviously, the two protein families share high structural homology. Membership of the B7 family is largely defined by the presence of a small intracellular cytoplasmic tail and a transmembrane region linked to extracellular IgV and IgC domains, which then confer receptor binding specificity that is mainly for other immunoglobulin superfamily members (148). Similarly, BTNs have two extracellular IgV and IgC domains, as well as a transmembrane region. In a divergence from the B7 family, most members of the BTN family further contain an intracellular B30.2 signaling domain (illustrated in Fig 1.5).

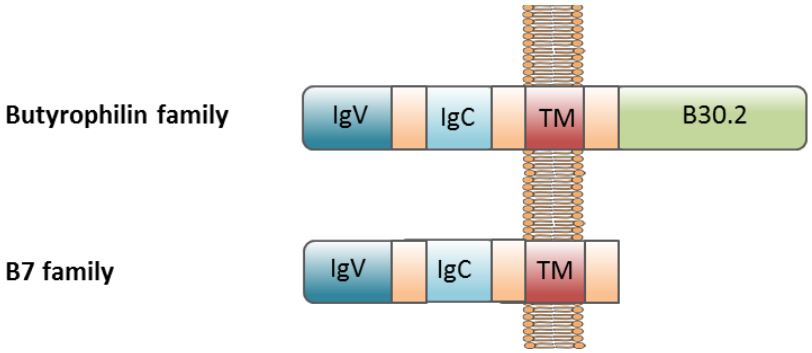

Fig. 1.5. Structural comparison of the B7 and BTN superfamilies. Both families share hallmark extracellular immunoglobulin domains, typically of IgV and IgC type. While the B30.2 domain is a common feature of BTNs, no known B7 members contain intracellular signaling domains. (TM, transmembrane domain)

Not a great deal is known about the specific cell surface binding partners of the BTN family, although it does not appear that these include the classic B7 targets of CD28, CTLA4, ICOS or PD1 (149,150). The intracellular binding interactions of BTNs are, meanwhile, governed by the B30.2 protein-binding domain. B30.2 binding partners can range from the enzyme xanthine oxidoreductase for BTN1A1 (151) to prenylated pAgs for BTN3A.1 (152). Interestingly, the B30.2 domains of these different BTNs do not appear cross-reactive in terms of binding partners. The B30.2 domain is defined by the presence of a PRY/SPRY element, which is composed of three specific sequence motifs, LDP, WEVE and LDYE. It derives its name from the inclusion of a SPRY domain, which is preceded by an amino-terminal PRY motif (153). The B30.2 domain is not exclusive to the BTN family, and has been identified in a total of 51 genes in the human genome, where it appears to play a role in mediating protein-protein interactions (154). Few specific binding partners are known.

The BTN3A (also known as CD277) subfamily of BTNs (BTNA.1, BTNA.2, BTNA.3) is expressed by most human immune cells, myeloid and lymphocytic in origin. These include T cells, B cells, NK cells, DCs and monocytes (149). A number of antibodies have been generated against all BTN3A subsets. Most of what we know about the importance of BTN3A in the functioning of γδT cells has been derived from these antibody studies, to which a significant contribution has been made by Dr. Daniel Olive's research group (Marseille, France). A summary of published anti-BTN3A antibodies can be viewed in Table 1.6.

Table 1.6 | Characteristics of published anti-BTN3A mAbs

Clone Identity	Effect on BTN3A	Known functional outcomes	Ref.
19.5	Agonistic to BTN3A.1	Binds to BTN3A directly on T cells, NK cells, monocytes and DCs, delivering an activatory signal. Results in enhanced proliferation, survival and cytokine production, incl. IFN-γ, IL-1, IL-8 and IL-12.	(200)
20.1	Agonistic to all isoforms		(201)
103.2	Antagonistic to all isoforms	Sterically blocks association of proteins engaged by BTN3A.1 during activation. Known to block Vγ9Vδ2 T cell BTN3A.1-mediated activation.	(202)
232.5	Agonistic to BTN3A.3	Phosphorylates BTN3A.3 and initiates inhibitory signalling. Inhibits T cell function.	(203)

Observations from the various anti-BTN3A antibody clones indicate that these can be both, agonistic and antagonistic. The specific binding site and mode of engagement are what appear to govern the functional outcome of mAb engagement of BTN3A, ranging from phosphorylation to steric blocking (Table 8 and associated references). It is interesting to note that, due to the wide lymphocytic expression of BTN3A, anti-BTN3A mAb can exert dual effects on T cell activation: i) indirect effects, such as altering BTN conformation on target cells (*e.g.* monocytes) or affecting bystander cell activation with consequent pro- or anti-inflammatory effects, and ii) direct effects, such as activatory cross-linking of BTN on T cells themselves.

Comparing the different BTN3A isoforms, it is known that each contains an extracellular N-terminal IgV and a membrane-proximal IgC domain, connected to a single-pass transmembrane domain. BTN3A.1 and BTN3A.3 further contain an intracellular B30.2 domain, which is absent in BTN3A.2 (159). Importantly, while all three isoforms mediate Vγ9Vδ2 cell activation if treated with the agonistic mAb 20.1, only BTN3A.1 mediates pAg-induced activation (100,157). This alludes to i) high extracellular homology of the BTN3A isoforms, and ii) the recognition by Vγ9Vδ2 cells of extracellular BTN3A conformational changes. A basic summary of BTN3A isoform differences can be viewed in figure 1.6. Curiously, BTN3A.3 was not able to induce Vγ9Vδ2 cell activation in a pAg-dependent manner, despite containing a B30.2 domain. Sandstrom *et al.* recently demonstrated that this is due to a single amino acid difference between BTN3A.1 and BTN3A.3-associated B30.2 domains, which, in the case of BTN3A.3, impedes effective pAg binding (152).

Upon co-crystallization of pAg and BTN3A.1-associated B30.2, Sandstrom *et al.* provided definitive evidence for the importance of the B30.2 domain in acting as an intracellular pAg 'sensor' that communicates stress to Vγ9Vδ2 cells via its extracellular BTN3A portion (152).

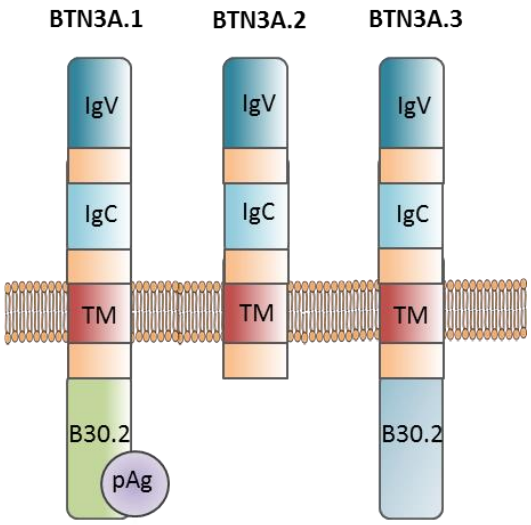

Fig. 1.6. Different isoforms of BTN3A. All isoforms share the same structure of the extracellular domain. Both BTN3A.1 and BTN3A.3 contain further intracellular B30.2 domains. Due to a single amino acid change, however, only BTN3A.1. B30.2 is capable of effectively binding pAg and, thus, transmitting the presence of intracellular pAg to Vγ9Vδ2 cells. (TM, transmembrane domain)

In contrast to the clarity of pAg-B30.2-BTN3A interactions, little is understood about how exactly the binding of pAg to BTN3A.1 B30.2 is recognized by Vγ9Vδ2 cells. Rhodes *et al.*, who were crucial in the discovery and characterization of human BTN3A, have recently described three different scenarios by which BTN3A.1 may mediate Vγ9Vδ2 cell activation (160). These are summarized in figure 1.7.

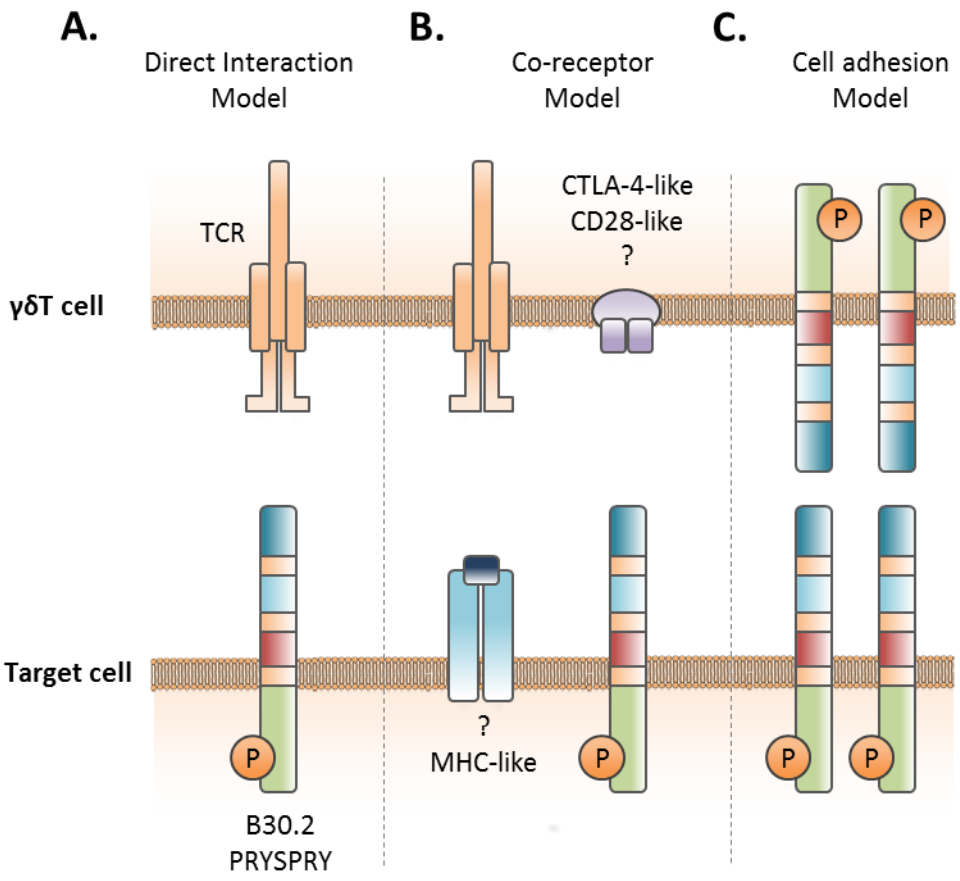

Fig. 1.7. Models of γδT cell activation by BTN3A. (**A**) The Direct Interaction model proposes that BTN3A.1 and its conformational changes are the ligand for the Vγ9Vδ2 TCR. (**B**) The Co-receptor model proposes that the Vγ9Vδ2 TCR interacts with an MHC-like molecule, while BTN3A interacts with a B7-like (e.g. CTLA-4-like or CD28-like) molecule. (**C**) The Cell-Adhesion model proposes that BTN3A engages in homotypic interactions, which promote cell contact between the Vγ9Vδ2 and potential APC.

While all of the models described by Rhodes *et al.* are based on the finding that it is specifically BTN3A.1 that is critical for activatory signal transmission to Vγ9Vδ2 cells, they highlight that data indicates there is a role for the BTN3A.2 and BTN3A.3 isoforms and the recruitment of periplakin to stabilize the complex in the cell membrane (161). The three models they describe are, briefly: I) the Direct Interaction model, in which BTN3A.1 (or specific conformation, therein) is a ligand for the γδTCR (Fig.1.7A), II) the Co-receptor model, which assumes that γδT cell activation occurs much more along the lines of canonical T cell – APC interactions; in this model, the γδTCR recognizes an MHC-like molecule, while BTN3A acts as a co-receptor, likely binding to CD28-like or CTLA-4-like molecules; importantly, no such target molecules for the γδTCR or BTN3A have (yet) been identified (Fig.1.7B), III) the Cell Adhesion model, whereupon BTN3A molecules, in given conformation, simply act as adhesion factors promoting cell-cell interation in a pAg-dependent manner (Fig.1.7C). Whether the γδTCR is engaged directly by BTN3A, thus, remains unclear.

In fact, so little is understood about the direct nature of BTN3A-Vγ9Vδ2 contact, that one is forced to expand the intracellular pAg-B30.2-BTN3A model. Acknowledging that pAg binding to B30.2 of BTN3A.1 is indeed activatory to Vγ9Vδ2 cells *in vitro,* Harly *et al.* hypothesize that the reality may be more complicated than that (162). One hypothesis put forth argues that extracellular pAgs are imported into target cells by unidentified transporter(s), or are produced intracellularly. Potentially, both are the case. These pAgs then bind the intracellular B30.2 domain of BTN3A.1, which induces non-exclusive key modifications in the receptor protein, which may include conformational changes, modifications of its membrane topology, recruitment or exclusion of molecular patterns, etc. Any or all of these changes can then be sensed by Vγ9Vδ2 cells. This is, perhaps, the current most popular model among researchers interested in Vγ9Vδ2 cell activation. Alternatively, Harly *et al.* argue, pAg binding could occur intracellularly *as well as* the extracellularly. Accepting that the B30.2-pAg interaction (and BTN3A.1 conformational changes, therein) are important does not exclude the possibility that also a kind of 'antigen presentation' occurs, whereupon the conformationally-changed BTN3A.1 can therefore present pAg to the Vγ9Vδ2 TCR. In contrast to Rhodes *et al.* and their proposed hypothetical mechanisms of activation, Harly and group appear to place significantly more

emphasis on the specific involvement of the TCR in BTN3A.1-triggered activation of Vγ9Vδ2 cells. A basic summary of Harly's different hypotheses can be viewed in figure 1.8. The idea that prenyl pyrophosphates may be presented to γδT cells on extracellular portions of BTN3A.1 has garnered experimental and intellectual support from a number of groups and researchers, including Vavassori *et al.*, Kabelitz *et al.*, De Libero *et al.* and Esser *et al.* (163–165).

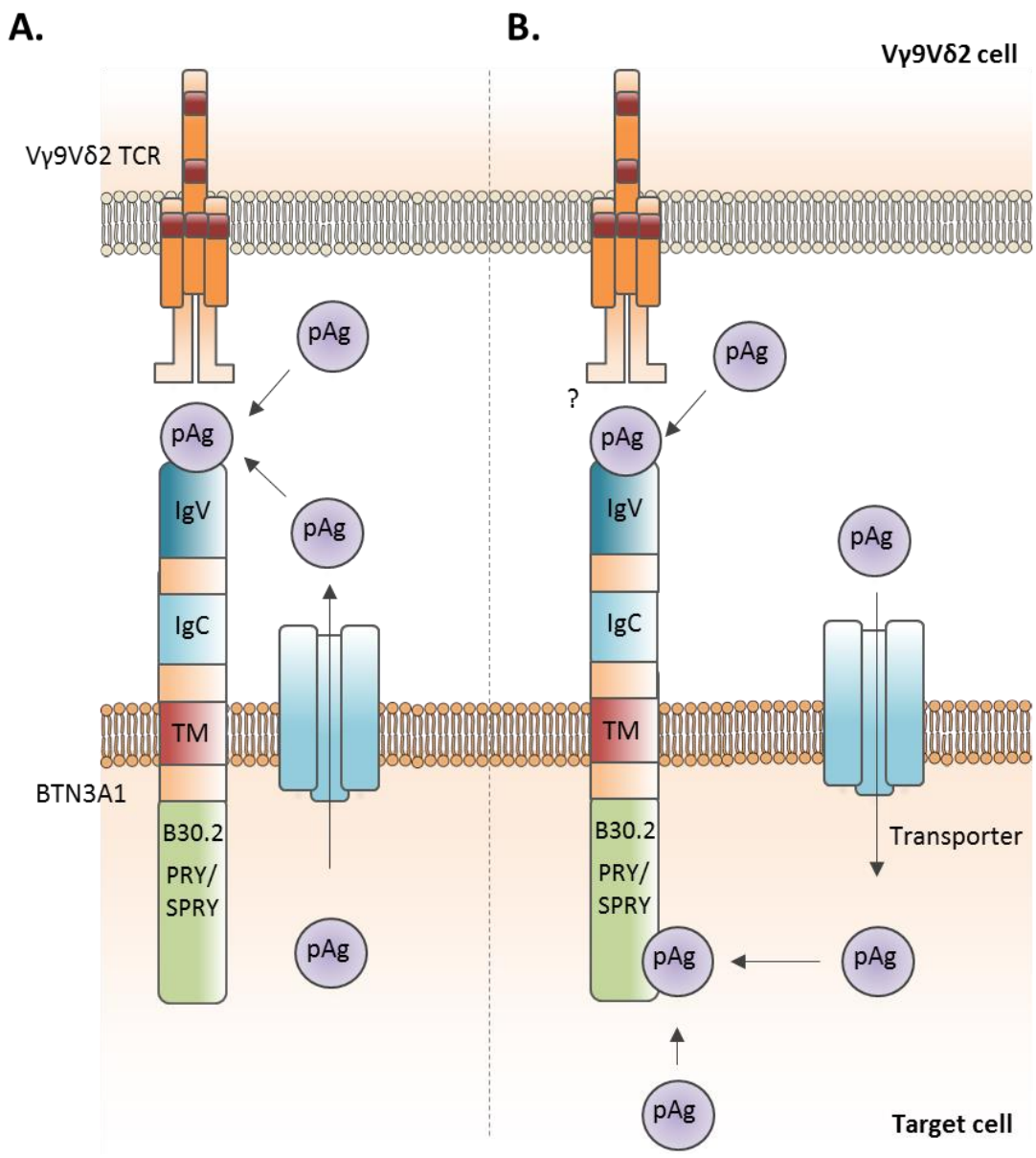

Fig. 1.8. Alternate models of pAg sourcing for Vγ9Vδ2 cell activation via BTN3A.1. Several competing and, possibly, complementary hypotheses exist of Vγ9Vδ2 cell activation by pAg via BTN3A.1 as put forth by Harley *et al*. (**A**) One hypothesis argues that intracellular pAg are exported from the cell by unidentified transporter(s), or are found in the extracellular environment, or both. These pAgs then bind BTN3A.1 on its extracellular portion (e.g. via the IgV). It is then the combination of pAg + BTN3A.1 'carrier' that is recognized by the Vγ9Vδ2 TCR. (**B**) Another hypothesis stipulates that pAgs are produced intracellularly and sourced from therein or imported from the extracellular space via unknown transporters, or both. Intracellular pAg then binds the BTN3A.1 B30.2 domain, inducing key modifications to the to the receptor protein, such as changes to the membrane topology, recruitment/exclusion of molecular patterns or conformational changes to the receptor. Possibly, extracellular pAg then further binds BTN3A.1 extracellularly. These changes are then recognized by the Vγ9Vδ2 TCR.

Experimental clarification is necessary to establish whether BTN3A extracellular domains are engaged by the Vγ9Vδ2 TCR directly. Even if this is achieved, reconciliation of the various BTN3A-TCR engagement theories and the observation that cross-linking of all BTN3A isoforms with agonistic mAb (*e.g.* clone 20.1) mimics Vγ9Vδ2 cell pAg stimulation is necessary. It is possible that all proposed scenarios are true in particular settings and that significant mechanistic redundancy is in place for the activation of γδT cells by pAg. It is not inconceivable that γδT cells may be capable of sensing pAg-related cellular stress via receptors other than the TCR. As described in earlier sections, it is known that human Vδ2 cells express a range of non-TCR receptors that sense stressed or transformed self, *e.g.* NKG2D.

An important aspect of placing a pivotal role on BTNs in Vγ9Vδ2 cell activation is the consequent apparent divergence of γδT cells from other innate-like human T cels. Like invariant NKT (iNKT) cells or MAIT cells, human γδT cells appear to originate preferentially prior to birth and express semi-invariant TCRs: Vγ9Vδ2 for γδT cells, Vα24-Jα14-Vβ11 for iNKT cells and Vα7.2-Jα22-oligoclonal Vβ for MAIT cells (166). iNKT and MAIT cells do, however, both recognize antigen presented on MHC class I-like molecules, CD1d and MR1, respectively. Thus, unlike nearly all other T cell types, including innate-like αβT cells, Vγ9Vδ2 cell activation appears indepdendent of MHC class-like molecules. As outlined earlier, γδT cells do not appear to share the αβT cell dependence on the presence of professional antigen presentation or specific antigen presenting molecules. And when and if a dependence on a cell surface molecule for activation is observed, it may be instead on B7-like BTNs.

1.9. γδT cells as a tool in immunotherapy

As outlined above, elevated counts of activated, functional γδT cells correlate with improved health outcomes in human infection and malignancy, both solid and non-solid in nature. The physiological rationale for the apparently beneficial presence of γδT cells may derive from their production of inflammatory cytokines, direct target lysis, recruitment and activation of innate immune components, maintenance of epithelial barrier integrity and tissue healing, regulation of B cell immunity and even priming and modulation of the αβT cell response.

Such an expansive array of effector phenotypes for any one cell type carries complications in its use and manipulation in the clinic. It remains unclear whether these functions can be carried out by all human γδT cell subsets and are stimulation-dependent. Is it crucial we focus on Vδ1, Vδ2 or $Vδ1^{-}Vδ2^{-}$ γδT cells? Does sourcing γδT cells from peripheral blood bear consequences on their putative eventual clinical effects at epithelial surfaces? Can the same γδT cells act as pAPCs and killers simultaneously? Questions such as these, among others, need to be resolved prior to the safe and effective introduction of γδT cells into the clinic. If, however, we are able to gain a firm grasp of understanding on this complex and potentially heterogeneous set of $γδTCR^{pos}$ cells, their clinical expediency would be significant. Perhaps the most useful property of γδT cells as an immunotherapeutic tool is the range of effector functions they may offer, uniting current clinical efforts with αβT cells, DCs and other myeloid cells under one cell type. A significant advantage of using γδT cells is their lymphoid capacity for rapid, extensive and sustained expansion *in vivo* and *in vitro,* in contrast to other pAPCs in immunotherapy development, such as DCs. We know, furthermore, that zoledronate – perhaps the most routinely used expansion stimulus for peripheral Vδ2 γδT cells – is safe to use in humans systemically. Zoledronic acid (zoledronate) is already used in the clinic as a treatment for osteoarthritis. It's proposed mechanism of action involves the reduction of osteoclast activity (167).

1.10. Aims of the project

The observations of *in vivo* γδT cell expansion upon clinical infectious disease, and the *ex vivo* work by Brandes, *et.al.* and our own laboratory on the pAPC function of human γδT cells prompted us to investigate how Gram-negative bacteria may impact the effector phenotype of this atypical T cell subset. I modeled the events that may occur during systemic infection by exposing whole, freshly-isolated PBMC to UV-irradiated *E.coli. E.coli* is a common cause of sepsis and bacteremia in humans, and resides as a commensal in the gastrointestinal mucosa highly populated by γδT cells. As mentioned above, *E.coli* further expresses known γδT cell targets such as HMBPP.

I ventured to document freshly-isolated γδT cell effector responses to acute *E.coli* exposure and subsequent responses to re-exposure of expanded cells. I further set out to compare γδT cell effector phenotype in response to two radically different stimuli: i) *E.coli*, representing a target-rich, complex, highly pro-inflammatory non-self stimulus, and ii) zoledronic acid, a small molecule pyrophosphate accumulation-inducing bisphosphonate drug, representing a stressed self stimulus.

2. MATERIALS AND METHODS

2.1. Study Design

This study was designed to investigate how Gram-negative bacteria may impact development of γδT cell effector phenotype. The experimental setup was modeled to reflect events that may occur during systemic infection via stimulating whole, freshly-isolated PBMC with UV-irradiated *E.coli*. γδT cell responses were recorded upon primary stimulation of fresh PBMC and subsequent re-stimulation of expanded cells. I further addressed which γδT cell effector functions are dependent on the γδT cell receptor and butyrophilin 3A.

The sample number for most expansions and experiments was five different donors; donor number is listed for all data in the corresponding figure legend. All donors used were healthy adults, between the ages of 21 and 55 years old, representing a roughly equal mixture of male and female participants. I note that recent research by Pennington and colleagues (106) indicates that gender and age of peripheral Vδ2 cell donors are unlikely to exert significant effects on observed cell behaviour. The number of five donors was chosen from previous experience in the study of known phenotypic variations between donors, in order to accurately reflect these variations. All experiments were repeated at least once, with the exception of DNA sequencing. DNA sequencing was, however, done as a separate experiment and eachclonotype sequencing included in the analysis was represented by multiple individual reads. In addition, spectratyping was carried out on two donors, both of whom yielded similar results.

All experiments using peripheral blood-derived cells were performed in accordance with relevant guidelines and regulations, and were approved by UCL Research Ethics Committee. Informed consent was obtained from all volunteer blood donors.

2.2. Statistical analysis

Where relevant, acquired data was evaluated statistically with paired or unpaired t tests without assumed consistent standard deviation. Statistical significance was assessed through the Holm-Sidak method of correcting for multiple comparisons. The results referred to as "significant" further in the text entail a P value of 0.05 or lower. The statistical and graphic analysis software employed was Prism 6.0. All bar graps indicate the mean and respective error bars indicate standard deviation of the data sets. Individual significant P values are indicated in each figure as appropriate. The individual donor number used to generate the data in each figure is indicated in accompanying figure legends. All figures are indicative of at least two experiments.

2.3. Sample acquisition and preparation

PBMCs from healthy adult donor peripheral blood were routinely extracted via Ficoll density gradient separation. Briefly, peripheral blood was obtained by venipuncture and collected in EDTA-treated containers to prevent coagulation. It was then layered onto Lymphoprep solution and centrifuged at 800g for 35min at room temperature, leading to a layer separation, according to constituent density. Plasma and red blood cell layers were discarded, while the PBMC buffy coat was processed by washing twice in phosphate-buffered saline (PBS; Invitrogen) with centrifugation at 800g for 10min. The PBMCs were then counted, suspended in complete RPMI 1640 at the desired concentration, and either stained immediately or cultured overnight for subsequent investigation.

Cells were cultured in supplemented RPMI 1640 medium at a density of $1.5x10^6$ cells/mL at 37^{o}C and 5% CO_2. Supplemented culture medium contained RPMI 1640-GlutaMax

(Life Technologies), 10% foetal calf serum, 1% Penicillin/Streptomycin (Life Technologies), 10mM HEPES buffer (Life Technologies), 1mM Sodium Pyruvate (Life Technologies) and 1x MEM non-essential amino acids (Life Technologies) (Table 2.1). All stimulation studies, unless explicitly specified, further included 100 IU/mL recombinant human IL-2 (MACS Miltenyi); medium was supplemented every two to three days.

Table 2.1 | **Recipe for complete γδT cell culture medium**

Reagent	**Concentration**
RPMI 1640 GlutaMax	1x
Foetal Calf Serum	10%
Penicillin / Streptomycin	100IU/mL
HEPES buffer	10mM
Sodium Pyruvate	1mM
MEM Non-Essential Amino Acids	1x

2.4. Growth and preparation of *E.coli* DH5α

E.coli (Thermo Fisher) were grown overnight at 37^{o}C shaking culture in 1mL ampicillin (Life Technologies)-supplemented LB medium (Sigma) from cryogenically preserved aliquots. Once grown, *E.coli* culture was washed thoroughly and assessed for colony forming unit (CFU) count via duplicate measurement of suspension optical density (OD), which typically equalled OD ~0.5 after overnight growth. With the exception of killing assays, all bacteria employed in co-culture experiments were irradiated in a trans-illuminator chamber (UVITEC), equipped with eight UV-C (250-280nm) lamps, for a period of at least 7min. Irradiation times were decided based on the minial irradiation period necessary to prevent growth of irradiated *E.coli* in subsequent overnight liquid culture. With the exception of *E.coli*-pHrodo acidification assays, all assays employed freshly-grown *E.coli* DH5α.

2.5. *E.coli* opsonisation

E.coli were opsonized with commercially available highly purified anti-*E.coli* rabbit serum IgG (*Escherichia coli* BioParticles Opsonizing Reagent from Thermo Fisher) according to commercial protocol. Briefly, irradiated *E.coli* were re-suspended in PBS to OD ~0.5, mixed with the recommended saturating dose of purified anti-*E.coli* rabbit serum IgG and incubated for 30min at room temperature. *E.coli* were then washed twice in 30mL of sterile PBS.

2.6. PBMC stimulation with UV-irradiated *E.coli*

Freshly isolated or expanded 1.5x10^{6} cells/mL PBMC were co-cultured with *E.coli* (MOI 10) in supplemented RPMI 1640 medium, and cultured overnight (16-18h) or left to expand for 14 days. Re-stimulation of expanded PBMC with *E.coli* was carried out by mixing 14-day *E.coli*-expanded PBMC with irradiated *E.coli* at MOI 10. Re-stimulation

of expanded PBMC with *E.coli* in the presence of freshly-isolated autologous PBMC was carried out by mixing fluorescence-assicted cell sorting (FACS)-stained 14 day *E.coli*-expanded PBMC with freshly-isolated unstained autologous PBMC at a ratio of 1:10 prior to the addition of irradiated *E.coli* at MOI 10.

2.7. PBMC stimulation with zoledronate

Freshly isolated $1.5x10^6$ cells/mL PBMC were cultured in 5μM zoledronic acid monohydrate (zoledronate; Sigma-Aldrich) in supplemented RPMI 1640 medium, and cultured for 14 days. Re-stimulation of expanded PBMC with *E.coli* was carried out by mixing 14 day *E.coli*-expanded PBMC with irradiated *E.coli* at MOI 10.

2.8. FACS staining and processing

PBMC were stained for cell viability, surface markers, intracellular cytokines and cell surface CD107a throughout stimulation and expansion as indicated in supplied commercial protocols. Intracellular cytokine and CD107a staining was carried out on overnight stimulated PBMC that were cultured for a further 4h in the presence of monensin (BioLegend). Colour compensation was carried out using OneComp eBeads (eBioscience). FACS analysis was performed on the Becton Dickinson (BD) LSR II and data processing - on FlowJo vX.07 software.

The following antibody conjugates were used in PBMC staining: CD3-PE/Dazzle594 (BioLegend; clone: UCHT1), αβTCR-PE (BioLegend; clone: IP26), αβTCR-PE/Vio770 (MACS Miltenyi; clone: BW242/412), γδTCR-PE/Vio770 (MACS Miltenyi; clone: 11F2), Vδ1-FITC (Thermo Fisher; clone: TS8.2), Vδ1-APC (MACS Miltenyi; clone: REA173), Vδ2-PerCP (Biolegend; clone: B6), Vδ2-PE (Biolegend; clone: B6), IFN-γ-PE (BioLegend; clone: B27), TNF-α-APC (BioLegend; clone: MAb11), IL-17-Brilliant Violet 605 (BioLegend; clone: BL168), CD69-PerCP (BioLegend; clone: FN50), IL-10-

FITC (Affymetrix eBiosciences; clone: BT-10), granulysin-PE (BioLegend; clone DH2), CD107a-FITC (BioLegend; clone: H4A3), CCR7-PE (R&D Systems; clone: 150503), CD62L-Brilliant Violet 605 (Biolegend; DREG-56), CD4-FITC (Miltenyi Biotec; clone: M-T466), CD8-APC (Miltenyi Biotec; clone: BW135/80), CD56-PE (Biolegend; clone: MEM-188), CD14-FITC (Miltenyi Biotec; clone: TÜK4), CD16-Brilliant Violet 605 (Biolegend; clone: 3G8), CD19-PE/Cy7 (Biolegend; clone: HIB19), CD33-APC (Biolegend; WM53), CD1c-PerCP/Cy5.5 (Biolegend; clone: L161), CD27-APC/Vio770 (Miltenyi Biotec; clone: M-T271), CD45RA-FITC (BioLegend; clone: HI100), HLA-DR (MHC II)-APC/Cy7 (BioLegend; clone: L243), CD86-APC (MACS Miltenyi; clone: FM95), CD277-PE (clone: BT3.1; Biolegend), Vγ9–APC/Vio770 (clone: REA470; MACS Miltenyi). Mouse IgG1κ of known, irrelevant, non-human specificity served as isotype control (BioLegend; clone: MG1-45). All FACS data presented subsequently is on singlet, live lymphocytes. All further references to 'MFI' denote median fluorescence intensity. The gating strategy employed in analysis is shown below in Fig.2.1. The use of a gating strategy that targets $CD3^{pos}\alpha\beta TCR^{neg}$ cells instead of $CD3^{pos}$ $\gamma\delta T^{pos}$ cells is justified and examined in Fig.2.2. No significant difference in γδT cell content was found using either strategy. $CD3^{pos}\alpha\beta TCR^{neg}$ cells were used to identify γδT cells due to the much brighter expression of αβTCR *versus* γδTCR, thus permitting clearer delineation of the two cell populations, and ensuring that all γδT cells are included in the analysis.

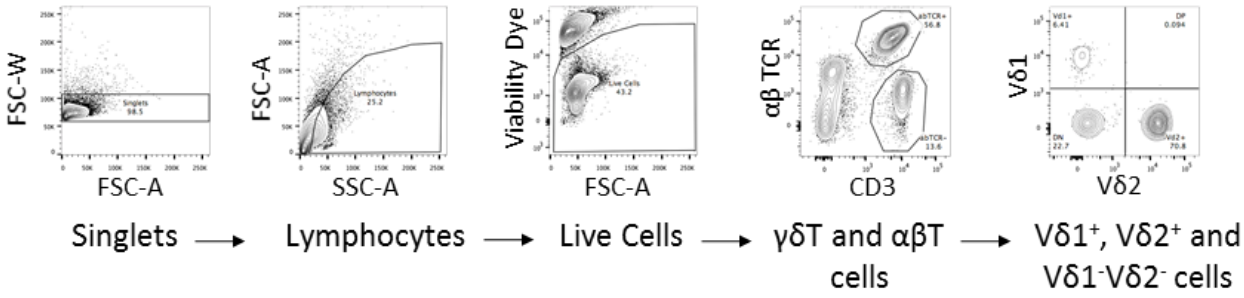

Fig. 2.1. Gating strategy. All FACS data shown for γδT cells is gated on singlet lymphocyte live $CD3^{pos}$αβTCR^{neg} and Vδ chain-determined subsets thereof. αβT cells are designated as singlet lymphocyte live $CD3^{pos}$αβTCR^{pos} cells, respectively.

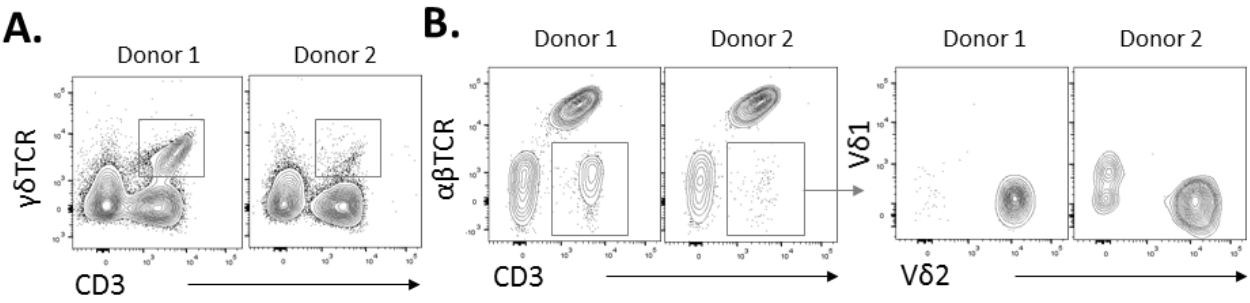

Fig. 2.2. Gating on γδT cells according to γδTCR versus αβTCR. Two methods of gating on γδT cells within whole, live PBMC, were compared. Two donor data is shown on freshly-isolated PBMC; one donor is representative of relatively high resting γδT cell content (~10% of total PBMC), and the other – of relatively low resting γδT cell content (~0.5% of total PBMC). The same anti-CD3 fluorochrome-antibody conjugate is used for both stains. Both anti-γδTCR and anti-αβTCR fluorochrome-antibody conjugates were purchased from the same vendor and are of the same fluorochrome (PE/Vio770). (**A**) γδT cells, gated as $CD3^{pos}$γδTCR^{pos} cells; (**B**) γδT cells, gated as $CD3^{pos}$αβTCR^{neg} cells, with a sub-gate of $CD3^{pos}$αβTCR^{neg} cell expression of Vδ1 and Vδ2.

2.9. Confocal imaging of γδT cell uptake of *E.coli*

Acknowledgement: This preliminary work was carried out by Dr. Anne Marijn Kramer, another PhD student in the laboratory, in collaboration with Dr. Dale Moulding of GOS ICH Imaging Facility. It is included in the present thesis, with her consent, to illustrate the evolution of thought that produced the experimental narrative.

Imaging was performed on a Zeiss AxioObserver LSM 710 confocal microscope. FACS-purified 14 day zoledronate-expanded γδT cells were incubated with IgG-opsonized, IPTG-inducible green fluorescent (GFP)-expressing *E.coli* (Thermo Fisher) for 60min, placed on ice and fixed. Cells were then fluorescently labeled, deposited on cleaned coverslips and mounted on glass slides using ProLong Gold antifade mountant (Thermo Fisher) and cured in the dark at room temperature for 24 hours. Images of cell conjugates were acquired with a 63× Plan-Apochromat oil objective, numerical aperture 1.4. Acquisition was optimized for subsequent deconvolution with Huygens software, using appropriate voxel sizes according to the Huygens Nyquist calculator.

2.10. Confocal imaging of γδT cell uptake of *E.coli*

Acknowledgement: This preliminary work was carried out by Dr. Anne Marijn Kramer. It is included in the present thesis, with her consent, to illustrate the evolution of thought that produced the experimental narrative.

Imaging was performed on an Image StreamMark II flow cytometer (Amnis). Prior to analysis, 14 day zoledronate-expanded γδT cells were incubated with protease-sensitive DQ-Green (Thermo Fisher), BSA-labelled opsonized or non-opsonized polystyrene beads 0.5μm or 1.0μm in size (Polysciences) for 60min, fixed and stained for cell surface markers. The opsonin used was Rituximab, a monoclonal, chimeric human-mouse IgG (Hoffman La Roche). The mode of opsonisation was passive adsorption of a saturating concentration of antibody to the bead, according to commercial protocol as supplied by

Thermo Fisher. Post- acquisition data analysis was performed using IDEAS software (Amnis). ImageStream internalisation scores (IS) were generated by IDEAS software as described in commercially supplied protocol. Briefly, IS is defined as the ratio of fluorescence intensity inside the cell to the intensity of the entire cell. The inside *versus* outside of the cell is judged by application of an internal mask based on the brightfield image that covers the inside of the cell, the thickness of the cell membrane in pixels and the fluorescence channel of interest, while the external region is determined by dilating the internal mask by the membrane thickness and combining this with the object mask of the channel of interest.

2.11. *E.coli* FITC uptake assay

A FITC-Trypan blue quenching assay was employed to assess PBMC uptake of *E.coli*. Briefly, UV-irradiated *E.coli* DH5α were FITC labeled by a gentle shaking in a saturated FITC isomer I (Sigma)-PBS solution for 1h at 37^{o}C, followed by washing prior to co-culture with fresh or expanded PBMC at MOI 10. PBMC were co-cultured in triplicate 60min at 37^{o}C in a 5% CO_2 incubator. Cells were then fixed in cold fixation buffer (Biolegend) before quenching with 0.4% Trypan blue solution (Sigma-Aldrich) to remove extracellular FITC signal. After quenching, PBMC were washed three times in a large volume of PBS and analyzed using flow cytometry. Each sample of quenched PBMC-*E.coli* mixture was treated in parallel to a non-quenched sample of the same origin to ensure that quenching had taken place. A quenched PBMC sample incubated with non-FITCylated *E.coli* was used as a control for background FITC fluorescence. In order to determine the involvement of actin polymerization in *E.coli* uptake, PBMC were pre-incubated in 0.2mM CyD (Sigma), vessel control, DMSO (Sigma), or normal medium.

Trypan blue quenching as a method to study phagocytosis has been described previously by Busetto *et al.* (168). Figure 3.1. illustrates the effect that Trypan blue quenching exerts on green fluorescent dyes, such as FITC (ex/em at 495/519) or close homologue, 5(6)-FAM-SE (ex/em at 494/519), which Busetto *et al*. employed in the development of this

uptake assay. The principles that the assay is based upon, briefly, are that Trypan blue i) absorbs light emitted by green dyes and emits it at a far red frequency, and ii) does not penetrate into the interior of live cells. Fig.2.3A and B show a Trypan blue quenched polymorphonuclear cell (PMN)-green *Candida albicans* co-culture analyzed via confocal microscopy and flow cytometry, respectively.

2.12. *E.coli* pHrodo acidification assay

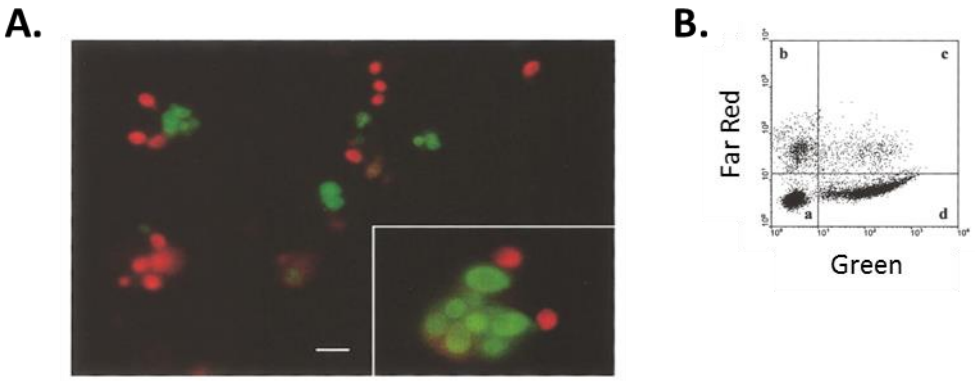

Fig. 2.3. Trypan blue (TB)-quenching as a method to study uptake of green fluorescent material. This figure is an adaptation from Busetto *et al.* (97) who describe a quantifiable assay to study uptake of fluorescently-labeled target particles, which employs Trypan blue quenching and flow cytometry. Polymorphonuclear (PMN) cells were incubated with 5(6)-FAM-SE-labeled *Candida albicans,* washed thoroughly, fixed, quenched with Trypan blue and analayzed via confocal microscopy or flow cytometry. 5(6)-FAM-SE is a FITC homologous fluorescent dye with an absorbance peak at 494nm and an excitation peak at 519nm. (**A**) PMN were examined via confocal microscopy, following Trypan blue quenching. Extracelular *C.albicans* particles can be seen in far red, while intracellular particles – in green. A magnified single PMN is shown, with both extracellularly adherent and internalized *C.albicans.* (**B**) The same sample was analyzed via flow cytometry. Gated on PMN, one can observe: i) negative, ii) Green single positive, iii) Far Red single positive or iv) double positive cells, corresponding to i) no *C.albicans,* ii) internalized *C.albicans* only, iii) extracellularly-adherent *C.albicans* only, or iv) PMN, which have both adhered and internalized *C.albicans.*

A commercial *E.coli*-pHrodo assay was employed to assess PBMC acidification of internalized bacteria according to supplied protocol (available from "pHrodoTM Red and Green BioParticles® Conjugates for Phagocytosis" by Thermo Fisher Scientific). Briefly,

supplied PFA-fixed, pHrodo-dyed *E.coli* (strain: K-12; product code: P35366) was re-suspended via sonication in a pH neutral isotonic buffer, HBSS (Life Technologies), and co-cultured with PBMC in triplicate in a 96-well plate for 60min at 37^{o}C and 5% CO_2. The medium used in this assay was pre-warmed, pH neutral HBSS, sans IL-2. After co-culture, the sample was removed into cold commercial fixation buffer (Biolegend). After fixation, PBMC were washed thoroughly and FACS stained for cell surface markers. Fixed and stained PBMC were then analyzed using flow cytometry. pHrodo dyes do not fluoresce at basic or neutral pH, but fluoresce strongly in proportion to pH drop below pH of 7. *E.coli*-pHrodo alone served as control for background pHrodo fluorescence. In order to determine the involvement of actin polymerization in bacterial acidification, PBMC were pre-incubated in 0.2mM CyD, DMSO or normal medium.

2.13. Meso Scale Discovery electrochemiluminescence assay

The following work was carried out in collaboration with and guidance by Dr. James Bonner and Dr. Intan Yeop, of ICH GOS Infection, Immunity and Rheumatology section. Levels of various cytokines in the medium of freshly-isolated, overnight-stimulated PBMC were measured and analyzed using a multiplex assay, the Meso Scale Discovery electrochemiluminescence assay (MSD). MSD analysis is a highly reproducible and accurate analysis platform, described by some as a more precise and reliable multiplex cytokine measurement system compared to the Cytometric Bead Array (169). Freshly-isolated PBMC were co-cultured overnight with irradiated *E.coli* (MOI 10) or zoledronate (5μM) and pelleted. Culture supernatant was removed and analyzed as described in commercial protocol. Samples were analyzed in duplicate using two MSD® Multi-Spot Assay System kits: i) V-Plex^{TM} Cytokine Panel 1 (human) kit, which included the cytokines GM-CSF, IL-1α, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF, and ii) V-Plex^{TM} Proinflammatory Panel 1 (human) kit, which included the cytokines / inflammatory mediators IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α.

2.14. Sequencing of γδTCR CDR3

The following work was carried out in collaboration with Dr. Yvonne Majani, of ICH GOS Developmental Biology and Cancer section. RNA was extracted from $1x10^6$ freshly isolated, 14 day *E.coli* or zoledronate-expanded PBMC. cDNA synthesis and PCR amplification of the gamma and delta chain sequences was performed using a commercial kit (Irepertoire Illumina human gamma delta kit; Cat. No. HTDGI-01-P). Amplified, barcoded fragments were sequenced on an Illumina MiSEq to a depth of 250 paired ends. Analysis was performed using a commercial platform provided by Irepertoire®.

2.15. Spectratyping of γδTCR CDR3

The following work was carried out in collaboration with Dr. Stuart Adams, of GOSH Department of Bone Marrow Transplantation. Spectratyping was used to determine CDR3 lengths for functional V-gamma 9 and V-delta 2 genes. RNA was extracted from 14 day *E.coli*-expanded or zoledronate-expanded PBMC. Following this, cDNA was synthesized using High Capacity RNA to cDNA (Thermo Fisher Scientific). With cDNA as the template, TCR V-delta 2 specific forward primer (TGAAAGGAGAAGCGATCGGT) or TCR V-gamma 9 specific forward primer (TGGAATGTGTGGTGTCTGGA), and a fluorescently labeled TCR C-delta (GACAAAACGGATGGTTTGG) or TCR C-gamma (GGGGAAACATCTGCATCAAG) reverse primer were used to amplify fragments of interest by PCR. Fragments were separated by size using capillary electrophoresis and analyzed by GeneMapper v3.7 software (Applied Biosystems).

2.16. Confocal imaging of *E.coli*-expanded γδT cell morphology

γδT cells were expanded from PBMC with *E.coli* for 14 days. Expanded PBMC were not purified, but were checked via FACS staining for 90+ % $CD3^{pos}$ cell content. PBMC were

then stained with Vδ2-PE mAb (clone: B6), washed, re-suspended at $1x10^6$ cells/mL in Live Cell Imaging Solution (Thermo Fisher Scientific) and plated in a 96-well glass-bottom plate (Thermo Fisher Scientific). After live imaging, cells were stained with DAPI and Hoechst dyes to visualize the cell nucleus and outer cell membrane. Imaging was performed on a Zeiss AxioObserver LSM 710 confocal microscope. Images of live cells were acquired with a 63× Plan-Apochromat oil objective, numerical aperture 1.4.

2.17. γδT cell sorting by flow cytometry

Day 14 expanded PBMC, with a predominantly γδT cell content, were purified further using flow sorting on the LSR II to >98% purity. Prior to the sort, PBMC were stained for expression of CD3 with CD3-PE/Dazzle594 (BioLegend; clone: UCHT1) and αβTCR with αβTCR-PE/Vio770 (MACS Miltenyi; clone: BW242/412). γδT cells were sorted for as $CD3^{pos}$αβTCR^{neg} PBMC, gated as shown in Fig.2.1 in the general Materials and Methods chapter (Chapter II). Bright fluorophores were used to maximize the clear separation of expanded αβT and γδT cells. Samples of purified γδT cells were stained post-sort for further γδT cell markers including Vδ2 and γδTCR to establish purity. γδT cells were sorted into 50% foetal calf serum and cultured overnight in complete RPMI 1640 prior to use in functional assays.

2.18. γδT cell antigen presentation assay

γδT cells were purified from 14 day zoledronate-expanded PBMC using FACS sorting to >98% purity; >90% of zoledronate-expanded γδT cells express a Vδ2 TCR, as described earlier in Chapter III. To mitigate the stress and potential TCR internalization in response to purification, purified γδT cells were rested overnight in supplemented, antibiotic-free RPMI 1640 medium. Zoledronate-expanded PBMC were used instead of *E.coli*-expanded PBMC to avoid the possibility that *E.coli* or debris thereof may still be present in culture medium or even intracellularly, if taken up by PBMC. Overnight rested, purified γδT cells were then incubated overnight with irradiated, IgG-opsonized *E.coli* DH5α at MIO 10 to

allow time for phagocytosis, antigen processing and presentation at the cell surface. γδT cells were then washed thoroughly. In parallel, αβT cells were purified via positive magnetic selection (Anti-αβTCR MicroBead selection kit, Miltenyi Biotec) from autologous, freshly-isolated PBMC and rested overnight. Purified, opsonized *E.coli*-stimulated, expanded γδT cells were then co-cultured for 2 days with purified, autologous fresh αβT cells at αβT:γδT cell ratios of 5:1, 10:1 and 20:1. After 2 day co-culture, αβT cells were blocked with monensin, permeabilized and stained via FACS for intracellular IFN-γ. The experimental setup is illustrated below in figure 2.4.

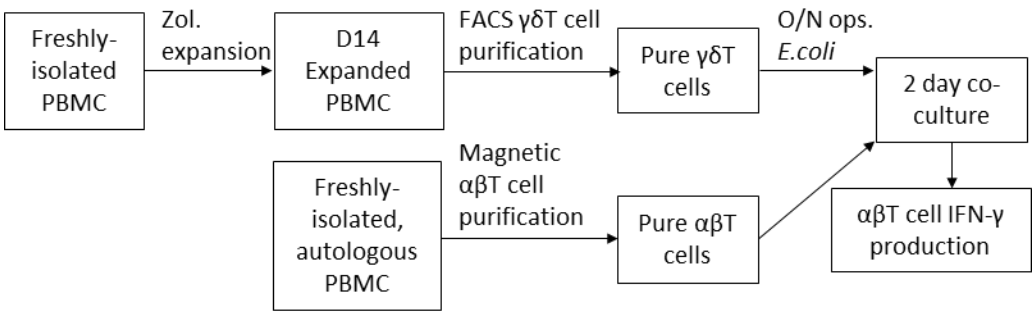

Fig. 2.4. Assay to assess expanded γδT cell antigen presentation to freshly-isolated, autologous αβT cells. γδT cells were expanded for 14 days from freshly-isolated PBMC using zoledronate (5μM). On day 14, γδT cells were purified from expanded PBMC using FACS. FACS purified γδT cells were then incubated overnight IgG-opsonized, irradiated *E.coli* at MOI 10 and washed thoroughly. In parallel, αβT cells were purified via positive magnetic selection from autologous, freshly-isolated PBMC and rested overnight. Purified, *E.coli*-stimulated, expanded γδT cells were then co-cultured for 2 days with purified, autologous fresh αβT cells. After 2 day co-culture, αβT cells were blocked with monensin, permeabilized and stained via FACS for intracellular IFN-γ.

2.19. Expanded γδT cell re-stimulation with *E.coli* in the presence of autologous 'helper' cells

Freshly-isolated or expanded $1.5x10^6$ PBMC/mL were co-cultured with *E.coli* (MOI 10) or zoledronate (5μM) in supplemented RPMI 1640 medium, and left to expand for 14 days. Re-stimulation of expanded PBMC with *E.coli* in the presence of freshly-isolated autologous PBMC was carried out by mixing FACS-stained 14 day *E.coli*-expanded PBMC with freshly-isolated, unstained autologous PBMC at a ratio of expanded PBMC : freshly-isolated PBMC of 1:10 prior to the addition of irradiated *E.coli* at MOI 10. The experimental setup is illustrated below in Figure 2.5.

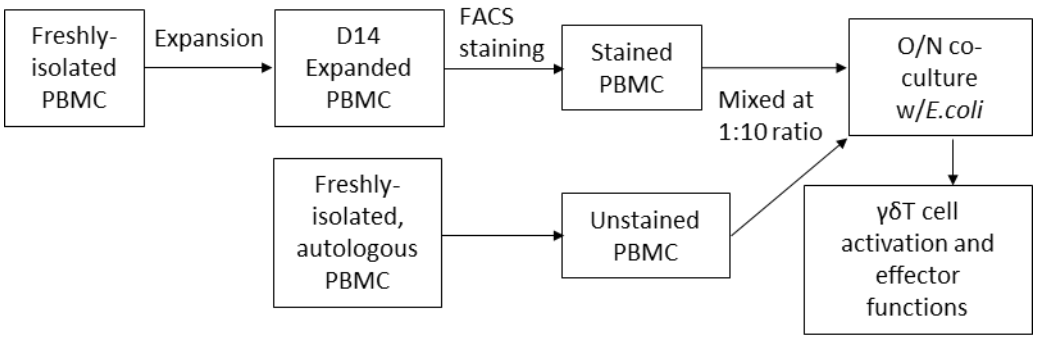

Fig. 2.5. Assay to assess expanded γδT cell responses to E.coli in the presence of autologous, freshly-isolated PBMC. γδT cells were expanded for 14 days from freshly-isolated PBMC using zoledronate (5μM) or *E.coli* (at MOI 10). On day 14, expanded PBMC were FACS-stained for γδT cell surface markers. In parallel, PBMC were isolated from autologous donors. Freshly-isolated, unstained PBMC were then mixed with stained, autologous 14 day-expanded PBMC (1 expanded cell per 10 freshly-isolated cells), prior to the addition of irradiated, IgG-opsonized *E.coli* at MOI 10. After overnight co-culture, PBMC were blocked with monensin, permeabilized and stained via FACS for CD107a, CD69, as well as intracellular IFN-γ and TNF- α.

2.20. γδT cell bactericidal activity against *E.coli*

γδT cells were purified from 14 day *E.coli* or zoledronate-expanded PBMC using FACS sorting to >98% purity. To mitigate the stress and potential TCR internalization in response to purification, purified γδT cells were rested overnight in supplemented, antibiotic-free RPMI 1640 medium. $1.5x10^6$ cells/mL γδT cells were then co-cultured with live, non-irradiated *E.coli* DH5α at MOI 10 for 15, 30, 60 or 90 minutes. At designated time points, PBMC-bacterial suspension was removed in duplicate into sterile-filtered, room temperature distilled H_20, allowed to hypotonically lyse for 10 minutes (to account for possible uptake of bacteria by γδT cells) and then serially diluted in H_20 to 10^{-6} of the original concentration. The dilution series was plated in duplicate onto LB agar plates and grown overnight. The number of colony forming units (CFU) was as a measure of bactericidal activity.

2.21. γδT cell bead phagocytosis assay

A green fluorescent bead-Trypan Blue quenching assay was employed to assess PBMC uptake of opsonized beads. Briefly, streptavidinylated (SA) 'Dragon Green' beads (Bangs Laboratories) were incubated with anti-SA rabbit mAb (GeneScript), washed and co-cultured in triplicate with PBMC for 60min at 37^{o}C in a 5% CO_2 incubator. Cells were then fixed in cold fixation buffer (Biolegend) before quenching with 0.4% Trypan Blue solution (Sigma-Aldrich) to reduce extracellular Dragon Green signal. After quenching, PBMC were washed three times in a large volume of PBS and analyzed using flow cytometry. Each sample of quenched PBMC-bead mixture was treated in parallel to a non-quenched sample of the same origin to ensure that quenching had taken place.

2.22. γδTCR blocking

PBMC were co-incubated for 2h with 10μg/mL LEAF-purified anti-γδTCR mouse IgG1κ mAb (Biolegend; clone: B1), the blocking properties of which have been described by Correia, *et al.*(170) or isotype-matched LEAF purified mouse IgG1κ mAb of known nonhuman specificity (BioLegend; clone: MG1-45). PBMC were than washed in PBS and utilized in the functional assay of interest.

2.23. BTN3A staining and blocking

BTN3A.1 (CD277) was stained for analysis via flow cytometry with CD277-PE (Biolegend; clone: BT3.1). BTN3A functionality was blocked using anti-BTN3A mAb (clone: 103.2), which was kindly supplied by Dr. Daniel Olive (Marseille, France). To achieve blocking, PBMC stimulator cells were co-incubated for 2h with 1.5μg/mL anti-BTN3A mouse IgG2a mAb or LEAF purified mouse mAb of known nonhuman specificity (BioLegend; clone: MG1-45). PBMC were than washed in PBS and utilized in the functional assay of interest.

2.24. PBMC responder stimulation with autologous cells in the presence of BTN3A and γδTCR blocking

Freshly-isolated PBMC were depleted of γδT cells using a positive magnetic selection kit for γδTCR (MACS Miltenyi). Labeled PBMC were run through two magnetic columns in sequence to ensure total γδT cell removal from PBMC. PBMC were then stimulated overnight in complete RPMI 1640 medium with one of the following stimuli: irradiated *E.coli* at MOI 10, HMBPP at 10μM (highly purified (E)-1-Hydroxy-2-methyl-2-butenyl 4-pyrophosphate lithium salt, dissolved for storage in ddH_20; Sigma), IPP at 10μM (highly purified isopentenyl pyrophosphate lithium salt, dissolved for storage in ddH_20; Sigma), zoledronate at 5μM (zoledronic acid; Sigma) or mock stimulated with medium only. After overnight stimulation PBMC were washed thoroughly in a large volume of PBS and blocked with either anti-BTN3A mAb (clone: 103.2) or isotype-matched control (clone: MG1-45). These PBMC served as BTN3A blocked or non-blocked stimulator cells. Stimulator cells were then pre-incubated with autologous, non-depleted PBMC responder cells. Responder cells were either pre-blocked for 2h with anti-γδTCR mAb (clone: B1) or isotype-matched control (clone: MG1-45). Responder cells and stimulator cells were then co-cultured at a ratio of 2:1. Responder γδT cell intracellular IFN-γ and cell surface CD107a were measured via FACS after overnight co-culture. The experimental setup is illustrated in figure 2.6.

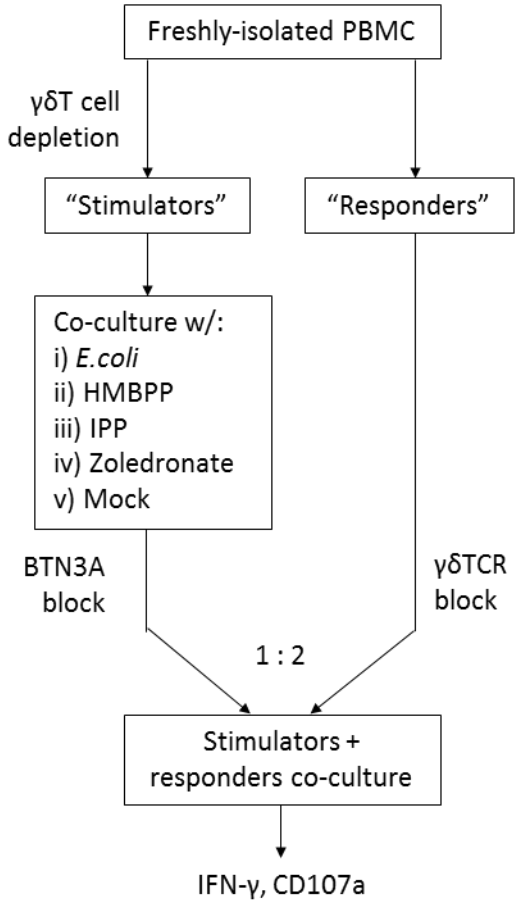

Fig. 2.6. Assay to assess γδT cell effector responses in the presence of autologous stimulator cell pre-incubation with anti-BTN3A mAb and responder cell pre-incubation with anti-γδTCR mAb. Freshly-isolated PBMC were split into "stimulator" and "responder" cells. Stimulator PBMC were depleted of γδT cells using a magnetic positive γδT cell selection kit. Stimulator cells were then co-cultured overnight with *E.coli*, HMBPP, IPP, zoledronate or mock-stimulated with media only, washed thoroughly and cultured for 2h with anti-BTN3A mAb or isotype-matched control. Responder cells were rested overnight during stimulator cell preparation, and then cultured for 2h with anti-γδTCR mAb or isotype-matched control. Stimulator and responder cells were then co-cultured at a ratio of 1 stimulator per 2 responders. Responder γδT cell intracellular IFN-γ and cell surface CD107a were measured via FACS after overnight co-culture.

2.25. PBMC responder stimulation with THP-1 cells in the presence of BTN3A and γδTCR blocking

THP-1 cells were stimulated overnight in complete RPMI 1640 medium with one of the following stimuli: irradiated *E.coli* at MOI 10, HMBPP at 10μM (highly purified (E)-1-Hydroxy-2-methyl-2-butenyl 4-pyrophosphate lithium salt, dissolved for storage in ddH_20; Sigma), IPP at 10μM (highly purified isopentenyl pyrophosphate lithium salt, dissolved for storage in ddH_20; Sigma), zoledronate at 5μM (zoledronic acid; Sigma) or mock stimulated with medium only. After overnight stimulation THP-1 cells were washed thoroughly in a large volume of PBS and blocked with either anti-BTN3A mAb (clone: 103.2) or isotype control (clone: MG1-45). These THP-1 cells served as BTN3A blocked or non-blocked "stimulator" cells. Freshly-isolated PBMC served as "responder" cells. Responder cells were either pre-blocked anti-γδTCR mAb (clone: B1) or isotype-matched control (clone: MG1-45). Stimulator THP-1 cells were incubated with freshly-isolated responder PBMC at a ratio of 1:2. Responder γδT cell intracellular IFN-γ and cell surface CD107a were measured via FACS after overnight co-culture. The experimental setup is illustrated in figure 2.7.

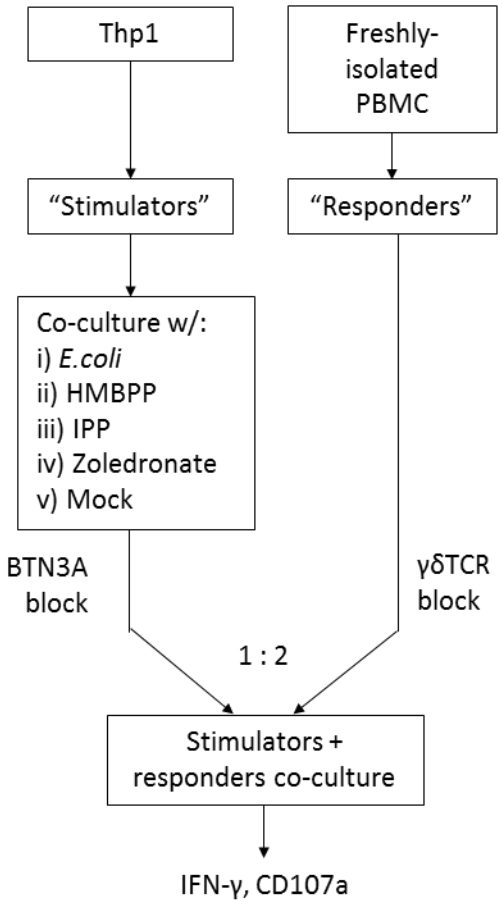

Fig. 2.7. Assay to assess γδT cell effector responses in the presence of THP-1 stimulator cell pre-incubation with anti-BTN3A mAb and responder cell pre-incubation with anti-γδTCR mAb. THP-1 stimulator cells were co-cultured overnight with *E.coli*, HMBPP, IPP, zoledronate or mock-stimulated with media only, washed thoroughly and cultured for 2h with anti-BTN3A mAb or isotype-matched control. Freshly-isolated PBMC responder cells cultured for 2h with anti-γδTCR mAb or isotype-matched control. Stimulator and responder cells were then co-cultured at a ratio of 1 stimulator per 2 responders. Responder γδT cell intracellular IFN-γ and cell surface CD107a were measured via FACS after overnight co-culture.

3. γδT CELL ACQUISITION OF PHAGOCYTIC CAPACITY

3.1. Aims

My intent in this chapter was to examine whether γδT cells are capable of target uptake in a fashion which bears relevance to their putative pAPC function. As described above, phagocytosis and receptor-mediated endocytosis can both contribute material to pAPC antigen processing and eventual presentation. Both processes are also dependent on actin polymerization. I shall not strive further in the text to determine the particular type of antigen uptake γδT cells may engage in, but rather to establish whether γδT cells are capable of target uptake in a pAPC function-permissive manner, and what cellular conditions are necessary for this to take place.

I set out to determine i) whether human peripheral γδT cells are capable of phagocytosis, and ii) what the optimal conditions for this phagocytosis may be. The conditions examined included single *versus* repeated exposure to antigen, cells freshly-isolated from blood *versus* cells expanded *in vitro* and target opsonisation or non-opsonisation with antibody. Various aspects of phagocytosis, such as uptake and acidification of material, were examined.

3.2. Zoledronate-expanded γδT cells take up IgG-opsonized 1.0μm beads and *E.coli*

We have previously reported limited phagocytosis by freshly-isolated peripheral γδT cells. Specifically, Wu *et al.* were able to induce γδT cell uptake of IgG-opsonized 1.0μm beads, which was inhibitable by cell pre-incubation with anti-CD16 (FcγR) mAb and was

sensitive to Cytochalasin D (CyD), an inhibitor of actin polymerization (50). Herein, I examined the effect of cell expansion on γδT cell phagocytic capacity in greater detail. I highlight that the data visible in Fig.3.1 and Fig.3.2 was generated predominantly by another PhD student in the Gustafsson laboratory, Anne Marijn Kramer, who has given her consent to the inclusion of the relevant data into the present thesis. This data was not further utilized by her, and is included in the present thesis to illustrate the evolution of thought and experimental narrative that lead to the completion of the work therein.

14 day zoledronate-expanded γδT cells were co-cultured with protease-activated DQ-Green fluorescent, bovine serum albumin (BSA)-labeled polystyrene beads (0.5µm or 1.0µm in size), with or without IgG opsonization. DQ-Green, BSA-labeled beads have been employed previously as an indicator of phagosome maturation and antigen processing in macrophages (171). Internalization of fluorescing, i.e. protease exposed, beads was quantified using an ImageStream internalization score (Fig.3.1A). Expanded γδT cell incubation with non-opsonized beads revealed significant uptake of 0.5µm, but not 1.0µm beads. Opsonization with Rituximab (monoclonal, chimeric human-mouse IgG against CD20) significantly enhanced 1.0µm bead uptake - to a level statistically indistinguishable from the uptake of beads 0.5µm in size (Fig.3.1B). Internalization scores indicated that ~9% of γδT cells associated with opsonized beads, of which ~86% showed internalization.

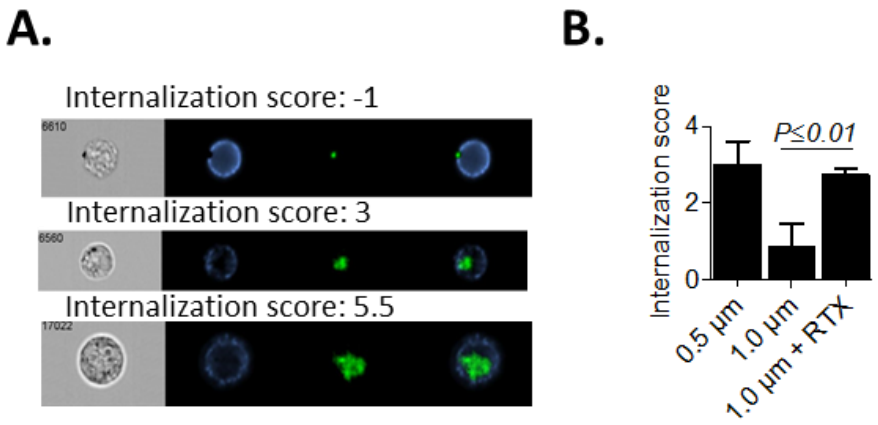

Fig. 3.1. Zoledronate-expanded γδT cells take up IgG-opsonized 1.0µm beads. 14 day zoledronate (5µM)-expanded γδT cells (n=3) were incubated with IgG-opsonized or non-opsonized protease-sensitive DQ BSA polystyrene beads, and analyzed for internalized material. γδT cell uptake of beads was assessed with an internalization score generated via ImageStream analysis. (**A**) Representative data is shown from one donor, with γδTCR in blue and beads in green. Bead fluorescence was an indicator of DQ BSA exposure to proteases. (**B**) Zoledronate-expanded PBMC (n=3) were cultured for 60min with non-opsonized 0.5µm and 1.0µm beads, as well as IgG (Rituximab; RTX)-opsonized 1.0µm beads. PBMC were then stained for ImageStream analysis; internalisation scores, as generated by ImageStream software, are shown for γδT cells. Bar graphs indicate the mean ± standard deviation.

The observation that a significant portion of expanded γδT cells internalize opsonized beads into a protease-rich environment (as indicated by fluorescence of the DQ Green dye) prompted us to investigate γδT cell uptake of bacteria, such as *E.coli*. Confocal microscopy allowed detection of whole and partially-degraded *E.coli* in the interior of zoledronate-expanded γδT cells incubated with IgG-opsonized, GFP-expressing *E.coli* (Fig.3.2). As exemplified in Fig.3.2, virtually all γδT cells within the field of vision were associated with multiple adherent *E.coli*, whereupon only a minor fraction of the bacteria were found to be intracellular.

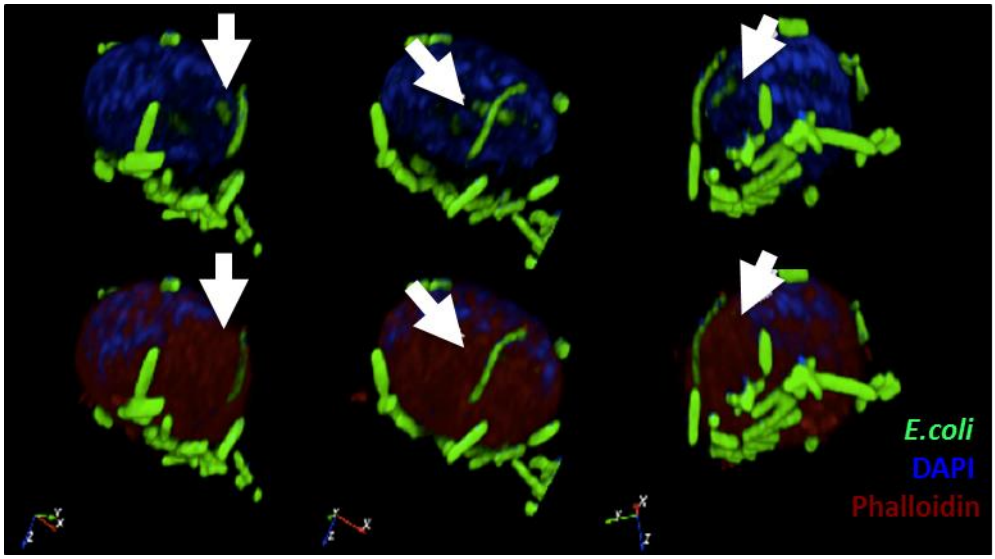

Fig. 3.2. Zoledronate-expanded γδT cells take up IgG-opsonized E.coli. 14 day zoledronate (5μM)-expanded, FACS-purified γδT cells were stained with phalloidin (red), DAPI (blue), incubated with IgG opsonized, GFP-expressing *E.coli,* and analyzed via confocal microscopy. Representative data is shown of a single cell in 3D-rotation with or without phalloidin. Internalised *E.coli* is indicated with white arrows.

3.3. *E. coli*-expanded, but not freshly-isolated γδT cells, take up IgG-opsonized *E.coli*

I next quantified the uptake of IgG opsonized *versus* non-opsonized *E.coli* by freshly isolated *versus* *E.coli*-expanded γδT cells. Freshly-isolated PBMC from healthy laboratory donors were co-cultured with UV-irradiated *E.coli* and left to expand for 14 days. Expansion resulted in a marked increase in $CD3^{pos}$ cells (Fig.3.3A), with a preferential (>200-fold) expansion of γδT cells (Fig.3.3B, C). It was interesting to note that a population of αβT cells persisted with minimal expansion (Fig.3.3C).

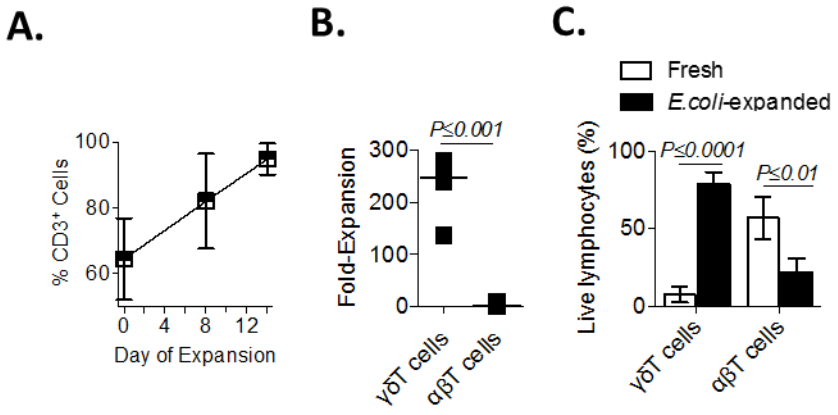

Fig. 3.3. PBMC stimulation with E.coli induces preferential γδT cell expansion. Freshly-isolated PBMC (n=5) were stimulated with irradiated *E.coli* at MOI 10 and left to expand in IL-2-supplemented media for 14 days. Rates of expansion were compared between γδT and αβT cells. (**A**) The proportion of $CD3^{pos}$ cells of total live PBMC was tracked. (**B**) Fold-expansion of γδT and αβT cells, assessed by FACS and Trypan Blue exclusion, was compared. (**C**) PBMC were compared via FACS for γδT and αβT cell content of total live lymphocytes. Bar graphs indicate the mean ± standard deviation.

Vδ2 γδT cells displayed the highest rate of expansion (~250-fold), followed by Vδ1-Vδ2- cells (~40-fold) and Vδ1 γδT cells, which instead contracted (Fig.3.4A). Donor-matched, parallel expansions of PBMC in IL-2 medium with zoledronate or *E.coli* induced similar rates of expansion of subsets (Fig.3.4B). Of note, IL-2 medium alone failed to induce expansion of γδT or αβT cells.

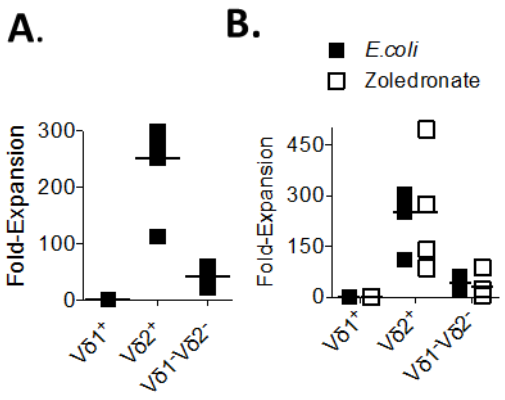

Fig. 3.4. PBMC stimulation with E.coli and zoledronate induces preferential Vδ2 γδT cell expansion. Freshly-isolated PBMC (n=5) were stimulated with irradiated *E.coli* at MOI 10 or zoledronate (5μM) and left to expand in IL-2-supplemented media for 14 days. Rates of expansion were compared between Vδ1, Vδ2 and Vδ1^{neg}Vδ2^{neg} γδT cell subsets. (**A**) Fold-expansion over 14 day stimulation was compared between γδT cell subsets in *E.coli*-expanded PBMC only, or (**E**) compared between *E.coli* and zoledronate-stimulated PBMC.

To measure phagocytosis, freshly-isolated or expanded PBMC were incubated with IgG-opsonized or non-opsonized FITCylated-*E.coli* for 60min, and analysed via Trypan Blue quenching and flow cytometry (Fig.3.5). Freshly-isolated γδT and αβT cells failed to show notable bacterial uptake; in contrast ~25% and ~45% of freshly isolated $CD3^{neg}$ PBMC (predominantly monocytes) internalized non-opsonized and opsonized *E.coli*, respectively; both processes were found to be sensitive to CyD (Fig.3.6). More than 50% of *E.coli* expanded γδT cells took up opsonized *E.coli* in a CyD-sensitive manner. Interestingly, a subpopulation (mean 35%) of the residual αβT cells following *E.coli* expansion also took up opsonized *E.coli*, but this uptake was not significantly inhibited by CyD (Fig.3.6).

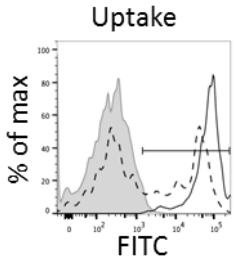

Fig. 3.5. γδT cell uptake of E.coli was investigated using a FITC-Trypan Blue quenching assay. PBMC were incubated for 60min with FITC-labeled *E.coli,* washed and quenched post-culture with Trypan Blue. PBMC were then stained for cell surface markers and analyzed via FACS. Shown are representative stains, gated on γδT cells: i) non-quenched co-culture indicating total FITC fluorescence (black, solid, unshaded), ii) quenched co-culture indicating intracellular FITC fluorescence (black, dotted, unshaded), iii) co- culture with non-FITCylated *E.coli* (gray, shaded).

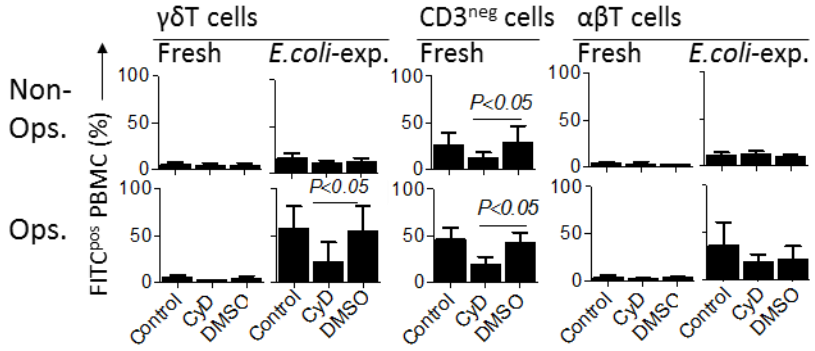

Fig. 3.6. E.coli-expanded, but not fresh, γδT cells take up IgG-opsonized E.coli. Freshly-isolated or *E.coli*-expanded PBMC (n=5) PBMC were incubated for 60min with FITC-labeled *E.coli* at MOI 10, washed and quenched post-culture with Trypan Blue. PBMC were then stained for cell surface markers and analyzed via FACS. Remaining post-quench FITC fluorescence was interpreted as intracellular in origin. The proportion of $FITC^{pos}$ PBMC was tallied, and compared between fresh and *E.coli*-expanded γδT and αβT cells, as well as fresh $CD3^{neg}$ cells. Intracellular FITC fluorescence was evaluated in the presence of PBMC pre-culture with normal media (control), Cytochalasin D at 0.2mM (CyD) or DMSO at 0.2mM (DMSO). *E.coli* were UV-irradiated prior to co-culture with PBMC and left non-opsonized (non-ops.) or opsonized with purified rabbit anti-*E.coli* IgG (ops.). Bar graphs indicate the mean ± standard deviation.

Phagocytosis promotes fusion of the phagosome with the lysosomal compartment. To determine whether *E.coli* uptake by *E.coli*-expanded γδT cells resulted in bacterial acidification (implying phagolysosome formation), freshly-isolated and 14 day expanded PBMC were co-cultured for 60min with IgG-opsonized pH-sensitive pHrodo-*E.coli* (Fig.3.7). *E.coli*-expanded γδT cells, but not αβT cells or freshly isolated γδT cells, showed notable acidification of *E.coli*, which increased further upon opsonization. As a positive control, freshly isolated $CD3^{neg}$ PBMC (largely monocytes) also acidified non-opsonized and opsonized *E.coli.* Only acidification by $CD3^{neg}$ PBMC was CyD-sensitive (Fig.3.8).

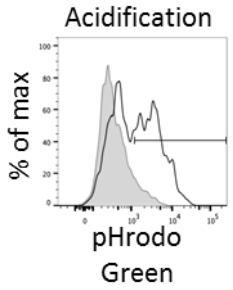

Fig. 3.7. γδT cell acidification of E.coli was investigated using a pHrodo-E.coli assay. PBMC were incubated for 60min with pH sensitive pHrodo-labeled *E.coli* and washed post-culture. PBMC were then stained for cell surface markers and analyzed via FACS. Shown are representative stains, gated on γδT cells, of: i) PBMC, gated on γδT cells, co-cultured with pHrodo-*E.coli* (black, solid, unshaded), ii) pHrodo-*E.coli* only control (gray, shaded).

It remains unclear as to why bacterial uptake but not acidification appeared CyD-sensitive. One possible explanation may be the difference in bacterial preparations employed, as fresh exponentially-grown *E. coli* were irradiated just prior to uptake studies whilst

lyophilized, *E.coli*-pHrodo conjugates were utilized to examine acidification. Lyophilisation may lead to bacterial acquisition of a spherical rather than rod shape, with consequential changes to the involvement of the actin cytoskeleton in the uptake process (172,173). Nonetheless, this series of experiments provides evidence for the first time that, upon expansion, γδT cells can phagocytose and direct bacteria to an acid-rich environment.

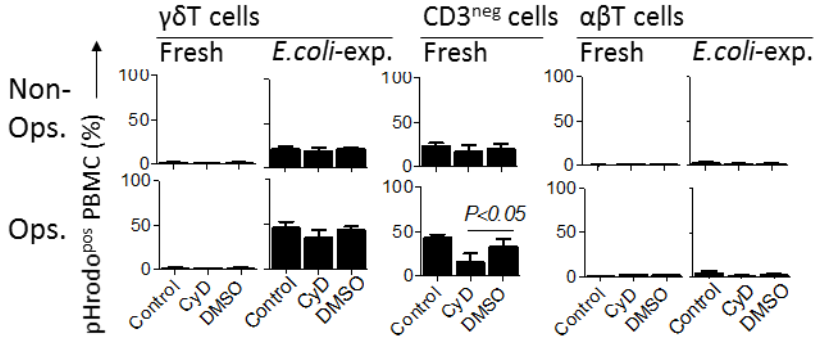

Fig. 3.8. E.coli-expanded, but not fresh, γδT cells acidify IgG-opsonized E.coli. Freshly-isolated or *E.coli*-expanded PBMC (n=5) PBMC were incubated for 60min with pHrodo-labeled *E.coli* and washed. PBMC were then stained for cell surface markers and analyzed via FACS. pHrodo positivity was interpreted as labeled *E.coli* exposure to acidic pH. The proportion of pHrodo^{pos} PBMC was tallied, and compared between fresh and *E.coli*-expanded γδT and αβT cells, as well as fresh CD3^{neg} cells. pHrodo fluorescence was evaluated in the presence of PBMC pre-culture with normal media (control), Cytochalasin D at 0.2mM (CyD) or DMSO at 0.2mM (DMSO). pHrodo-*E.coli* were left non-opsonized (non-ops.) or opsonized with purified rabbit anti-*E.coli* IgG (ops.). Bar graphs indicate the mean ± standard deviation.

A similar degree of opsonized *E.coli* uptake and CyD-sensitivity by expanded γδT cells was seen regardless of whether PBMC were expanded with *E.coli* or zoledronate (Fig.3.9). The same was true for expanded γδT cell acidification of opsonized *E.coli* (Fig.3.10).

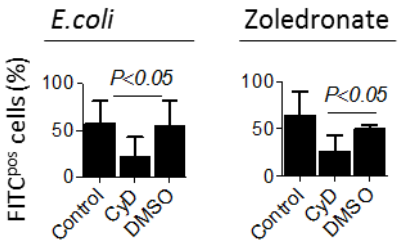

Fig. 3.9. E.coli and zoledronate-expanded γδT cells take up IgG-opsonized E.coli with similar dynamics. 14 day *E.coli* or zoledronate-expanded PBMC (n=5) PBMC were incubated for 60min with FITC-labeled *E.coli* at MOI 10, washed and quenched post-culture with Trypan Blue. PBMC were then stained for cell surface markers and analyzed via FACS. Remaining post-quench FITC fluorescence was interpreted as intracellular in origin. The proportion of $FITC^{pos}$ PBMC was tallied, and compared between *E.coli* or zoledronate-expanded γδT cells. Intracellular FITC fluorescence was evaluated in the presence of PBMC pre-culture with normal media (control), Cytochalasin D at 0.2mM (CyD) or DMSO at 0.2mM (DMSO). *E.coli* were UV-irradiated prior to co-culture with PBMC and opsonized with purified rabbit anti-*E.coli* IgG. Bar graphs indicate the mean ± standard deviation.

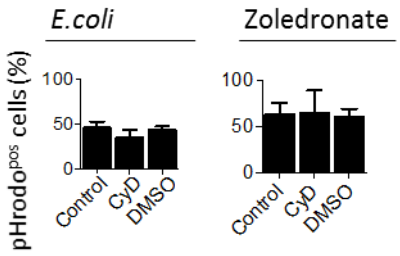

Fig.3.10. E.coli and zoledronate-expanded γδT cells acidify IgG-opsonized E.coli with similar dynamics. 14 day *E.coli* or zoledronate-expanded PBMC (n=5) PBMC were incubated for 60min with pHrodo-labeled *E.coli* and washed. PBMC were then stained for cell surface markers and analyzed via FACS. pHrodo positivity was interpreted as labeled *E.coli* exposure to acidic pH. The proportion of $pHrodo^{pos}$ PBMC was tallied, and compared between *E.coli* or zoledronate-expanded γδT cells. pHrodo fluorescence was evaluated in the presence of PBMC pre-culture with normal media (control), Cytochalasin D at 0.2mM (CyD) or DMSO at 0.2mM (DMSO). *E.coli* were opsonized with purified rabbit anti-*E.coli* IgG. Bar graphs indicate the mean ± standard deviation.

Interestingly, the magnitude of bacterial acidification varied between expanded γδT cells and freshly-isolated $CD3^{neg}$ PBMC (Fig.3.11), suggesting cell-specific pathways may be involved in bacterial uptake and processing.

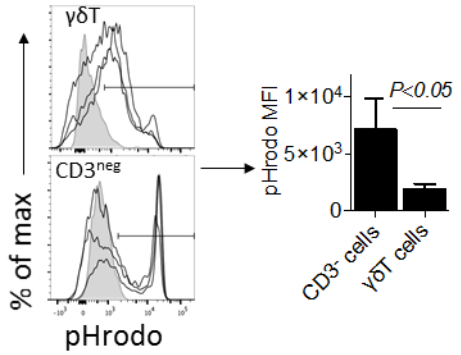

Fig. 3.11. Expanded γδT cells acidify bacteria with different dynamics than $CD3^{neg}$ fresh PBMC. Freshly-isolated or *E.coli*-expanded PBMC (n=5) were incubated with IgG-opsonized pHrodo-*E.coli* for 60min, washed and stained for cell surface markers for subsequent analysis via FACS. Shown are representative stains of three donors, gated on *E.coli*-expanded γδT cells and freshly-isolated $CD3^{neg}$ PBMC, of: i) PBMC, gated on either cell type, co-cultured with pHrodo-*E.coli* (black, solid, unshaded), ii) pHrodo-*E.coli* only control (gray, shaded). A compilation of donor pHrodo MFI is shown, comparing *E.coli*-expanded γδT cells and freshly-isolated $CD3^{neg}$ cells.

3.4. Discussion

In this chapter, I sought to address whether human peripheral γδT cells are capable of phagocytosis, to quantify this phenomenon and to establish what the conditions permissive of this phenotype may be. My findings included:

i) Zoledronate-expanded γδT cells are capable of phagocytosing 1.0μm polystyrene beads, but only if the beads are opsonized with IgG, as indicated by ImageStream quantification.

ii) Intracellular *E.coli* can be visualized using confocal microscopy after 60min zoledronate-expanded γδT cell co-culture with opsonized *E.coli.*

iii) Freshly-isolated PBMC stimulation with *E.coli* and IL-2 leads to a preferential expansion of Vδ2 γδT cells, at rates similar to those achieved by PBMC stimulation with zoledronate and IL-2.

iv) A novel application of a FITC-Trypan blue quenching FACS-based assay for the quantification of *E.coli* uptake by γδT cells. Some 50% of expanded, but not fresh, γδT cells can take up *E.coli* at the same rate as freshly-isolated $CD3^{neg}$ PBMC (mostly monocytes).
   a. This uptake is significantly enhanced if the *E.coli* is opsonized with IgG.
   b. The uptake of opsonized *E.coli* is sensitive to cytochalasin D (CyD), an inhibitor of actin polymerization.
   c. Expanded αβT cells appear capable of some uptake of opsonized *E.coli*, but this uptake is not CyD sensitive.

v) A novel application of a pHrodo Green FACS-based assay for the study and quantification of *E.coli* acidification by γδT cells. Some 50% of expanded, but not fresh, γδT cells can acidify *E.coli* at the same rate as freshly-isolated $CD3^{neg}$ PBMC (mostly monocytes).
   a. This acidification is significantly enhanced if the *E.coli* opsonized with IgG.
   b. In γδT cells this acidification is, curiously, not significantly sensitive to CyD, an inhibitor of actin polymerisation, but is CyD-sensitive in freshly-isolated $CD3^{neg}$ PBMC.

c. Expanded αβT cells appear capable of some uptake of opsonized *E.coli*, but it is not CyD sensitive.

vi) *E.coli* and zoledronate-expanded γδT cells take up and acidify opsonized *E.coli* with similar efficiency.

vii) γδT cell acidification of opsonized *E.coli* is different from acidification by freshly-isolated $CD3^{neg}$ PBMC. $CD3^{neg}$ PBMC appear to acidify opsonized *E.coli* with greater intensity to a lower pH, as indicated by higher fluorescence of the pH-sensitive pHrodo dye.

As mentioned previously, work by our lab and others has reported limited phagocytosis by freshly-isolated peripheral γδT cells. Wu *et al.* were the first to publish an account of phagocytosis by peripheral human γδT cells (50). They were able to induce CyD-sensitive γδT cell uptake of IgG-opsonized 1μm beads in a process that was inhibitable by cell pre-incubation with anti-CD16 (phagocytic FcγR) mAb. Their work further documented some uptake of opsonized *E.coli.* Himoudi *et al.* have since demonstrated that γδT cells are seemingly able to cross-present antigen from IgG-opsonized tumour cells (49), implying phagocytosis (or an alternative uptake process) of target material. While establishing that γδT cells are indeed capable of phagocytosis, these experiments crucially lacked quantification of the phenomena observed.

Important questions such as I) "What proportion of γδT cells are capable of phagocytosis?", II) "How does γδT cell phagocytosis of targets quantifiably differ from that carried out by established myeloid phagocyets?", III) "Are particles uptaken by γδT cells shuttled into phagolysozomes for subsequent processing?", IV) "Does the phagocytic capacity of freshly-isolated *versus* expanded γδT cells differ significantly?" and V) "Is γδT cell phagocytosis enhanced by previous encounter with specific antigen?" have not been addressed comprehensively in published research. Therein, I carried out the experiments described in this chapter.

Preliminary ImageStream work by Anne Marijn Kramer quantified for the first time γδT cell uptake of material; she was able to perform statistical analysis on zoledronate-

expanded γδT cell uptake of pH-sensitive IgG-opsonized beads. Some 7% of expanded γδT cells presented with intracellular, pH<7-exposed beads (Fig.3.1). While this presented an advance in the examination of γδT cell phagocytic capacity, it was carried out using a highly non-physiological target - plastic beads - and used a single phagocytosis stimulus, IgG, neglecting the potential necessity TCR, TLR and other PRR stimulation (or co-stimulation) that may be necessary to stimulate γδT cell uptake of antigenic material. Using confocal microscopy, she was further able to document *E.coli* in the interior of zoledronate-expanded γδT cells. This was not quantified. The desire to quantify and explore in detail γδT cell phagocytosis of a physiologically relevant target, I set out to characterize and quantify γδT cell uptake of *E.coli.*

My initial attempts to quantify γδT cell phagocytosis of *E.coli* were via gentamicin protection assays, considered the 'gold standard' in classic phagocytosis literature (174–177). These assays are based on the principle that gentamicin, a potent bactericidal Gram negative antibiotic, does not penetrate the cell membrane of mammalian cells. Cells are incubated with live *E.coli* for a given time period, washed thoroughly, treated with gentamicin, washed once more and lysed with hypotonic pressure. The cell lysate is then plated and cultured on agar, providing optimal growth conditions for surviving *E.coli.* If any *E.coli* CFU are seen, one concludes that these *E.coli* must have been protected from the bactericidal effects of gentamicin by the mammalian cell membrane - therein implying intracellular location.

My attempts at γδT cell gentamicin protection assays consisted of FACS-purified *E.coli* or zoledronate-expanded γδT cells co-cultured with *E.coli* at various MOIs (ranging from 1-100). None of my attempts yielded viable *E.coli* CFU post gentamicin treatment. The obvious conclusion that γδT cells must, therefore, not engage in phagocytosis of *E.coli,* was overturned when I began examining the capacity of γδT cells to kill live *E.coli* in culture. Data on γδT cell killing of bacterial targets is further discussed in Chapter V. Briefly, expanded, FACS-purified γδT cells exhibited a surprisingly rapid and potent bactericidal phenotype: within less than an hour γδT cells reduced *E.coli* CFU by >90%. Given that a gentamicin protection assay involves hours-long incubation periods – during

which time γδT cells may have effectively killed present *E.coli* – I decided to pursue different phagocytosis quantification methodology.

As outlined in the 'Materials and Methods' chapter, I employed a FITC-Trypan blue quenching assay, described by Busetto *et al.* (168). In addition to permitting quantification of phagocytosis, the use of a FACS-based assay further permitted staining for cell surface markers, which omitted the need for cell purification. I could, thus, examine *E.coli* uptake by various PBMC populations simultaneously and in parallel (Fig.3.6). The pH-sensitive pHrodo dye phagocytosis assay further indicated that, within the 60min of observed *E.coli*-FITC uptake, *E.coli*-pHrodo is also acidified (Fig.3.7). Using this as an indirect readout of phagolysosome formation allowed me to state with added confidence that the phenomenon I am observing is indeed phagocytosis.

It was interesting to note the difference in the intensity of acidification between expanded γδT cells and freshly-isolated $CD3^{neg}$ PBMC (Fig.3.11). While this may allude to cell-specific phagocytic and antigen processing mechanisms, it is important to address the possibility that - given the potent cytotoxicity that γδT cells displayed upon exposure to *E.coli* - it cannot be ruled out that *E.coli*-pHrodo may also be acidified by γδT cells extracellularly. The bactericidal mechanisms of γδT cells are poorly understood. It is conceivable that one route of killing involves γδT cell release of acidic lysosomal contents onto nearby bacteria, which may activate the pH-sensitive pHrodo dyes. Further work, involving visualizing techniques such as confocal microscopy, is necessary to determine whether pH-sensitive dyes are an adequate means of studying post-phagocytosis phagolysosomal formation in γδT cells. I stipulate that a likely scenario is that γδT cells acidify *E.coli* via cytotoxicity extracellularly *and* via phagolysosome formation intracellularly. Cytotoxicity data (Chapter V) indicated potent anti-*E.coli* CD107a-mediated degranulation by freshly-isolated γδT cells, which nonetheless failed to show acidification of *E.coli*-pHrodo. This observation lends strength to the idea that expanded γδT cells take up and subsequently acidify opsonized *E.coli* intracellularly. It is nonetheless important to clarify this question prior to drawing meaningful conclusions from data concerning γδT cell acidification intensity of internalized bacterial targets.

Tt is also important to further examine the CyD-insensitivity of γδT cell acidification of *E.coli.* A potential cause of this may be the different preparations of *E.coli* used. One way to address this would be to label freshly-grown *E.coli* with FITC and a pHrodo dye in parallel, ensuring that both types of labeled *E.coli* have undergone the same treatment. Thus, the two preparations could also have more closely controlled MOIs, omitting the reliance on commercially-available *E.coli*-pHrodo protocols that do not specify bacterial cell number. It is possible that the difference in CyD sensitivity between uptake of *E.coli*-FITC and acidification of *E.coli*-pHrodo was caused by differences in phagocyte-to-target ratio.

The necessity for IgG opsonisation of targets for γδT cells has been reported previously (178). What was surprising and a novel finding herein was the stark difference I found between the phagocytic capacity of expanded *versus* freshly-isolated γδT cells, whereupon expanded γδT cells phagocytosed *E.coli* at a rate statistically similar to that observed in freshly-isolated $CD3^{neg}$ PBMC (largely monocytes and B cells). The marked difference in phagocytic capacity between freshly-isolated and *E.coli*-expanded γδT cells implies a significant phenotypic shift of γδT cells after initial exposure to *E.coli*. This prompted us to examine whether the acquired extensive phagocytic capacity for *E.coli* may be antigen specific. I enquired whether expansion with a particular antigen followed by a re-challenge by that same antigen (*E.coli* in this case) was responsible for the vast difference in phagocytic γδT cells that I observed.

I quantified the phagocytic capacity for opsonized *E.coli* of zoledronate-expanded γδT cells, and compare it to that of *E.coli*-expanded γδT cells. To my surprise, no significant difference between the two expansion conditions could be found in either uptake of *E.coli*-FITC (Fig.3.9) or acidification of *E.coli*-pHrodo (Fig.3.10). The importance of this finding lies in the different mechanisms that have been proposed by which *E.coli* and zoledronate induce γδT cell proliferation. As outlined in the thesis introduction, both stimuli are known to activate Vδ2 γδT cells. General consensus argues, however, that *E.coli* activates Vδ2 cells via HMBPP, Lipid A and other bacterial antigens via stimulation of the TCR and various PRRs (93,179). Zoledronate, meanwhile, is a small molecule aminobisphosphonate drug, which does not contain any conceivable targets for γδT cell

antigen receptors. Instead, zoledronate is thought to disrupt the mevalonate pathway during cholesterol synthesis, and lead to the accumulation of toxic intermediates, such as isoprenyl pyrophosphate (IPP). IPP accumulation then leads to cellular stress and consequent conformational changes in cell surface butyrophilin 3A (BTN3A) molecules (180). IPP itself and its effect on BTN3A conformation have both been proposed as mediators of γδTCR activation (100,181). *E.coli* and zoledronate, thus, represent two separate types of antigen: i) non-self and ii) 'stressed' self. The observation that these differential stimuli induced γδT cell populations with equivalent phagocytic capacity - which was nonetheless different from that of freshly-isolated γδT cells - prompted us to compare the effect of *E.coli versus* zoledronate expansion on freshly-isolated γδT cells in greater detail.

3.5. Summary

Analysis of the data laid out in this chapter lead us to the conclusion that *E.coli* or zoledronate-expanded γδT cells are capable of effective phagocytosis of IgG-opsonized targets, including *E.coli*, while freshly-isolated γδT cells are not.

4. *E.COLI* AND ZOLEDRONATE PROMOTE EXPANSION OF PHENOTYPICALLY EQUIVALENT γδT CELL POPULATIONS

4.1. Aims

Surprised to find that *E.coli* and zoledronate-expanded, but not fresh, γδT cells display equivalent efficiency in opsonized *E.coli* phagocytosis, I set out to compare the phenotype of these expanded γδT cells in greater detail. Particular emphasis was placed on antigen presenting cell (APC) phenotype expression and T cell receptor (TCR) repertoires.

In the characterization of *E.coli versus* zoledronate-expanded Vδ2 cells, I placed a particular emphasis on their expression patterns of APC molecules, MHC class II (HLA-DR, specifically) and co-stimulatory CD86, as well as homing receptors, chemokine receptor 7 (CCR7) and CD62L (also known as L-selectin). HLA-DR and CD86 expression has been documented on human γδT cells in various contexts, ranging from *in vitro* stimulation to *in vivo* infectious disease. Both endogenous pAgs and Gram-negative bacteria-stimulated γδT cells have been reported to upregulate expression of HLA-DR and CD86 (75,106). The dynamics, time scale and stability of this upregulation has, however, not been reported in detail.

4.2. *E.coli* and zoledronate stimulation induces sustained γδT cell upregulation of HLA-DR and CD86

In the previous chapter I outlined phagocytosis by zoledronate and *E.coli*-expanded peripheral γδT cells. Consequently, I hypothesized that opsonization-mediated phagocytosis is associated with acquisition of APC capabilities by γδT cells. To test this, expression of classic APC markers, HLA-DR and CD86, was investigated. *E.coli* mediated a steady increase in both markers, with a majority of expanding γδT cells developing an HLA-DR^{pos}CD86^{pos} phenotype within days of stimulation (Fig.4.1A and B). HLA-DR and CD86 expression on expanded γδT cells was similar to that observed on freshly-isolated monocytes (CD3^{neg}CD14^{pos} PBMC). αβT cells remained negative for both markers throughout expansion (Fig.4.1B).

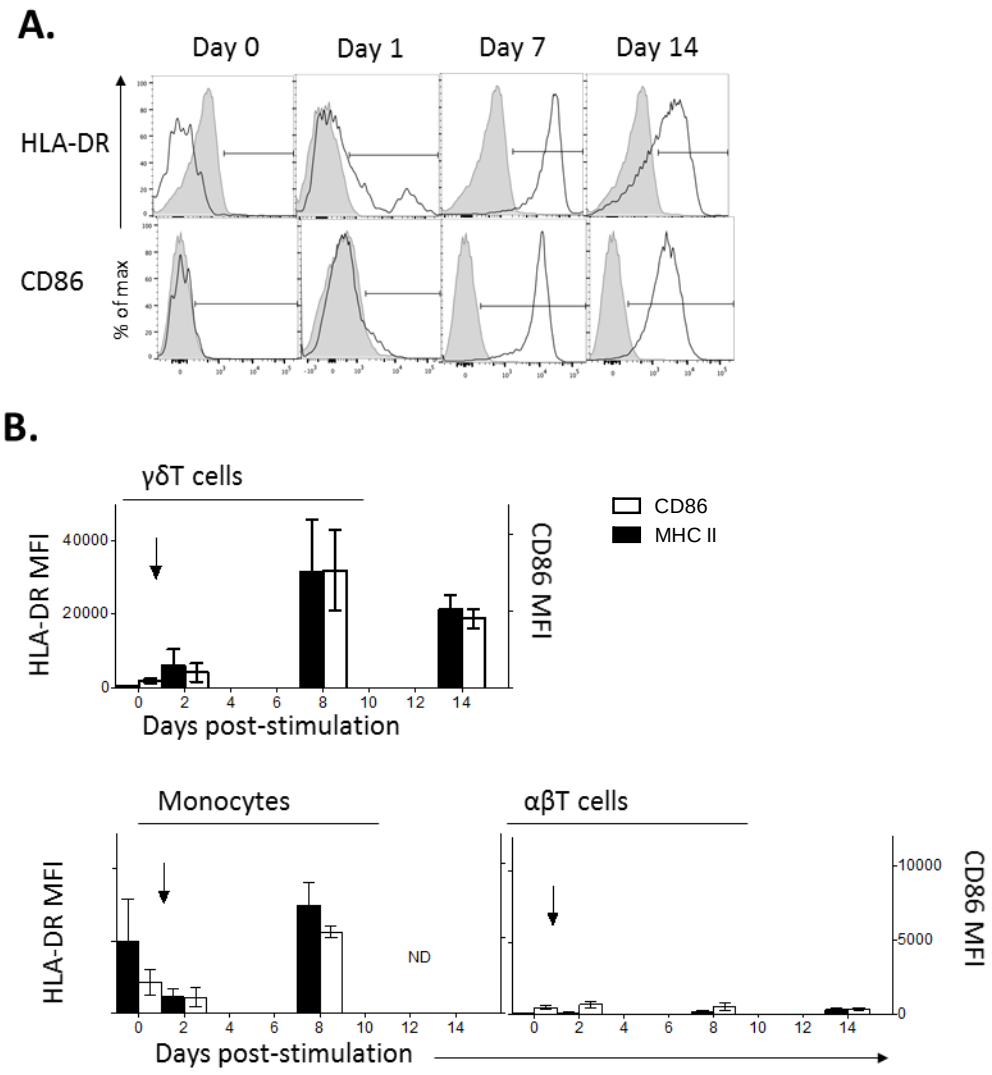

Fig. 4.1. PBMC stimulation with E.coli leads to γδT cell upregulation of cell surface HLA-DR and CD86. Freshly-isolated PBMC (n=5) were expanded for 14 days with irradiated *E.coli* at MOI 10, and FACS stained for cell surface HLA-DR and CD86 throughout expansion. (**A**) Representative stains of one donor γδT cells is shown, with specific antibody in black, unshaded, and isotype-matched control - in gray, shaded. (**B**) HLA-DR and CD86 of five donor compiled MFI over *E.coli* expansion was compared between γδT, αβT cells and monocytes ($CD3^{neg}CD14^{pos}$ PBMC). Points of *E.coli* stimulation are indicated with black arrows. Bar graphs indicate the mean ± standard deviation.

Data indicated, moreover, that PBMC stimulation with *E.coli* or zoledronate produced a similar and sustained upregulation of the two pAPC cell surface markers (Fig.4.2).

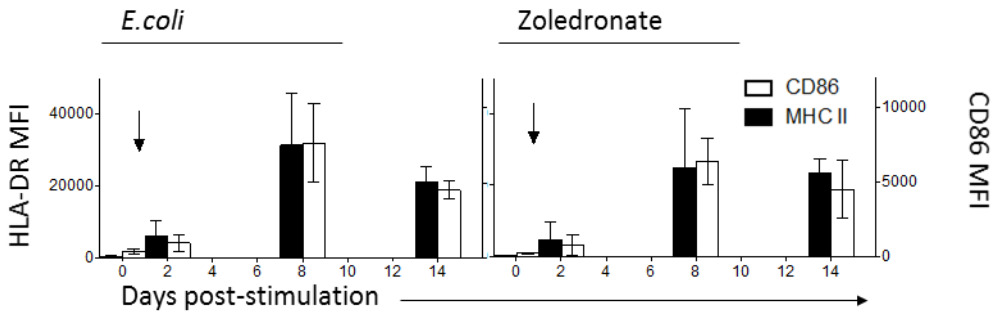

Fig. 4.2. PBMC stimulation with zoledronate leads to similar levels of γδT cell upregulation of cell surface HLA-DR and CD86 as stimulation with E.coli. Freshly-isolated PBMC (n=5) were expanded for 14 days with irradiated *E.coli*, and stained for cell surface HLA-DR and CD86 throughout expansion. (**A**) Representative stains of one donor γδT cells is shown, with specific antibody in black, unshaded, and isotype-matched control - in gray, shaded. (**B**) HLA-DR and CD86 MFI over expansion was compared between γδT, αβT cells and monocytes ($CD3^{neg}CD14^{pos}$ PBMC). Points of *E.coli* stimulation are indicated with black arrows. Bar graphs indicate the mean ± standard deviation.

4.3. *E.coli* and zoledronate stimulation induces transient γδT cell upregulation of CCR7 and downregulation of CD62L

In order to further explore the putative pAPC phenotype of expanded, but not fresh unstimulated γδT cells, I set out to document whether γδT cell expression of the lymphoid tissue homing markers, CCR7 and CD62L, mirror those of classic αβT cells or, rather - those of myeloid APCs, such as DC. Brandes, *et al*. have previously documented γδT cells as negative for CCR7 when freshly-isolated, followed by a rapid, potent and transient upregulation of CCR7 on short-term (overnight-36h) endogenous pyrophosphate-stimulated, peripheral human γδT cells, which then returned to baseline negativity during short term culture; up to 100% of peripheral γδT cells in their culture displayed a $CCR7^{pos}$ phenotype 24h post-stimulation. They specify further that maximal γδT cell upregulation of CCR7 took place prior to the initiation of cell proliferation (182). PBMC stimulation with *E.coli* in my model produced a similar result (Fig.4.3).

While Brandes and others interpreted CCR7 upregulation on freshly-isolated γδT cells as an indication of migration to lymph nodes as part of putative pAPC function, my data indicate that freshly-isolated γδT cells do not engage in en-masse phagocytosis of targets. This is supported by the findings of Wu *et al.*, discussed in the previous chapter, who were able to find only a small minority of short term-activated γδT cells engaging in active phagocytosis (50). Moreover, as laid out in this chapter, freshly-isolated or short term-activated γδT cells do not appear positive for the APC markers, HLA-DR and CD86. My data indicate that 50+ % of γδT cells, expanded with zoledronate or *E.coli*, engage in phagocytosis only upon expansion. Curiously, expanded, phagocytic γδT cells did not re-upregulate CCR7 after exposure to *E.coli* (Fig.4.3).

In order to establish whether γδT cell expression of CCR7 was similar to that on αβT cells during expansion with *E.coli*, CCR7 levels were compared between the two cell populations in parallel. During the transient upregulation by γδT cells, CCR7 levels were similar between the two T cell populations (Fig.4.4). Indeed, within 24h of PBMC

stimulation with *E.coli*, a vast majority of γδT cells expressed CCR7 at the same level as freshly-isolated αβT cells. However, while αβT cells – which did not expand after PBMC stimulation with *E.coli* – maintained their expression of CCR7, it was lost entirely on the rapidly-expanding Vδ2 cells, and was not rescued by *E.coli* re-stimulation.

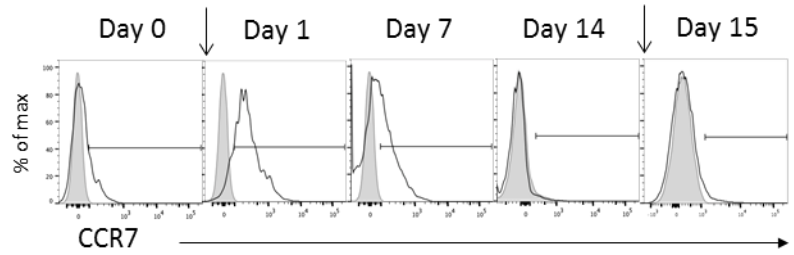

Fig. 4.3. PBMC stimulation with E.coli leads to a transient γδT cell upregulation of cell surface CCR7. Freshly-isolated PBMC (n=4) were expanded for 14 days with irradiated *E.coli*, re-stimulated overnight with *E.coli* at D14, and stained for cell surface CCR7. Representative stains of one donor γδT cells is shown, with anti-CCR7 antibody in black, unshaded, and isotype-matched control - in gray, shaded. Points of *E.coli* stimulation are indicated with black arrows.

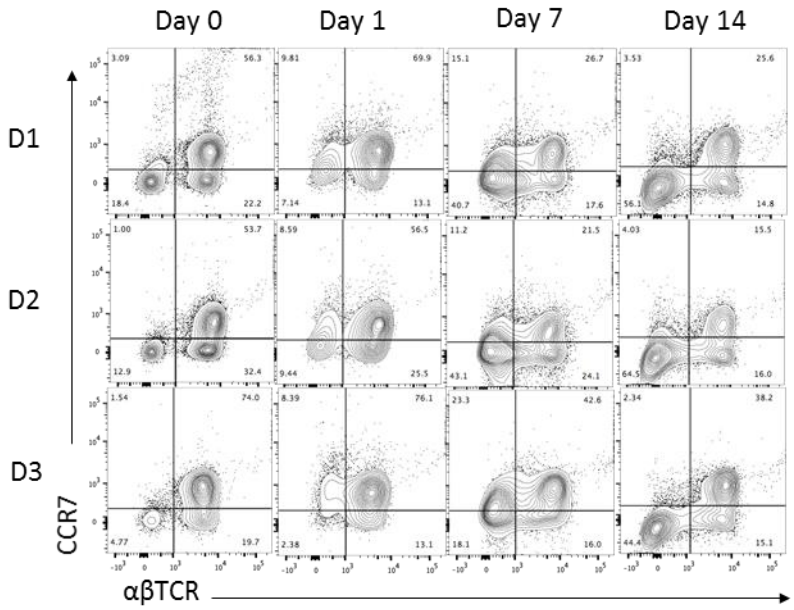

Fig. 4.4. CCR7 expression dynamics after PBMC stimulation with E.coli vary between αβT and γδT cells. Freshly-isolated PBMC (n=4) were expanded for 14 days with irradiated *E.coli*, re-stimulated overnight with *E.coli* at day 14, and stained for cell surface CCR7. CCR7 expression is shown in three representative donor (D1-D3) $CD3^{pos}$ PBMC, and compared in αβT *versus* γδT cells over 14 days of expansion.

I concurrently examined γδT cell expression of CD62L, which mediates αβT cell extravasation from blood into the lymphatic circulation – a step necessary for blood borne cell migration to sites of classic DC antigen presentation. Brandes *et al.* too examined γδT cell expression of this lymphoid migratory marker (182). Once again, my data relates closely to their findings. As discussed in their work, and unlike with CCR7, freshly-isolated γδT cells appear positive for CD62L (Fig.4.5).

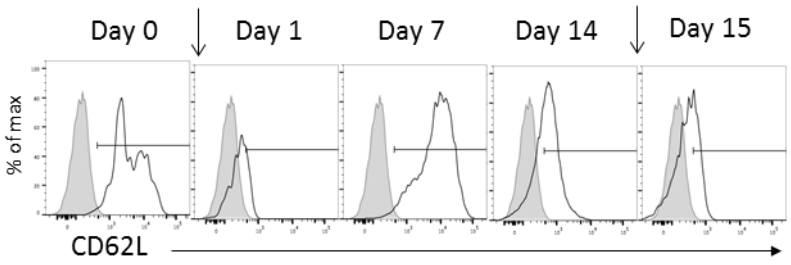

Fig. 4.5. PBMC stimulation with E.coli leads to a transient γδT cell downregulation of cell surface CD62L. Freshly-isolated PBMC (n=4) were expanded for 14 days with irradiated *E.coli*, re-stimulated overnight with *E.coli* at D14, and stained for cell surface CD62L throughout. Representative stains of one donor γδT cells is shown, with anti-CD62L antibody in black, unshaded, and isotype-matched control - in gray, shaded. Points of *E.coli* stimulation are indicated with black arrows.

PBMC stimulation with *E.coli* produced a transient downregulation of CD62L. Recovered CD62L expression was also decreased upon re-stimulation of *E.coli*-expanded γδT cells. In further contrast to CCR7, CD62L levels followed a similar pattern on both cell types, although levels on αβT cells were consistently higher than those on γδT cells (Fig.4.6).

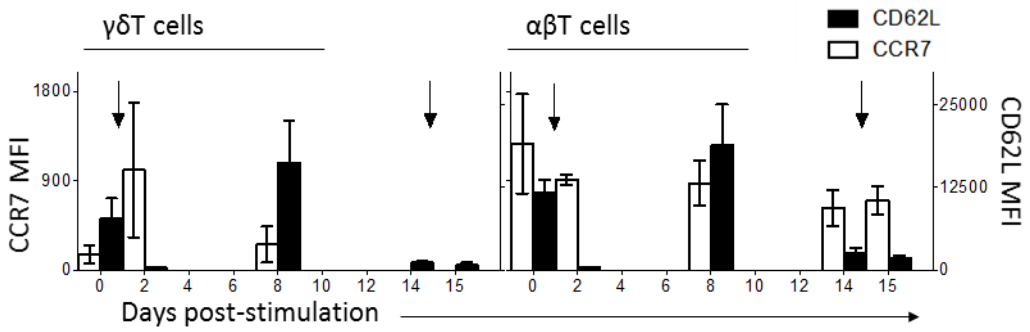

Fig. 4.6. PBMC stimulation with E.coli leads to a transient γδT cell upregulation of cell surface CCR7 and downregulation of CD62L. Freshly-isolated PBMC (n=4) were expanded for 14 days with irradiated *E.coli*, re-stimulated overnight with *E.coli* at D14, and stained for cell surface CCR7 and CD62L throughout. Donor CCR7 and CD62L expression MFI was compiled and compared between γδT and αβT cells throughout expansion and re-stimulation. Points of *E.coli* stimulation are indicated with black arrows. Bar graphs indicate the mean ± standard deviation.

As with other parameters examined, no significant difference in terms of CCR7 or CD62L expression could be seen between zoledronate and *E.coli*-expanded γδT cells (Fig.4.7).

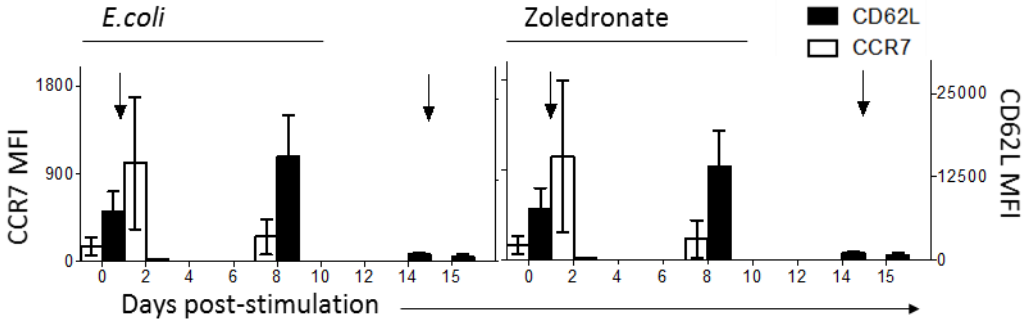

Fig. 4.7. PBMC stimulation with zoledronate leads to similar levels of CCR7 and CD62L on γδT cells and stimulation with E.coli. Freshly-isolated PBMC (n=4) were expanded for 14 days with irradiated *E.coli*, re-stimulated overnight with *E.coli* at D14, and stained for cell surface CCR7 and CD62L throughout. Donor CCR7 and CD62L expression MFI was compiled and compared between γδT and αβT cells throughout expansion and re-stimulation. Bar graphs indicate the mean ± standard deviation.

4.4. *E.coli* and zoledronate stimulation lead to expansion of similar populations of γδT cells, as well as αβT cells and $CD3^{neg}$ cells

I further endeavored to examine the non-APC effector markers routinely used to distinguish different γδT cell populations. As mentioned in the general introduction, peripheral as well as epithelia-resident γδT cells display a largely $CD4^{neg}CD8^{neg}$ phenotype, with small single positive $CD8^{pos}$ subpopulations. A similar phenotype was observed upon investigation of both *E.coli* and zoledronate-expanded γδT cells (Fig. 4.8).

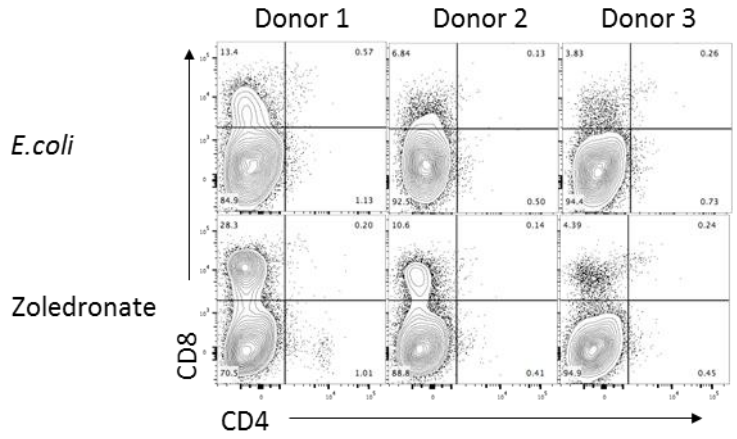

Fig. 4.8. PBMC expansion with E.coli or zoledronate leads to proliferation of predominantly $CD4^{-}CD8^{-}$ γδT cells. Day 14 *E.coli* and zoledronate-expanded PBMC (n=4) were examined for γδT cell surface marker expression via flow cytometry. Shown are representative stains from three donors, gated on total live γδT cells.

Similarly, *E.coli* and zoledronate-expanded γδT cells exhibited equivalent positivity for the cytotoxic marker CD56 and low affinity phagocytic FcγR, CD16, both of which are further classic markers of NK cells and NKT cells (Fig.4.9). Expression of CD16 and CD56 on freshly-isolated peripheral γδT cells and Vδ2 cells, in particular, has been documented in the literature previously (183,184). In summary, my data indicate that freshly-isolated peripheral *E.coli* and zoledronate-sensitive Vδ2 cells transition from a non-phagocytic, largely $CD4^{-}CD8^{-}$ HLA-DR^{neg} $CD86^{neg}$ $CCR7^{neg}$ $CD62L^{pos}$ $CD16^{pos}$ $CD56^{pos}$ phenotype to a phagocytic, largely $CD4^{-}CD8^{-}$ HLA-DR^{pos} $CD86^{pos}$ $CCR7^{neg/pos}$ $CD62L^{pos}$ $CD16^{pos}$ $CD56^{pos}$ phenotype as they expand in response to stimulus.

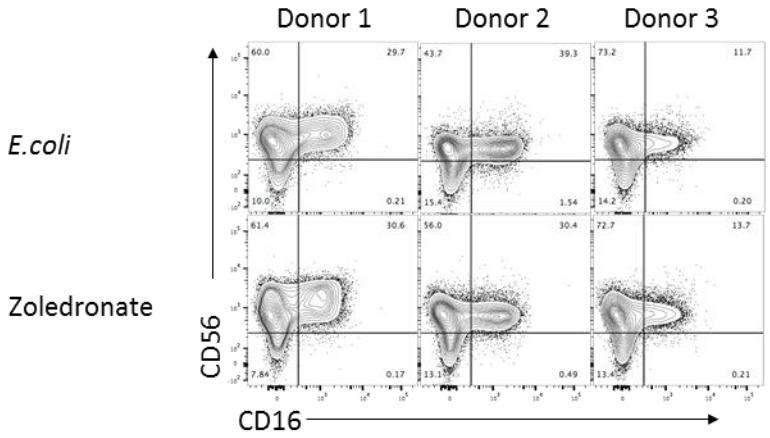

Fig. 4.9. PBMC expansion with E.coli or zoledronate leads to proliferation of γδT cells that are predominantly $CD56^{pos}$ and $CD16^{pos}$. Day 14 *E.coli* and zoledronate-expanded PBMC (n=4) were examined for γδT cell surface marker expression via flow cytometry. Shown are representative stains from three donors, gated on total live γδT cells.

The apparent homogeneity of *E.coli* and zoledronate-expanded γδT cells brought my attention to the phenotype of other cell types that may have been expanded or maintained with these expansion protocols. As discussed in the previous results chapter, while both conditions resulted in the preferential expansion of Vδ2 cells, αβT cells and a minor population of $CD3^{neg}$ cells persisted in culture throughout stimulation. αβT cells remaining in culture after two weeks of PBMC expansion with *E.coli* or zoledronate presented with similar proportions of single positive $CD4^{pos}$, $CD8^{pos}$ or double negative $CD4^{neg}CD8^{neg}$ T cells – with each phenotype representing roughly a third of all αβT cells (Fig.4.10).

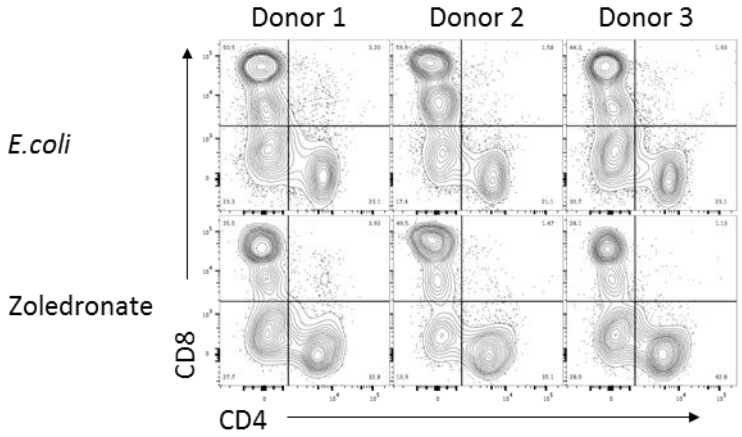

Fig. 4.10. PBMC stimulation with E.coli or zoledronate sustains $CD4^{pos}$, $CD8^{pos}$ and $CD4^{neg}CD8^{neg}$ αβT cells. Day 14 *E.coli* and zoledronate-expanded PBMC (n=4) were examined for αβT cell content via flow cytometry. Shown are representative stains from three donors, gated on total live αβT cells.

A minor difference could be observed between *E.coli* and zoledronate-maintained αβT cells in terms of CD56 expression, whence *E.coli*-expanded αβT cells contained a ~5% $CD56^{pos}$ subpopulation, whereas zoledronate-expanded αβT cells did not (Fig.4.11).

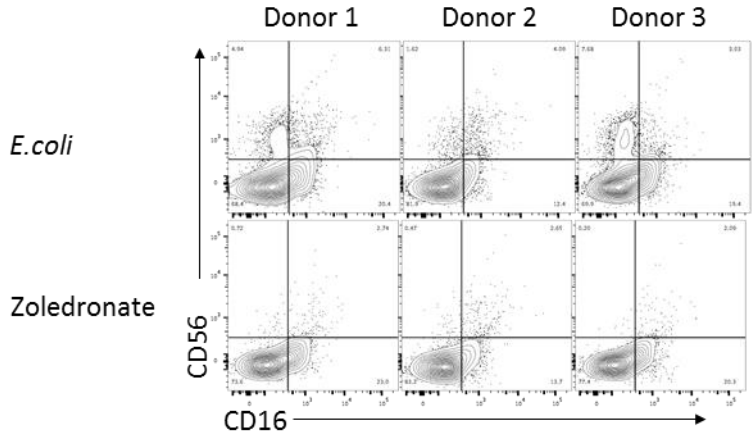

Fig. 4.11. PBMC stimulation with E.coli or zoledronate sustains $CD16^{neg}$ $CD56^{neg}$ αβT cells. Day 14 *E.coli* and zoledronate-expanded PBMC (n=4) were examined for αβT cell content via flow cytometry. Shown are representative stains from three donors, gated on total live αβT cells.

Perhaps, most crucial to my examination of non-γδT cell populations maintained within *E.coli* or zoledronate-expanded PBMC was to determine whether expanded PBMC cultures contain *bona fide* myeloid, phagocytic APCs. Published data of γδT cell APC characteristics have been met with great scepticism from large swaths of the T cell field, informed by justified concern for the effects observed being cause by 'contaminating' classic myeloid APC, rather than γδT cells. Staining whole, expanded PBMC for CD33 – a general myeloid marker (185) – suggested that both *E.coli* and zoledronate-expanded PBMC contained 0.1-0.8% $CD3^{neg}$ $CD33^{pos}$ cells (Fig.4.12).

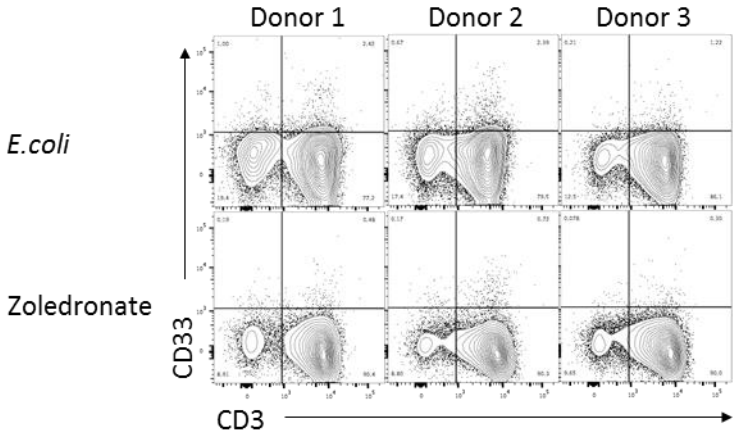

Fig. 4.12. PBMC stimulation with E.coli or zoledronate does not expand myeloid cells. Day 14 *E.coli* or zoledronate-expanded PBMC (n=4) were examined for $CD3^{neg}$ cell content via flow cytometry. Shown are representative stains from three donors, gated on total live PBMC.

While such a low proportion of myeloid cells cannot be deemed a significant potential contaminant set against my observation that nearly 100% of expanding γδT cells express CD86 and HLA-DR, while ~50% display phagocytic activity, it was further found that the 0.1-0.8% proportion of $CD3^{neg}$ $CD33^{pos}$ cells was not significantly different from that of $CD3^{neg}$ isotype control^{pos} cells. The minor $CD33^{pos}$ population observed is, thus, likely to represent non-specific binding of the antibody, rather than a real subset of $CD3^{neg}$ cells within expanded PBMC. Further staining revealed that these $CD3^{neg}$ cells are also negative for cell surface, CD14 and CD1c, excluding the possibility that expanded PBMC may contain B cells, monocytes or DCs, respectively, all of which possess well-documented pAPC capacity (Fig.4.13).

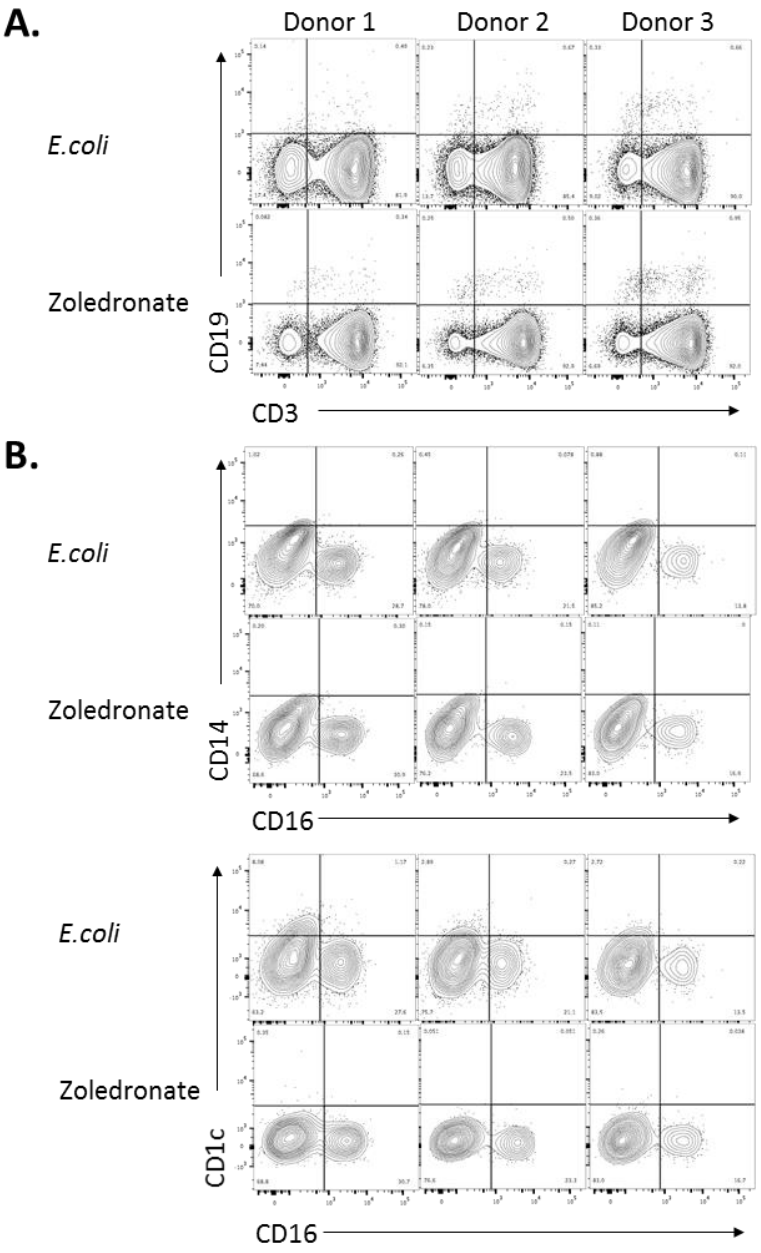

Fig. 4.13. PBMC stimulation with E.coli or zoledronate does not expand B cells, dendritic cells or monocytes. Day 14 *E.coli* or zoledronate-expanded PBMC (n=4) were examined for $CD3^{neg}$ cell content via flow cytometry. Shown are representative stains from three donors (**A**) of total live PBMC expression of CD3 *versus* CD19, or (**B**) of $CD3^{neg}$ cell CD14 *versus* CD16 or CD1c *versus* CD16 expression.

Notably, however, a significant portion of $CD3^{neg}$ PBMC presented with a $CD16^{pos}$ phenotype. Closer examination revealed that the vast majority of these $CD16^{pos}$ cells were also positive for CD56 (Fig.4.14). I, thus, stipulate that the minor $CD3^{neg}$ population that is sustained during γδT cell expansion with *E.coli* or zoledronate consists largely of natural killer (NK) cells.

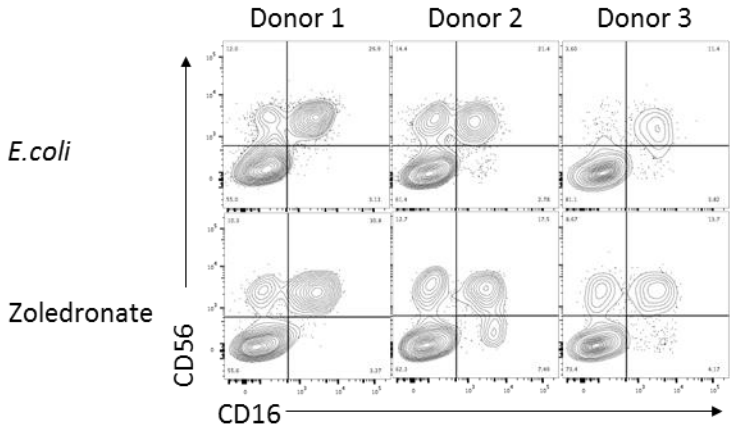

Fig. 4.14. PBMC stimulation with E.coli or zoledronate and IL-2 sustains NK cells. Day 14 *E.coli*-expanded PBMC (n=4) were examined for non-γδT cell content via flow cytometry. Markers employed in the analysis were: CD3, CD16 and CD56. Shown are representative stains from three donors, gated on total live $CD3^{neg}$ PBMC.

4.5. *E.coli* and zoledronate induce significantly different early cytokine environments within stimulated PBMC

While both *E.coli* and zoledronate have been linked to pAg-mediated activation of γδT cells directly and indirectly, respectively, both stimuli nonetheless represent vastly different antigenic challenges to the PBMC compartment (64,103). As covered briefly in the general introduction, zoledronate is a small molecule bisphosphonate drug that that inhibits farnesyl pyrophosphate synthase of the eukaryotic cell mevalonate cholesterol synthesis pathway and, consequently, leads to the accumulation of endogenous pyrophosphate metabolic intermediates, such as isopentenyl pyrophosphate (IPP) (180). IPP is then thought to induce conformational changes in the MHC-like butyrophilin (BTN) molecules at the cell surface, signalling cell 'stress'. T cell receptor (TCR)-engagement of BTN3A.1, specifically, is thought to play a central role in Vγ9Vδ2 T cell recognition of transformed or damaged cells (100). Phosphoantigens within *E.coli,* meanwhile, include some of the most potent documented Vγ9Vδ2 T cell stimuli, such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (known as 'HMBPP'), which is synthesized by some bacterial and parasitic species via isoprenoid biosynthesis (64). The potency of HMBPP in activating γδT cells, by some estimates, exceeds that of IPP by up to a 1000-fold. The structural differences between IPP and HMBPP are briefly addressed in Fig.1.2, while the various routes by which IPP and HMBPP may be activating γδT cells – in Fig.1.3.

In addition to HMBPP, *E.coli* expresses a plethora of further immunogenic signals that are thought to be recognized by γδT cells via a number of pathogen-sensing mechanisms, such as i) Lipid A, thought to engage the γδTCR directly (93), ii) LPS, which triggers γδT cell TLR-4 (93) or iii) a series of alkyl amines, including isobutylamine, which too activate γδT cells in a TCR-dependent manner (66,124). I note that, while γδT cell use and expression of TLRs has been studied and reviewed before (186,187), a comprehensive understanding of the role this receptor family plays in the physiology of various γδT cell subsets is lacking.

In addition to *E.coli* representing a significantly different stimulus from zoledronate to γδT cells directly, the introduction of a bisphosphonate drug *versus* a bolus of Gram-negative bacteria induces starkly different responses in other non-γδT cell PBMCs. To assess the collective early PBMC response to *E.coli*, zoledronate or a non-specific T cell stimulus, such as IL-2, freshly-isolated PBMC were cultured overnight in IL-2 medium (100 IU/mL) with the addition of *E.coli* (MOI 10) or zoledronate (5μM), or medium only (mock). After overnight culture, supernatant was collected and analysed for cytokine content via the cytokine multiplex assay, meso scale discovery electrochemiluminescence (MSD) (Fig.4.15). An examination of the lymphocyte growth factors, IL-7 and IL-15, revealed that neither cytokine was produced by zoledronate or *E.coli*-stimulated PBMC. High levels of IL-2 among all conditions served as an internal technical control for MSD, as all cell culture medium in the experiment contained added IL-2 at a pre-specified, equal amount. Significant differences in the cytokine response could be observed in terms of IFN-γ, TNF-α and TNF-β – cytokines associated with a pro-inflammatory Th1-type response. It is, perhaps, unsurprising that PBMC stimulation with *E.coli,* rather than zoledronate, induced a potent Th1 response and production of cytokines linked to activated monocytes. Such responses by PBMC to Gram-negative bacteria have been documented previously (66,102,188–190). Although Th2-type cytokines (IL-4, IL-5 and IL-13) could not be detected at high levels, *E.coli* nonetheless elicited higher levels than zoledronate. Unlike PBMC stimulation with zoledronate, stimulation with *E.coli* induced high levels of pyrogens and systemic inflammatory cytokines, such as IL-6, IL-1α and IL-1β, as well as IL-8 and GM-CSF. Interestingly, while the mean amount produced was higher in *E.coli* compared to zoledronate-stimulated PBMC, the same vast differences in statistical significance between the two stimuli couldn't be observed when examining antigen presentation and priming-associated cytokines, such as IL-12p40, IL-12p70 and IL-16. The same was true for the anti-inflammatory, regulatory mediator, IL-10. Freshly-isolated PBMC stimulation with IL-2 medium alone (mock) failed to induce a response in any of the cytokines examined.

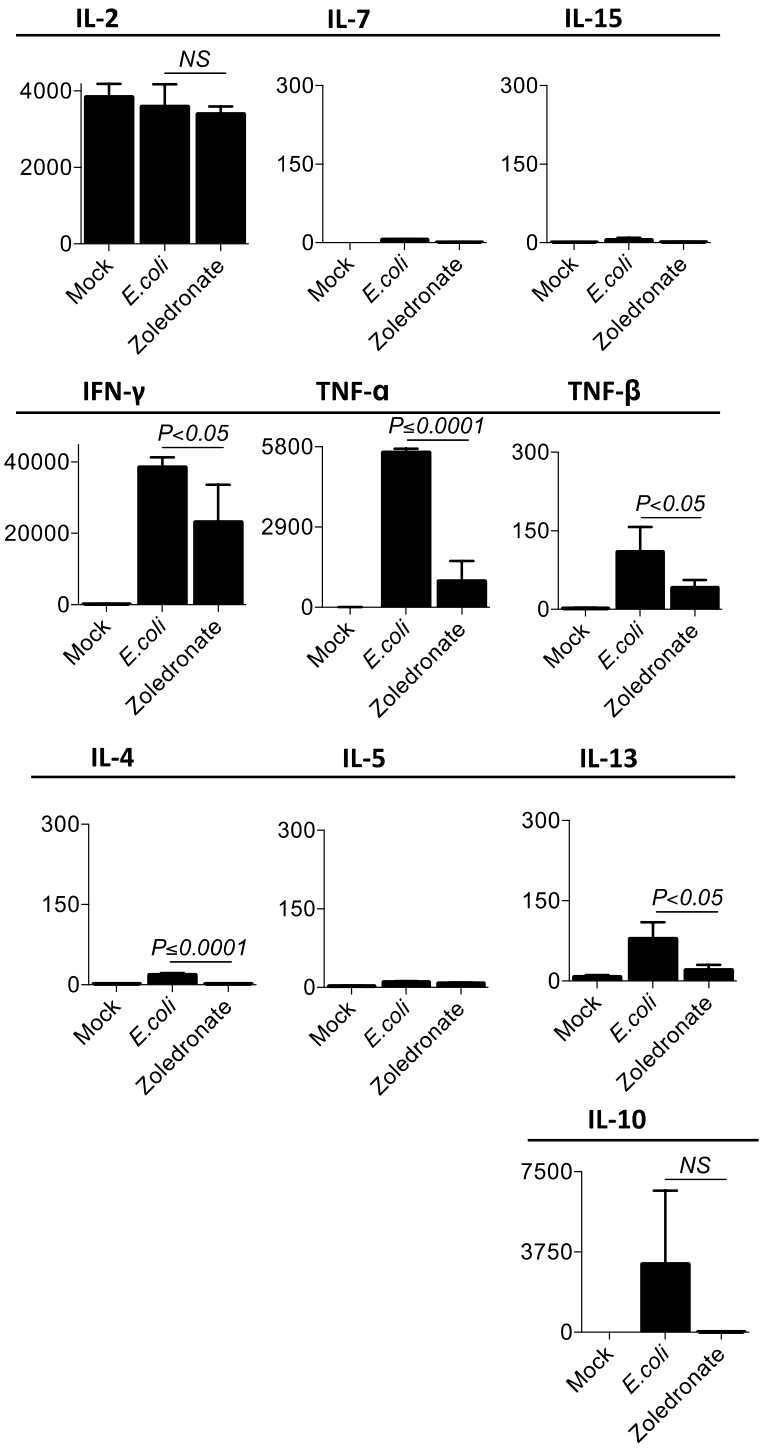

Figure 4.15 is continued on the next page.

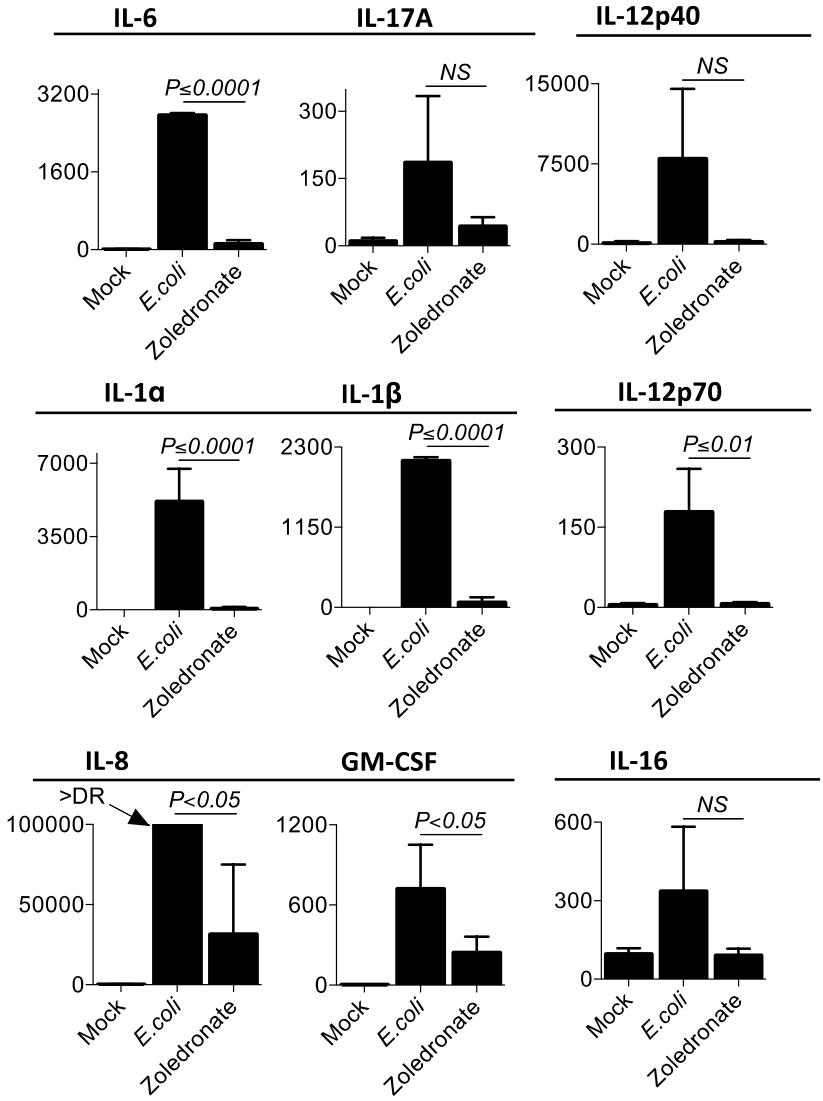

Fig. 4.15. Fresh PBMC stimulation with E.coli or zoledronate leads to significantly different early cytokine environments. Freshly-isolated PBMC (n=4) were stimulated overnight with irradiated *E.coli,* zoledronate (5μM) or mock-stimulated with IL-2 media alone. Supernatants were collected and analyzed using a meso scale discovery electrochemiluminescence assay (MSD). The x-axis on the graphs indicates the stimulus applied to each set of PBMC; the y-axis indicates the amount of cytokine detected in media (pg/mL). '>DR' denotes that the cytokine amount was above the detection range of the MSD assay. Bar graphs indicate the mean ± standard deviation.

4.6. *E.coli* and zoledronate induce similar Vγ9Vδ2 TCR repertoires

The observation that *E.coli* and zoledronate stimulation of freshly-isolated PBMC induces largely equivalent γδT cell populations in terms of APC marker, homing receptor, CD4 and CD8, as well as CD16 and CD56 expression prompted us to examine the early cytokine environment within stimulated PBMC. Upon noting that the early cytokine environment is vastly different between zoledronate and *E.coli*-stimulated PBMC, I set out to characterise the T cell receptor (TCR) repertoire of the differentially expanded γδT cells. As described earlier, zoledronate and *E.coli* are antigenically vastly divergent stimuli – each representing one documented self TCR ligand (i.e. BTN3A) and multiple non-self TCR ligands (*e.g.* Lipid A, isobutylamine, HMBPP, etc.), respectively.

Freshly-isolated PBMC were expanded with *E.coli* or zoledronate for 14 days, and the resulting TCR repertoire was examined, using an Irepertoire Illumina human gamma delta CDR3 sequencing platform. The TCR CDR3 regions of (a) freshly-isolated unexpanded, (b) *E.coli*-expanded and (c) zoledronate-expanded γδT cells from one representative donor were sequenced. *E.coli* and zoledronate-expanded γδT cells showed significant overlap of their Vγ and Vδ CDR3 sequences; both expansions resulted in a predominantly Vγ9Vδ2 γδT cell population (Fig.4.16).

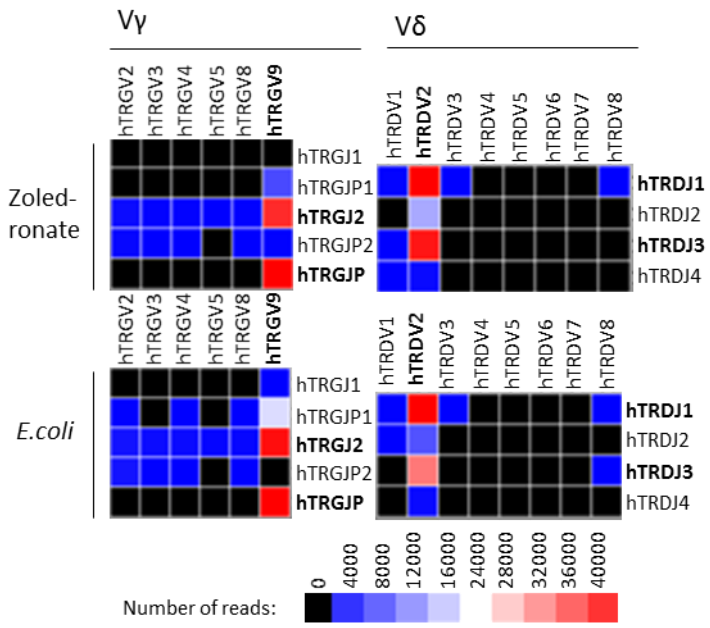

Fig. 4.16. E.coli and zoledronate induce proliferation of similar γδT cell clones. Freshly-isolated PBMC were expanded with UV-irradiated *E.coli* or zoledronate for 14 days. The resulting γδT cell CDR3 repertoire was examined. Representative heat maps of one donor expanded γδT cell Vγ (VG), Vδ (VD) and Vj (VJ) chain reads are shown.

CDR3 homology and frequency in selected sequences that represented 5000 or more CDR3 reads were investigated (Fig.4.17). The most prominent group in Vγ chain CDR3 sequences was shared between all three expansion conditions, suggesting a largely conserved expansion. The other prominent group was shared between *E.coli* and zoledronate-expanded γδT cells, with only low counts exclusive to either group. ~80-90% overlap in the Vγ and the Vδ chain CDR3 regions was observed between the two expansion protocols. Notably, the single exclusive group of Vδ chains was found in freshly isolated γδT cells, indicating a significant shift in Vδ chain CDR3 repertoire post expansion.

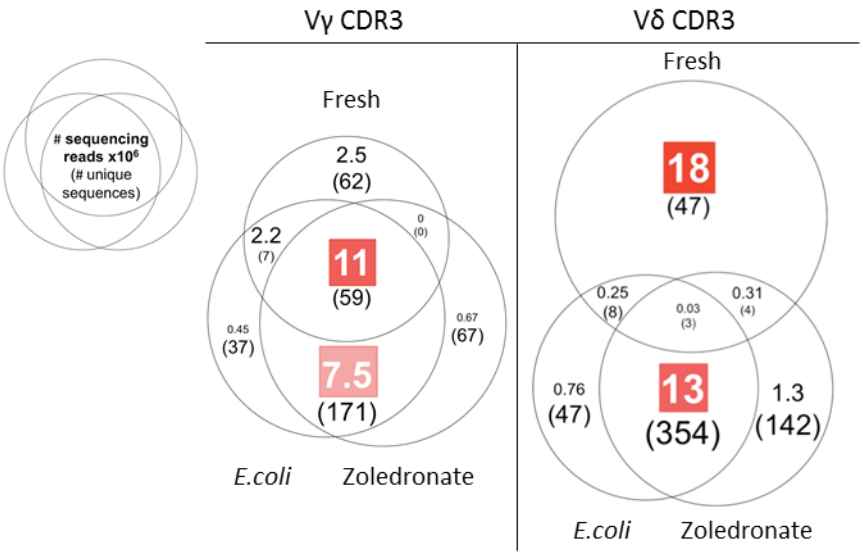

Fig. 4.17. E.coli and zoledronate focus the γδT cell CDR3 repertoire in a similar manner. Freshly-isolated PBMC were expanded with UV-irradiated *E.coli* or zoledronate for 14 days. The resulting γδT cell CDR3 repertoire was examined. The number of shared TCR CDR3 sequences was tallied and compared in expanded and fresh unexpanded γδT cells. A comparison of the number of sequencing reads shared as well as the number of unique sequences in each category is shown for both Vγ and Vδ TCR chains. For all samples, total depth of sequencing was equivalent. Only sequences with more than 5000 reads were tallied to select for the commonest clones.

To further examine the homology between the TCR repertoires of γδT cells expanded with *E.coli* or zoledronate, CDR3 spectratype analysis was performed for both populations. The donor used for spectratype analysis was different to the donor used for sequencing, so as to validate repertoire similarities or differences in more than one donor. Striking homology in CDR3 spectratypes was observed in the Vγ9 and Vδ2 chains of *E.coli* and zoledronate-expanded γδT cells, confirming the CDR3 overlap observed in CDR3 sequencing (Fig.4.18).

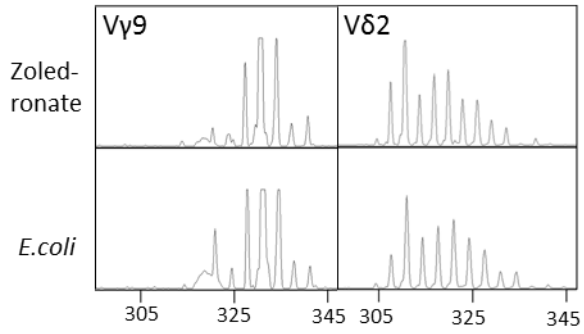

Fig. 4.18. E.coli and zoledronate-expanded γδT cells present with similar spectratypes. Freshly-isolated PBMC were expanded with UV-irradiated *E.coli* or zoledronate for 14 days. The resulting γδT cell CDR3 repertoire was examined. TCR CDR3 spectratyping was carried out for expanded Vγ9Vδ2 T cells. Representative spectratypes of one donor are shown.

4.7. Discussion

The experiments described in this chapter were designed to provide a detailed phenotypic analysis of fresh *versus E.coli* and zoledronate-expanded γδT cells. Particular focus was placed on expression of the APC molecules, CD86 and MHC class II (HLA-DR, specifically), and lymphoid tissue homing receptors, CCR7 and CD62L. Further analysis was performed on expanded and freshly-isolated Vγ9Vδ2 cell TCR repertoires. Findings included:

i) Freshly-isolated PBMC stimulation with *E.coli* or zoledronate induces sustained γδT cell (including Vδ2 cell) upregulation of HLA-DR and CD86.

ii) γδT cell upregulation of HLA-DR and CD86 is equivalent to that of freshly-isolated monocytes in terms fluorescence intensity.

iii) αβT cells do not significantly upregulate HLA-DR or CD86 in response to PBMC stimulation with *E.coli* or zoledronate.

iv) Freshly-isolated PBMC stimulation with *E.coli* or zoledronate induces transient γδT cell (including Vδ2 cell) upregulation of CCR7.

v) Expanding γδT cells return to a $CCR7^{neg}$ phenotype post-transient upregulation, which is not rescued by expanded γδT cell re-stimulation with *E.coli.*

vi) The period of maximal γδT cell surface HLA-DR and CD86 levels does not overlap with that of maximal cell surface CCR7 levels.

vii) Freshly-isolated γδT cells are positive for CD62L.

viii) Freshly-isolated PBMC stimulation with *E.coli* or zoledronate induces transient γδT cell (including Vδ2 cell) downregulation of CD62L.

ix) Expanding γδT cells return to a somewhat $CD62L^{pos}$ phenotype post-transient downregulation, although positivity eventually declines as the expansion progresses.

x) Some downregulation of γδT cell CD62L is still seen upon expanded γδT cell re-stimulation with *E.coli.*

xi) Of the markers examined, fluctuation levels of γδT cell surface CD86 and HLA-DR, pre- and post-stimulation, follow a similar pattern. CCR7 levels do not match the pattern of CD86 and HLA-DR positivity. CD62L levels do not match the pattern of either CD86 and HLA-DR, nor CCR7.

xii) Freshly-isolated PBMC stimulation with *E.coli* or zoledronate leads to the expansion of predominantly $CD4^{neg}CD8^{neg}$ Vδ2 cells that are $CD56^{pos}$ and $CD16^{pos}$.

xiii) The same expansions further sustain a mixture of $CD4^{pos}$, $CD8^{pos}$ and $CD4^{neg}CD8^{neg}$ αβT cells that are largely $CD56^{neg}$ and $CD16^{neg}$.

xiv) Freshly-isolated PBMC stimulation with *E.coli* or zoledronate does not sustain or expand myeloid cells (including DC and monocytes) or B cells.

xv) The stimulation does, however, sustain a minor population of $CD3^{neg}CD56^{pos}CD16^{pos}$ PBMC.

xvi) TCR sequencing of expanded γδT cells indicates that both *E.coli* and zoledronate expand Vγ9Vδ2 cells preferentially.

xvii) Sequencing further indicates that both expansion stimuli expand highly homologous TCR repertoires, in terms of CDR3, which are nonetheless significantly different from freshly-isolated γδT cell CDR3.

xviii) Focusing of the CDR3 repertoires in response to both stimuli compared to freshly-isolated γδT cells is largely located in the Vδ chain locus and not the Vγ chain locus.

xix) The homology of CDR3 repertoires between *E.coli versus* zoledronate-expanded Vγ9δ2 cells is further supported by spectratype analysis.

xx) Similar populations of γδT cells, αβT cells and $CD3^{neg}$ cells were expanded or sustained despite divergent early cytokine milieus present in PBMC stimulated with *E.coli versus* zoledronate.

The focus of this chapter was two-fold. Firstly, I aimed to characterize the dynamics of pAPC markers on γδT cells expanded with *E.coli* or zoledronate. Secondly, I aimed to establish whether the expansion of Vδ2 cells with apparently equivalent phagocytic

capacity in response to stimulation with *E.coli* or zoledronate, as described in the previous chapter, correlates to further phenotypic similarities in the two cell populations.

As outlined in the chapter introduction, the upregulation of MHC class II (HLA-DR, specifically) and CD86 on activated Vδ2 cells in response to diverse stimuli, ranging from IPP to Gram-negative bacteria as well as mycobacteria, has been described in the literature previously (102,105). I was able to reproduce these results and establish, moreover, that this upregulation is sustained and consistent among the various donors examined. This is strongly indicative of the putative pAPC role of activated, but not resting (i.e. freshly-isolated, unstimulated) peripheral Vδ2 cells. γδT cell upregulation of the lymphoid-homing receptor CCR7 in response to stimulation has been previously described by Brandes, Moser and colleagues (51,182). This finding was interpreted as an indication that γδT cell APC function is likely to resemble that of classic professional APCs, such as DC, in terms of localisation.

Curiously, I was unable to find an obvious correlation between these lymphoid homing cell surface markers and cell surface levels of HLA-DR and CD86. While transient upregulation of CCR7 could be observed in response to freshly-isolated γδT cell stimulation, it did not appear to correlate with maximal γδT cell upregulation of HLA-DR nor CD86, nor was I able to detect significant phagocytosis of opsonized *E.coli* by freshly-isolated γδT cells. It must be noted that staining for homing receptors on circulating γδT cells is significantly complicated by the fact that CCR7 and CD62L are homing markers with dual interpretations of function. On canonical, peripheral αβT cells these are markers of a naïve, unstimulated state and continued lymphatic re-circulation in search of cognate antigen. On classic APC, such as DC, meanwhile, these are markers of exit from tissue where an inflammatory target has been encountered, and shuttling to lymph nodes for presentation of phagocytosed antigenic material to naïve T cells. It remains unclear as to what relationship the 'innate-like' γδT cells bear to canonical αβT cell homing markers. The interpretation of Brandes *et al.* regarding γδT cell CCR7 ascribes it to the 'myeloid-like' properties of these 'innate T cells'. Due to a lack of obvious correlation and in the

absence of functional studies, I hesitate to make such a conclusion sans further investigation. It remains, moreover, unclear as to whether γδT cell antigen presentation occurs in lymph nodes *in vivo*.

Comprehensive further experimental investigation is necessary to determine the homing patterns of activated, phagocytic APC-like peripheral human Vδ2 cells. It cannot be ruled out, for example, that the phagocytic and antigen presenting capacity of these cells peaks at seven days post-stimulation and not the 14 days post-stimulation employed in the study. At seven days post-stimulation CCR7 positivity on stimulated γδT cells remained higher than compared to baseline expression. The phagocytic and antigen presenting capacity peak may even occur at any time before the seven-day mark after PBMC stimulation with *E.coli* or zoledronate. Perhaps 'maturation' into this phenotype occurs at different rates in a donor-dependent manner. The only way to effectively address this question is to carry out comparative γδT cell phagocytic assays and chemokine receptor staining at much shorter time intervals than the experiments described. A more thorough enquiry would warrant investigation of these phenomena every successive day post-stimulation, or even at hour intervals to ensure precision of the data generated. The resources required for such an undertaking are vast, which was a deciding factor in my not pursuing of this strategy. It is curious to note, however, that 14 day expanded γδT cells were capable of *E.coli* phagocytosis, but did not re-upregulate CCR7 in response to re-encounter with *E.coli*. CD62L was, furthermore, downregulated upon γδT cell re-encounter with *E.coli*. This may be indicative that γδT cell phagocytosis does not correlate with γδT cell migration to lymphoid tissue – at least in terms of CCR7 and CD62L.

A point of consideration in the context of examining APC phenotype in short term-expanded γδT cells concerns my investigative motivation, which is rooted in the potential use of γδT cells in the clinic. A requirement in the clinic for a large number of cells available for use, in combination with the relative scarcity of γδT cells in the periphery, indicates a likely necessity for clinic-destined γδT cells to be expanded *in vitro* for 14 days

or more. The phenotypic characterisation of γδT cells expanded for 14 days and more, thus, carries some value in and of itself.

A crucial step in the meaningful exploration of activated peripheral γδT cell homing properties is characterisation of homing receptors beyond CCR7 and CD62L. In addition to the above-mentioned CCR7 and CD62L, various groups have addressed γδT cell expression of CCR2, CCR5, CCR6, CXCR3 and CXCR4, among others (191,192). There persists, however, an overall lack of consensus on the significance of each of these receptors on γδT cells compared to canonical αβT cells. It is, moreover, unclear whether these investigations have examined both activated and non-activated γδT cells. Comprehensive review on γδT cell homing properties is necessary. Such an undertaking is significantly complicated by the ambiguous relationship between human and laboratory animal (*e.g.* mouse) γδT cells, making information on *in vivo* human γδT cells trafficking scarce. While some review on the subject is available in the literature, it is nearly 15-years-old and likely out of date (193). The chemokine receptors I propose ripe for investigation in the context of γδT cell APCs, before and after stimulation, are summarized in Table 4.1.

Table 4.1 | **Homing receptors of interest to γδT cell APC function**

Receptor	Immune cell expression	Key immune function
CXCR3	Th1, $CD8^{pos}$ TCM and T_{EM}, Treg, Tfh, NK, NKT, pDC, B cell	Th1-type adaptive immunity
CXCR5	Tfh, Tfr, $CD8^{pos}$ T_{EM}, B cells	T and B cell trafficking in lymphoid tissue to B cell zones/follicles
CCR2	Th1, monocyte, macrophage, iDC, basophil, NK	Th1-type adaptive immunity, monocyste trafficking
CCR4	Th2, skin- and lung-homing T, Treg, Th17, $CD8^{pos}$ T, monocytes, B cells, iDC	Homing of T cells to skin and lung, Th2-type immune response
CCR6	Th17, iDC, γδT, NKT, NK, Treg, Tfh	iDC trafficking, GALT development, Th17 adaptive immune responses
CCR8	Th2, Treg, skin T_{RM}, γδT, monocyte, macrophage	Immune surveillance in skin, type 2 adaptive immunity, thymopoiesis
CCR9	Gut-homing T, thymocytes, B cells, DC, pDC	Homing of T cells to gut, GALT development and function, thymopoiesis
CCR10	Skin-homing T, IgA-plasmablasts	Humoral immunity at mucosal sites, immune surveillance in skin
CXCR1	Cross-presenting $CD8^{pos}$ DC, thymic DC	Antigen cross-presentation by $CD8^{pos}$ DCs
CX3CR1	Resident monocytes, macrophages, microglia, Th1, $CD8^{pos}$ T_{EM}, NK γδT, DCs	Patrolling monocytes in innate immunity, microglial cell and NK cell migration, Th1-type adaptive immunity

Elements of this table have been adapted from Luster at. al. (2014) (103).

Abbreviations: *DC, dendritic cell; iDC, immature DC; GALT, gut-associated lymphoid tissue; NK, natural killer cell; NKT, natural killer T cell; T_{CM}, central memory T cell; T_{EM}, effectory memory T cell; T_{RM}, resident-memory T cell; Tfh, T follicular helper cell; Tfr, follicular regulatory T cell; Th, T helper cell; Treg, regulatory T cell.*

Upon considering non-γδT cell populations that were sustained during expansion post PBMC stimulation with *E.coli* or zoledronate, it is perhaps unremarkable that I found these consisted largely of αβT cells and $CD3^{neg}CD33^{neg}CD56^{pos}CD16^{pos}$ cells. While finding PBMC in culture that were $CD3^{neg}$ after 14 days of T cell expansion was initially surprising, learning that these are $CD33^{neg}$ (i.e. not of myeloid origin) as well as $CD56^{pos}CD16^{pos}$ leads us to postulate that these cells are likely NK cells. All γδT cell expansion media employed in these studies contained high concentrations of IL-2, which is a known mitogen of T cells (with γδTCRs or αβTCRs) as well as NK cells (194). As indicated by MSD data, PBMC exposure to *E.coli* or zoledronate further induced high levels of potent pro-inflammatory cytokines, such as IFN-γ. An intriguing possibility remains that the αβT cells remaining in culture after two weeks of *E.coli* or zoledronate expansion represent antigen-specific outgrowth of αβT cell clones rare at baseline, rather than a non-specific bystander effect. Significant further phenotyping and, importantly, functional assays are necessary to determine the nature of the αβT cells and likely NK cells remaining in γδT cell culture. It cannot be ruled out that these cells play a 'supportive' role in their engagement of expanding γδT cells. While a skewing of the functional phenotype is possible, expansion protocols with purified γδT cells have been published, suggesting that the presence of αβT cells or NK cells is not essential for γδT cell expansion (195).

The finding that both expansion conditions induced proliferation of predominantly $CD4^{neg}CD8^{neg}$ Vδ2 cells that are $CD56^{pos}$ and $CD16^{pos}$ is, perhaps, also unremarkable. The expansion of $CD56^{pos}$ and $CD16^{pos}$ Vδ2 cells using pAg stimulation has been described previously (184,196). More surprising was the finding that no significant differences could be found in terms of these important functional markers whether PBMC had been exposed to *E.coli versus* zoledronate. I postulated initially that, given the highly divergent stimuli that *E.coli* and zoledronate represent, proliferating Vδ2 cells may be skewed to divergent phenotypic paths. In terms of the parameters that I examined, this did not appear to be the case.

Expanded γδT cell TCR sequencing determined that both expansion stimuli induced proliferation of specifically Vγ9Vδ2 cells. CDR3 sequencing and spectratyping further revealed a striking similarity between *E.coli* and zoledronate-expanded Vγ9Vδ2 cell CDR3, which were nonetheless significantly different from freshly-isolated, unexpanded γδT cells. These finding are consistent with the focusing of the γδTCR repertoire on a common set of TCR ligands, regardless of stimulation with *E.coli versus* zoledronate. As described earlier, it has been proposed that zoledronate activates γδT cells by causing accumulation of endogenous pyrophosphates and, consequently, conformational changes in butyrophilin molecules, such as BTN3A. Like zoledronate, *E.coli* too has been documented to cause the accumulation of endogenous pyrophosphates (IPP), which then serve to drive activation and proliferation of Vγ9Vδ2 T cells (101,102). It may have to be considered, therefore, that Vγ9Vδ2 cell proliferation is regulated foremostly by recognition of 'self-stress', via *e.g.* BTN molecules, even if the stimulus is exogenous in nature (as addressed briefly in Fig.1.3); even if the stimulus contains a plethora of further pro-inflammatory molecules in and of itself, as is the case with *E.coli.* Related to this is the observation that the recognition of pAg signals appears to occur primarily through germline-encoded regions of the Vγ9Vδ2 TCR, and involves all CDR loops (181). Relative to the αβTCR, there are, moreover, relatively few germline genes available for assembly of the γδTCR (197). While some have postulated that the expansion of the Vγ9Vδ2 subset in the periphery after birth is driven by exposure to environmental microbial ligands, there remains a lack of consensus on the factors that drive Vγ9Vδ2 cell expansion *in vivo* (198).

Having observed striking similarities between Vγ9Vδ2 cell responses within PBMC stimulated with zoledronate or *E.coli,* I ventured to better characterise the functional responses of both freshly-isolated and expanded γδT cells. I set out, moreover, to determine whether the functional shift between a non-phagocytic to a phagocytic cell population pre- and post-expansion correlates with further effector function changes in γδT cells pre- and post-stimulation.

4.8. Summary

Data laid out in this chapter lead us to the conclusion that *E.coli* and zoledronate-expanded γδT cells bear a close resemblance in terms of functional cell surface marker expression, with both populations presenting with a predominantly HLA-DR^{pos} CD86^{pos} CCR7^{neg} CD62L^{low} CD4^{neg} CD8^{neg} CD16^{pos} CD56^{pos} phenotype. Striking homology could, moreover, be seen in the differentially expanded Vγ9Vδ2 TCR CDR3 sequences, which were similar to one another, but not to freshly-isolated TCR CDR3s. This suggests that PBMC stimulation with *E.coli* and zoledronate leads to Vγ9Vδ2 CDR3 focusing on a similar set of ligands.

5. γδT CELL ACQUISITION OF pAPC PHENOTYPE IS CONCURRENT TO A LOSS OF Th1 INFLAMMATORY PHENOTYPE

5.1. Aims

The previous results chapter documents the observation that expanded Vδ2 γδT cells acquire an opsonin-dependent phagocytic phenotype that correlates with upregulation of CD86 and MHC class II. This phenotypic shift resulted from PBMC stimulation with either *E.coli* or zoledronate. Surprisingly, in all parameters examined (including TCR repertoire) Vδ2 cells expanded with *E.coli* or zoledronate appeared highly similar. In this chapter I set out to document in further detail the impact of expansion on Vδ2 cell effector phenotype. Parameters such as antigen presentation, cytokine production and cytotoxic capacity were considered.

A number of publications describe the *in vitro* pAPC activity of human γδT cells (49–51,60). This phenotype has, however, not been correlated to corresponding output of cytokines, memory phenotype, expansion status or cytotoxic capacity of the γδT cells in question. In this chapter I set out to determine whether γδT cells expanded with *E.coli* or zoledronate do indeed act as APCs to naïve peripheral αβT cells. I explored, moreover, how this antigen presentation correlates to more accepted and classic γδT cell functions, such as production of IFN-γ and cytotoxicity.

5.2. γδT cells expanded with *E.coli* or zoledronate display a pAPC effector phenotype

The previous chapters described my observation that γδT cell expansion with *E.coli* or zoledronate produces a population of efficiently phagocytic HLA-DR^{pos}CD86^{pos} Vδ2 cells. While these observations provide evidence toward pAPC function by γδT cells, functional assays are necessary to establish whether expanded Vδ2 cells are indeed capable of antigen presentation.

E.coli-expanded PBMC examination with confocal microscopy revealed that, within ~30min of plating, Vδ2 cells had re-formed large, 'grape-like' clusters, observed typically with *in vitro* activated γδT and αβT cells (Fig.5.1A). These Vδ2 cells further displayed a single, regular, rounded nuclear shape, as indicated by DAPI staining (Fig.5.1B), another classic T cell morphological feature. Inadvertently, such clustering presents a significant technical challenge to imaging. Clear cell images via confocal microscopy require a low cell density and separation of single cells, so as to be able to document cell shape and movement precisely. Thus, detailed information about expanded cell morphology was acquired by examination of non-clustered Vδ2 cells (Fig.5.2). Low density, unclustered expanded Vδ2 cells revealed a shape highly atypical for normally-round and regular T cells - with dendritic-like cell shape and irregular membrane ruffles extending in all directions (Fig.5.2A, B).

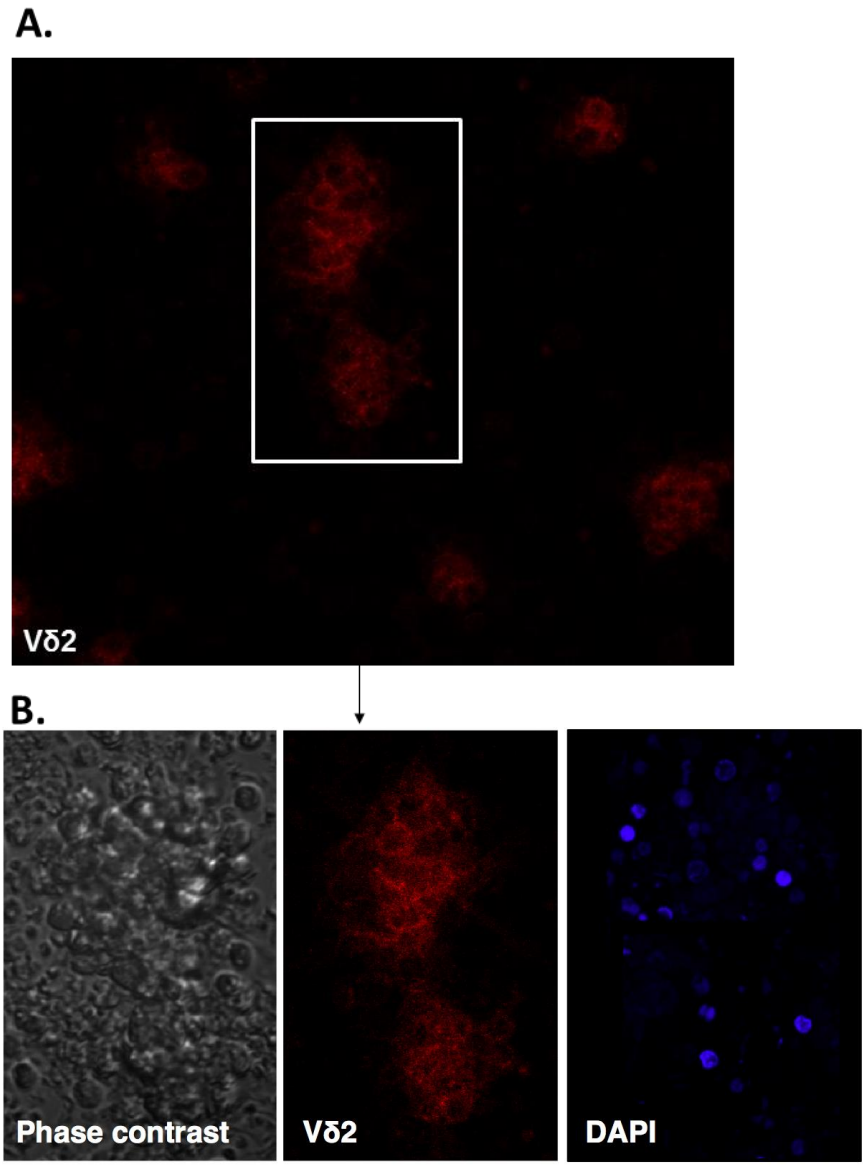

Fig. 5.1. γδT cell activation and expansion leads to Vδ2 cell clustering. Day 14 *E.coli*-expanded PBMC were stained with Vδ2-PE mAb, DAPI and examined via confocal microscopy. (**A**) Shown is a representative field of vision with Vδ2-PE seen in red. (**B**) A section of the field of vision, showing a Vδ2 cell cluster, was amplified to provide greater detail of cell membrane and nuclear shape.

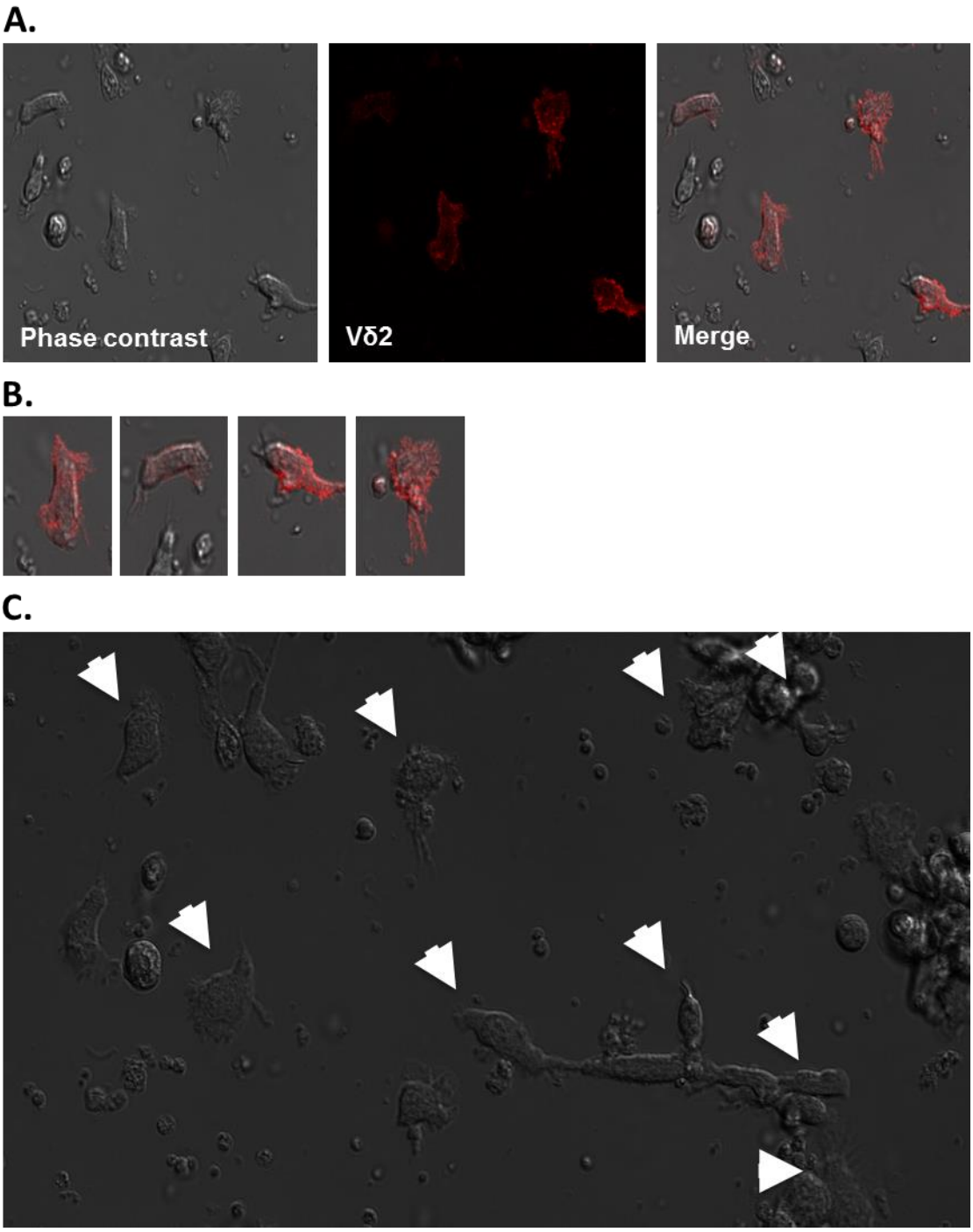

Fig. 5.2. Vδ2 cell expansion leads to their acquisition of a phagocyte-like morphology and probing behaviour. Day 14 *E.coli*-expanded PBMC were stained with Vδ2-PE mAb and examined via confocal microscopy. (**A**) Shown is a representative field of vision, with PBMC in light phase contrast, Vδ2-PE or a merge of the two, with (**B**) magnified Vδ2 PBMC from the representative field of vision. (**C**) Light phase contrast images from the same field of vision as shown in panel (**A**) were collected every 2 seconds for 5 minutes. Shown is a video sequence of the collected images. Vδ2 PBMC are indicated with white arrows. The video material is available for viewing in the CD attached to the thesis as supplementary media material.

Strikingly, the movement of expanded Vδ2 cells was strongly reminiscent of phagocyte probing (a screenshot from the video material is visible in Fig.5.2C; the full video is provided in a CD attached to the thesis in Supplementary Media Material). Every single Vδ2 cell in the field of vision appeared engaged in active, exploratory membrane ruffling. Grinstein and Botelho define 'probing' as "membrane extensions enriched in phagocytic receptors flail[-ing] about in an actin-dependent manner, enhancing the likelihood of particle contact and receptor-ligand engagement" (199). Such probing is characteristic of phagocytic cells, including dendritic cells, and constitutes the first stage of phagosome formation. The stages of phagosome formation, as described by Grinstein and Botelho, are i) probing via membrane ruffling followed by receptor-ligand binding, given presence of target, ii) early signalling and cup formation, mediated by receptor clustering and tyrosine kinase activation, iii) pseudopod extension via actin remodelling and localised membrane secretion, and iv) phagosome closure involving phagocytic signal termination.

To test whether expanded Vδ2 cells are capable of activating naïve αβT cells, zoledronate-expanded PBMC were FACS-sorted for Vδ2 cells to >98% purity. Zoledronate instead of *E.coli*-expanded PBMC were used to avoid the possibility that *E.coli* debris may be lingering in the cell media or has been internalized by cells. Purified Vδ2 cells were then incubated overnight with IgG-opsonized *E.coli.* In parallel, αβT cells were purified from freshly-isolated, unstimulated autologous PBMC. Expanded, opsonized *E.coli*-stimulated Vδ2 cells were then co-cultured for two days with purified αβT cells at γδT:αβT ratios of 1:20, 1:10 and 1:5. The experimental setup is illustrated in the Materials and Methods chapter. Normally, αβT cell antigen presentation assays require the use of a cell line with a known TCR specificity or αβT cell transduction with TCR of known specificity, and possibly even transduction of the APC with a specific HLA type. I was able to overcome the necessity for HLA transduction by employing 'responder' αβT cells isolated from the same donor as the 'stimulator' expanded Vδ2 cells. As for the necessity for TCR transduction of responder αβT cells, I stipulated there may be a relatively high baseline frequency of *E.coli*-reactive αβT cells in the peripheral blood of healthy human adults, due to the ubiquitous distribution of *E.coli* as both, a commensal and pathogen. I, therefore, speculated that there may be high-enough numbers of *E.coli*-reactive αβT cells

in unstimulated donor PBMC to be detectable using flow cytometry. Indeed, various groups have documented the relatively high numbers of *E.coli*-reactive αβT cells in humans at various anatomical sites, ranging from blood to synovial fluid, to lamina propria (200–203). The responders in this antigen presentation assay were, thus, non-transduced, normal adult PBMC αβT cells.

αβT cells co-cultured with mock-stimulated Vδ2 cells (Vδ2 cells stimulated with medium only) did not produce IFN-γ. Importantly, this indicates that HLA-DR^{pos}CD86^{pos} Vδ2 alone are not sufficient to induce an IFN-γ response from naïve αβT cells (Fig.5.3A, B). *E.coli*-stimulated Vδ2 cells, meanwhile, induced αβT cell IFN-γ production at all the γδT:αβT cell ratios examined. γδT:αβT ratios ranging from 1:5 to 1:20 did not produce a significant difference in the IFN-γ observed. In terms of subset-specificity of the response, IFN-γ production was observable only in CD4+ αβT cells (Fig.5.3C).

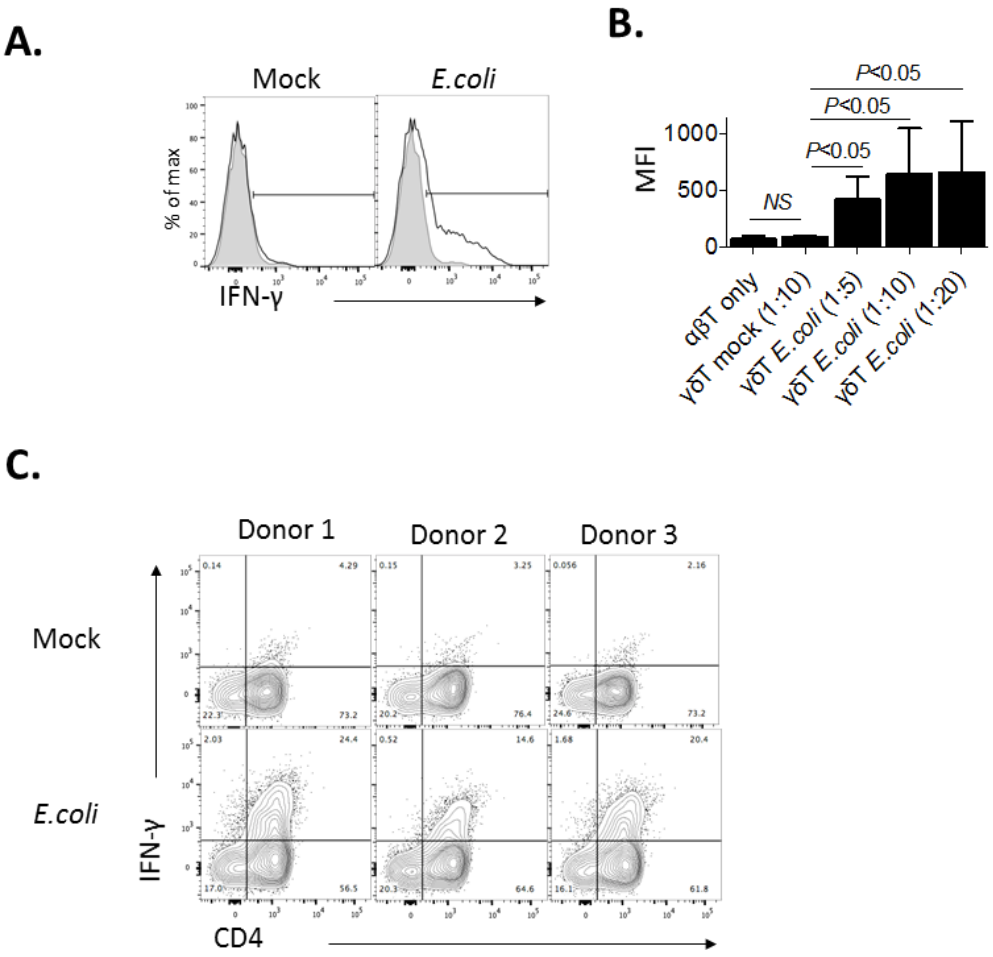

Fig. 5.3. Expanded γδT cells, co-cultured with opsonized E.coli, induce IFN-γ production in fresh autologous αβT cells. γδT cells were expanded for 14 days from freshly-isolated PBMC using zoledronate (5μM). On day 14, γδT cells were purified from expanded PBMC using FACS-sorting and rested overnight. Purified γδT cells were then incubated overnight with IgG-opsonized, irradiated *E.coli* at MOI 10 and washed thoroughly. In parallel, αβT cells were purified via positive magnetic selection from autologous, freshly-isolated PBMC and rested overnight. Purified, *E.coli*-stimulated, expanded γδT cells were then co-cultured for 2 days with purified, autologous fresh αβT cells. After 2 day co-culture, αβT cells were blocked with monensin, permeabilized and stained via FACS for intracellular IFN-γ. (**A**) Representative stains of one donor αβT cells is shown comparing co-culture with mock (media only)-stimulated or *E.coli*-stimulated expanded γδT cells at a γδT:αβT ratio of 1:10 (anti-IFN-γ antibody in black, unshaded, and isotype-matched control - in gray, shaded). (**B**) Three donor αβT cell IFN-γ MFI were compiled and compared between αβT cells stimulated with i) media only (αβT only), ii) mock-stimulated γδT cells at a γδT:αβT ratio of 1:10, iii) *E.coli*-stimulated γδT cells at a γδT:αβT ratio of 1:5, iv) *E.coli*-stimulated γδT cells at a γδT:αβT ratio of 1:10, v) *E.coli*-stimulated γδT cells at a γδT:αβT ratio of 1:20. (**C**) Representative stains of three donor αβT cells is shown comparing co-culture with mock (media only)-stimulated or *E.coli*-stimulated expanded γδT cells. αβT cell IFN-γ production is set against expression of CD4. Bar graphs indicate the mean ± standard deviation.

αβT cells within freshly-isolated, *E.coli*-stimulated whole PBMC did not produce IFN-γ (Fig.5.4). Freshly-isolated Vδ2 cells do not, thus, appear capable of professional antigen presentation to naïve αβT cells. Significant further experimental investigation is, however, necessary to draw this conclusion with certainty. The autologous stimulator-responder APC assay setup is experimentally convenient and interesting in some aspects, but is far from an all-encompassing experimental design. Of note, γδT cells within freshly-isolated, *E.coli*-stimulated whole PBMC, meanwhile, produced large amounts of IFN-γ (Fig.5.4).

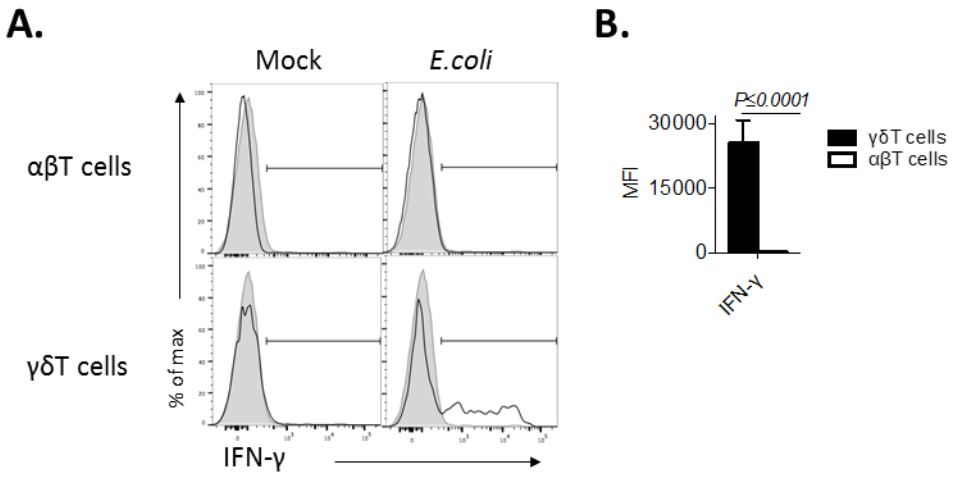

Fig. 5.4. αβT cells within whole, freshly-isolated, E.coli-stimulated PBMC do not produce IFN-γ. Freshly-isolated PBMC were stimulated overnight with *E.coli* or mock stimulated (media only). PBMC were then blocked with monensin, stained for cell surface markers, permeabilized and stained for intracellular IFN-γ. (**A**) Representative stains of intracellular IFN-γ levels from one donor are shown, comparing mock or *E.coli*-stimulated γδT cells and αβT cells (anti-IFN-γ antibody in black, unshaded, and isotype-matched control - in gray, shaded). (**B**) Five donor data were compiled, comparing IFN-γ production by γδT and αβT cells within overnight *E.coli*-stimulated, freshly-isolated PBMC. Bar graphs indicate the mean ± standard deviation.

5.3. γδT cell expansion leads to a loss of a Th1 inflammatory phenotype

Having documented the phagocytic and pAPC capacity of expanded γδT cells, I set out to further explore the *E.coli* response of freshly-isolated γδT cells. Freshly-isolated PBMC were co-cultured with irradiated *E.coli* at MOI 10. After overnight stimulation, responses within stimulated PBMC were characterised. *E.coli*, but not mock, stimulation resulted in significant γδT cell upregulation of CD69, production of IFN-γ and TNF-α, as well as CD107a-mediated cytotoxic degranulation in a manner that was highly consistent among all donors examined (Fig.5.5A). γδT cells presented with high baseline positivity for granulysin, which remained largely unchanged after stimulation. A mean ~50% of γδT cells within freshly-isolated PBMC responded to *E.coli* with the upregulation of CD69, production of IFN-γ and cytotoxic degranulation (Fig.5.5B). While αβT cells failed to upregulate CD69 or produce cytokines, a cytotoxic response could be observed, albeit at significantly lower levels than that of γδT cells (Fig.5.6).

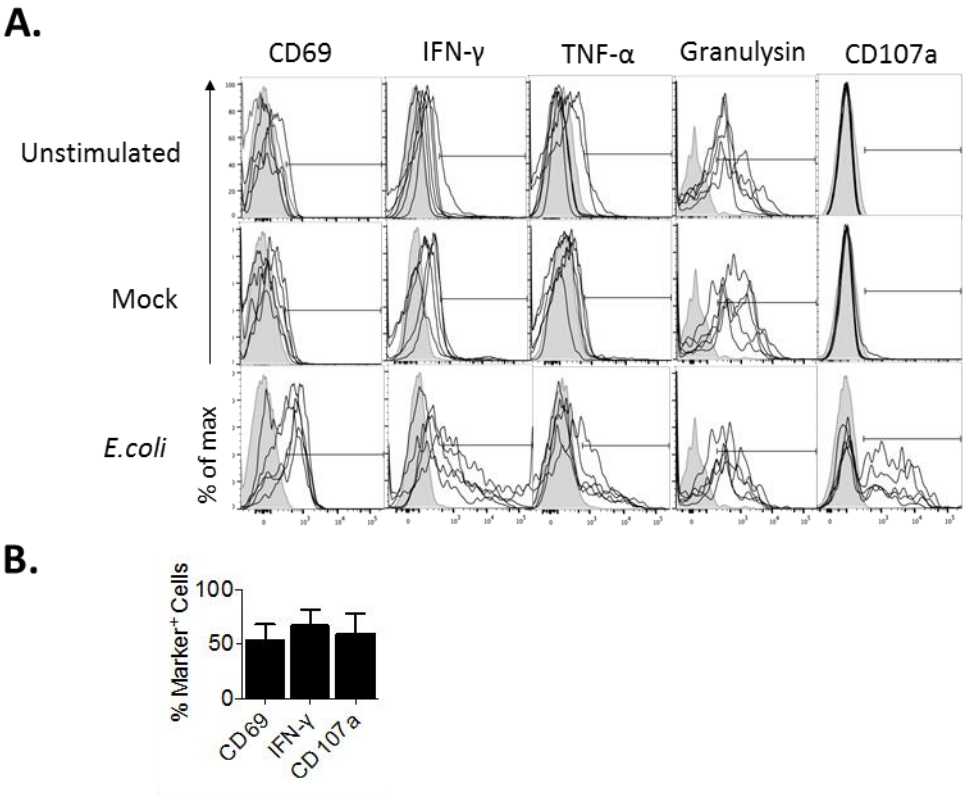

Fig. 5.5. Freshly-isolated PBMC stimulation with E.coli leads to potent γδT cell activation and effector responses. Freshly-isolated PBMC (n=5) were stimulated with irradiated *E.coli* at MOI 10 and analysed after overnight (16-18h) culture. (**A**) Shown are overlaid five donor sample stains of unstimulated, and overnight mock (IL-2 only) or *E.coli*-stimulated PBMC. The parameters examined were γδT cell surface CD69 and CD107a, as well as intracellular IFN-γ, TNF-α and granulysin (marker in black, unshaded; isotype control in gray, shaded). (**B**) The proportion of $CD69^{pos}$, $IFN\text{-}\gamma^{pos}$ and $CD107a^{pos}$ cells was examined in γδT cells of overnight *E.coli*-stimulated PBMC.

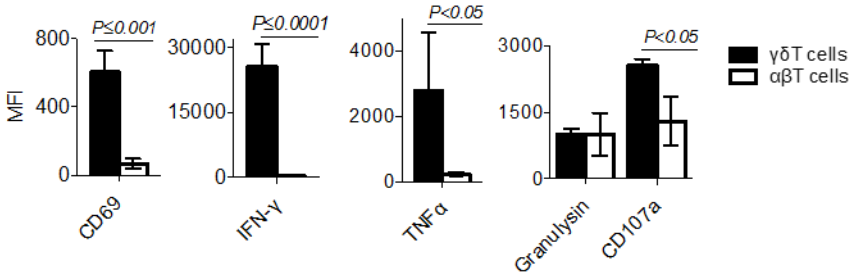

Fig. 5.6. Fresh PBMC stimulation with E.coli leads to γδT cell, but not αβT cell, effector responses. Freshly-isolated PBMC (n=5) were stimulated with irradiated *E.coli* at MOI 10 and analysed after overnight (16-18h) culture. The MFI of cell surface CD69, CD107a, as well as intracellular IFN-γ, TNF-α and granulysin were compared in γδT and αβT cells of overnight *E.coli*-stimulated PBMC. Bar graphs indicate the mean ± standard deviation.

A majority of γδT cells were IFN-γ^{pos}CD107a^{pos}; a significant fraction, nonetheless, exhibited a single-positive IFN-γ^{pos} or CD107a^{pos} phenotype (Fig.5.7). These were later found to be Vδ2 and Vδ1 cells, respectively (Fig.5.7B). While Vδ2 and Vδ1^{neg}Vδ2^{neg} γδT cell populations were major cytokine producers, Vδ1 cells exhibited a significantly more potent cytotoxic response as measured by granulysin and CD107a expression. CD69 expression was markedly higher on Vδ2 cells compared to other γδT cell subsets. No IL-17 or IL-10 production by γδT cells could be detected via FACS, ELISA or MSD prior to or after expansion, as indicated in the previous chapter.

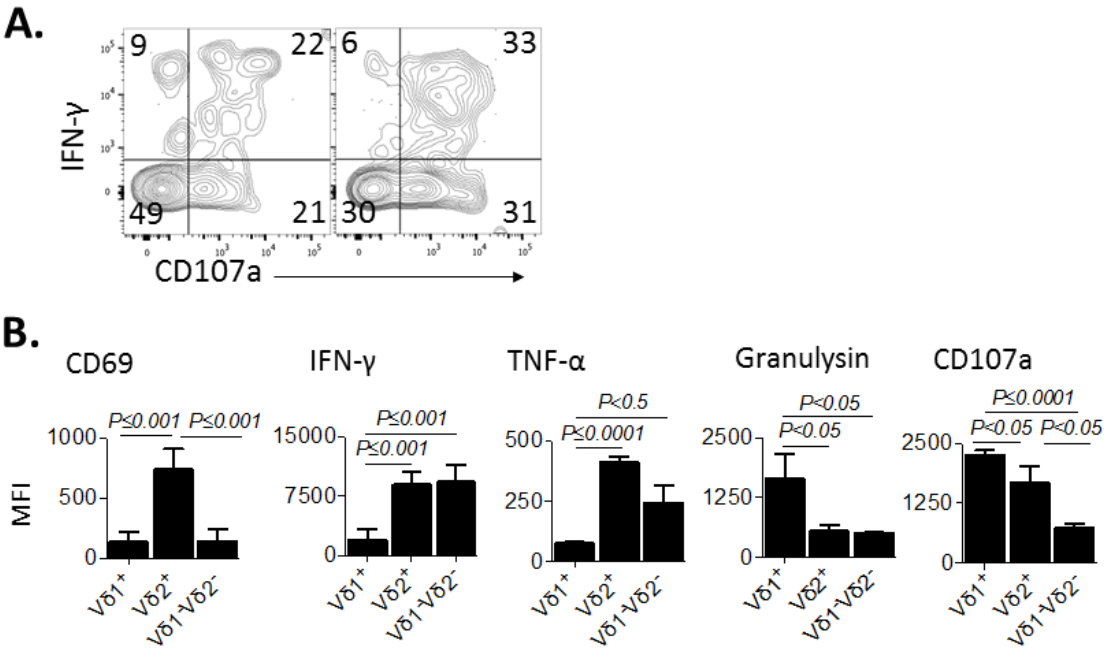

Fig. 5.7. Fresh PBMC stimulation with E.coli leads to predominantly Vδ2 cell activation and effector responses. Freshly-isolated PBMC (n=5) were stimulated with irradiated *E.coli* at MOI 10 and analysed after overnight (16-18h) culture. (**A**) Two representative donor stains are shown for *E.coli*-stimulated γδT cells, comparing cell surface CD107a and intracellular IFN-γ. (**B**) Cell surface CD69, CD107a, as well as intracellular IFN-γ, TNF-α and granulysin MFI were compared in Vδ1, Vδ2 and Vδ1^{neg}Vδ2^{neg} γδT cells. Bar graphs indicate the mean + standard deviation

I next hypothesized that γδT cell expansion with *E.coli* may enhance the anti-*E.coli* response upon re-challenge. Unexpectedly, following expansion with *E. coli*, γδT cells exhibited a significantly decreased cytokine (IFN-γ, TNF-α) response to *E.coli*, and decreased CD69 expression compared to that observed in freshly isolated γδT cells (Fig.5.8A). *E.coli*-expanded γδT cells did, however, continue to exhibit CD107a-mediated cytotoxic degranulation and presence of granulysin, albeit at lower levels compared to freshly isolated γδT cells (Fig.5.8A, B). These observations may partially be attributed to the loss of the highly cytotoxic Vδ1 subset after expansion. Further surprising was the finding that γδT cell expansion with zoledronate resulted in a similar loss of cytokine production capacity post-expansion (Fig.5.9), ruling out the possibility that the loss of Th1 cytokine production was a specific result of exposure to *E.coli*.

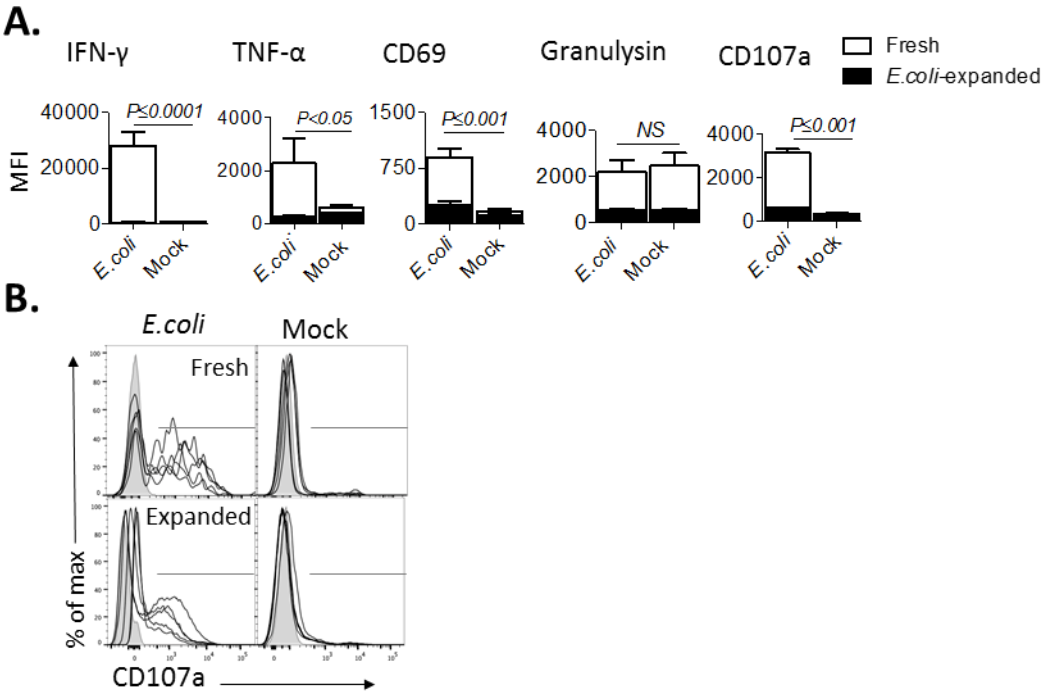

Fig. 5.8. Expanded γδT cells lose IFN-γ and TNF-α production capacity, but retain cytotoxicity, upon re-stimulation with E.coli. Freshly-isolated PBMC (n=5) were stimulated with *E.coli* and analyzed after overnight (16-18h) culture or left to expand for 14 days, and re-stimulated with *E.coli* or mock-stimulated (media only) overnight on day 14. γδT cell accumulation of intracellular IFN-γ, TNF-α, granulysin and cell surface CD69, as well as CD107a were assessed via FACS analysis. (**A**) γδT cell MFI in response to *E.coli* or mock stimulation were compared between freshly-isolated and 14 day *E.coli*-expanded γδT cells. (**B**) Shown are overlaid five donor sample stains of overnight mock or *E.coli*-stimulated γδT cell CD107a in fresh *versus* *E.coli*-expanded PBMC (marker in black, unshaded; isotype control in gray, shaded). Bar graphs indicate the mean ± standard deviation.

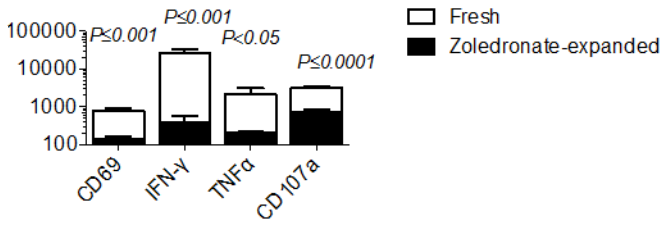

Fig. 5.9. γδT cell loss of early Th1 and reduction of cytotoxic phenotype occurs in response to expansion with zoledronate. Freshly-isolated PBMC (n=3) were stimulated with zoledronate, left to expand for 14 days, and re-stimulated with *E.coli* overnight on day 14. γδT cell intracellular IFN-γ, TNF-α and cell surface CD69 as well as CD107a were assessed via FACS analysis. Parameter MFI in response to *E.coli* stimulation were compared between freshly-isolated and 14 day zoledronate-expanded γδT cells. Bar graphs indicate the mean ± standard deviation.

The decrease in cytokine production by *E.coli*-expanded compared to freshly-isolated γδT cells was not associated with a shift in memory phenotype or exhaustion marker expression (Fig.5.10A-C).

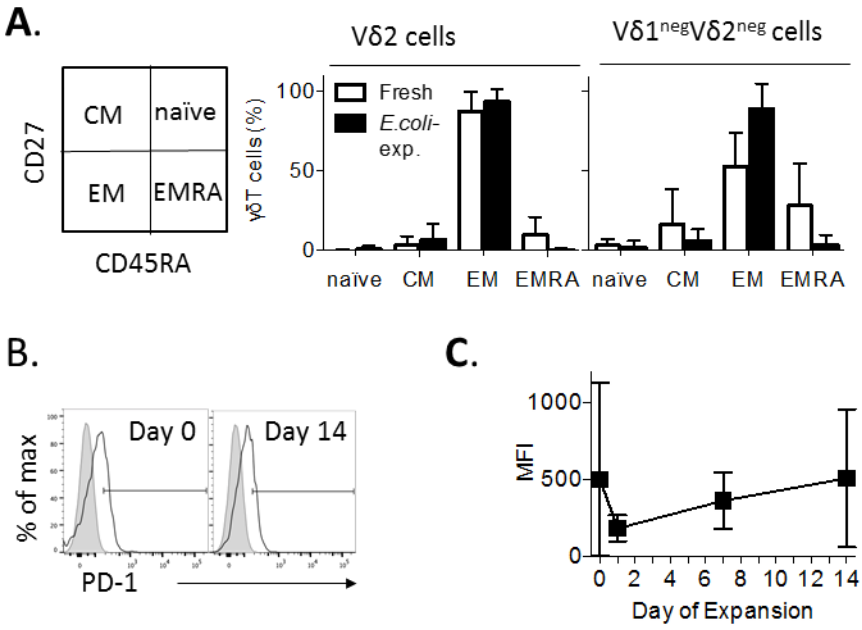

Fig. 5.10. Expansion in response to PBMC stimulation with E.coli does not lead to a significant change in γδT cell memory phenotype or PD1 expression. (**A**) γδT cell memory phenotype, as determined by cell surface expression of CD27 and CD45RA, was compared between fresh and *E.coli*-expanded Vδ2 and Vδ1^{nrg}Vδ2^{neg} γδT cells (n=7). (**B**) Representative donor stains are shown comparing PD-1 expression on fresh (D0) and expanded (D14) γδT cells with PD-1 in black, unshaded, overlaid with isotype control in gray, shaded. (**C**) Cell surface PD-1 MFI on γδT cell surface was tracked throughout PBMC expansion with *E.coli* (n=7).

In order to establish whether the failure of *E.coli* re-stimulation to induce Th1-type cytokine production in expanded γδT cells is due to a lack of non-T cell 'helper' cells, I set up an autologous expanded + freshly-isolated PBMC culture system (described in the materials and methods chapter). Briefly, *E.coli*-expanded γδT cells were mixed with autologous, freshly-isolated PBMC at a γδT cell:PBMC ratio of 1:10 prior to the addition of *E.coli*. Therefore, if γδT cell production of IFN-γ requires, for example, the presence of freshly-isolated monocytes, the presence of such 'helper' cells would be provided by autologous PBMC. This addition of autologous PBMC did not rescue the lost expanded γδT cell Th1-like phenotype (Fig.5.11). I, thus, concluded that the altered cytokine profile of expanded *versus* freshly isolated γδT cells represents a true phenotypic shift.

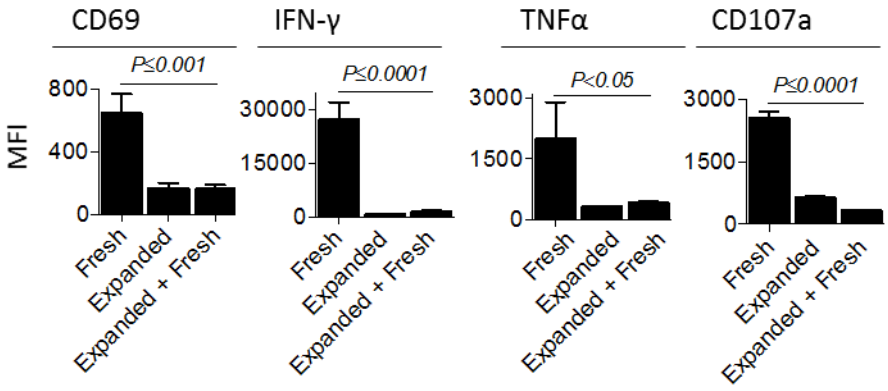

Fig. 5.11. γδT cell primary response effector phenotype is not rescued by the addition of fresh PBMC to expanded γδT cell culture. 14 day *E.coli*-expanded γδT cell culture (n=5) was stained for cell surface markers and supplemented with freshly-isolated, autologous, γδT cell-depleted PBMC at a ratio of 1:10 prior to overnight co-culture with *E.coli* at MOI 10. γδT cell responses were compared in terms of cell surface CD69, CD107a, as well as intracellular IFN-γ and TNF-α between i) freshly-isolated PBMC, ii) 14 day expanded PBMC, and iii) 14 day expanded PBMC supplemented with freshly-isolated PBMC.

Having established that expanded γδT cells do maintain some CD107a-mediated cytotoxic degranulation in response to *E.coli* re-stimulation, I evaluated direct expanded γδT cell cytotoxicity against *E.coli*. FACS-sorted *E.coli*-expanded γδT cells were exposed to live

bacteria for specified time intervals; cytotoxicity was inferred from residual colony numbers on agar plates. Remarkably, *E.coli* colony forming unit (CFU) count declined by >90% within 30 minutes (Fig.5.12). The high efficiency of bactericidal activity was observed upon use of either *E.coli* or zoledronate-expanded γδT cells.

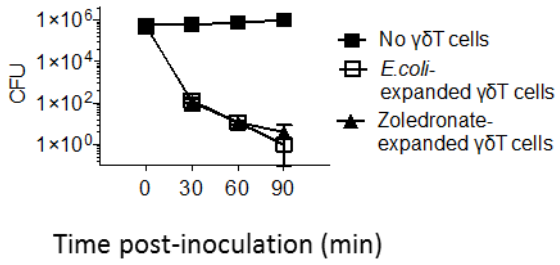

Fig. 5.12. Expanded γδT cells kill E.coli rapidly and effectively. Freshly-isolated PBMC (n=4) were stimulated with *E.coli* or zoledronate and left to expand for 14 days. γδT cells were then purified by twice-repeated positive magnetic selection and rested overnight. Rested γδT cells were co-cultured with live *E.coli* at MOI 10. At designated time points the co-culture was lysed in H_2O, and plated on agar for overnight growth; resulting *E.coli* CFU were counted.

5.4. Discussion

The data laid out in this chapter explores the pAPC capacity of expanded Vδ2 cells, and attempts to correlate it to more commonly-described γδT cell functions, such as Th1-type cytokine production and cytotoxicity. Findings included:

i) Expanding Vδ2 cells display classic T cell morphological features in terms of clustering and a single, round, regularly sized nucleus.

ii) Expanding Vδ2 cells also display morphological features characteristic of myeloid phagocytes, such as an irregular cell shape with numerous membrane protrusions, and probing behaviour via guided membrane ruffling and motility.

iii) Expanded Vδ2 cells, co-cultured with IgG-opsonized *E.coli* (but not medium alone), induce IFN-γ production in naïve, autologous αβT cells.

iv) *E.coli*-pulsed Vδ2 cell-induced IFN-γ production is specific for CD4 αβT cells.

v) IFN-γ production by αβT cells is not seen when *E.coli* is added to whole freshly-isolated PBMC. This indicates that expanded (phagocytic, HLA-DR^{pos} CD86^{pos}) Vδ2 cells are necessary for the triggering of an αβT cells IFN-γ response.

vi) Freshly-isolated PBMC stimulation with irradiated *E.coli* induces potent γδT cell Th1-like cytokine (IFN-γ and TNF-α) production that is consistent among all donors examined.

vii) Freshly-isolated γδT cells within PBMC are highly positive for granulysin at baseline. While stimulation with *E.coli* induces potent CD107a-meditaed cytotoxic degranulation, intracellular granulysin levels are not significantly altered.

viii) These responses within PBMC are seen in freshly-isolated γδT cells, but not αβT cells.

ix) γδT cell response to *E.coli* is subset-specific. Vδ2 cells are significantly more activated and are the predominant producers of Th1-type cytokines, while Vδ1 cells display the most potent cytotoxicity.

x) The potent Th1-type, cytotoxic response to *E.coli* is seen in freshly-isolated γδT cells, but not expanded Vδ2 cells. This loss of phenotype does not appear to be due to exhaustion, memory phenotype shift, nor absence of myeloid 'helper' cells (*e.g.* monocytes) within expanded PBMC.

xi) The loss of a Th1-type phenotype to *E.coli* challenge results from γδT cell expansion with *E.coli* as well as zoledronate.

xii) An effector function that is preserved, albeit at lower level than when freshly-isolated, is Vδ2 cell CD107a-mediated cytotoxic degranulation.

xiii) γδT cells expanded with *E.coli* and zoledronate display remarkably effective and rapid anti-*E.coli* bactericidal capacity.

Establishing that HLA-DR^{pos} CD86^{pos} phagocytic Vδ2 cells display myeloid-like morphology and probing behaviour, combined with the observation that *E.coli*-pulsed Vδ2 cells are capable of inducing IFN-γ production in naïve, autologous αβT cells in a CD4-dependent manner provides a strong case for the pAPC capacity of peripheral human γδT cells. I concede, however, that the antigen presentation assay described in this chapter is non-standard and far from exhaustive. While there are indeed published statistics on the frequency of *E.coli*-reactive αβT cell clones in the periphery, they are significantly lower than the proportion of IFN-γ^{pos} CD4 αβT cells I observed in our antigen presentation assay (14-24% of all αβT cells). This casts doubt on the reliability of the findings described. I stipulate that the high proportion of activated αβT cells observed is likely a consequence of the purification method employed: αβT cells were isolated from PBMC using positive magnetic selection, a method known to cross-link the αβTCR. A longer 'resting' period sans stimulation (*e.g.* two days instead of one) may be necessary to fully mute this initial stimulus. Alternatively, other cell purification methods may be employed, such as FACS sorting for *e.g.* CD3^{pos} γδTCR^{neg} PBMC. It is interesting to note, however, that αβT cells co-cultured with non-pulsed Vδ2 cells failed to show any IFN-γ production. It is unclear as to why that is. It is conceivable that the high proportion of IFN-γ^{pos} CD4 αβT cells post stimulation with *E.coli*-plused Vδ2 cells was the consequence of a necessary double stimulus from anti-αβTCR-coated magnetic beads, followed by TCR stimulation via HLA-DR-presented *E.coli* antigen and co-stimulation from γδT cells. The data obtained

in these experiments further requires a significant enlargement of the panel of target antigens used. *E.coli* on its own is insufficient as a model to test the pAPC capacity of γδT cells. The use of whole bacteria complicates the investigation in a number of manners, including the possibility that the observed αβT cell activation is due, in some part, to triggering of TLRs or other PRRs by *E.coli* that have been carried over from the γδT cell pulsing co-culture. Such issues may be overcome by the use of protein targets that still require pAPC processing for presentation on MHC class II, as was the case with previous work in my laboratory by Himoudi *et al.* (49). A peptide target stimulus does, however, require αβT cell responder cells with a known TCR specificity and, therefore, transduction or the use of a cell line. Microfluidic platforms for the examination of single cell pair Ca^{2+} flux as the result of TCR triggering (as described by Dura, *et al.* (204)) would doubtless provide fascinating insight into Vδ2 cell activation of naïve αβT cells. A further matter to be considered in a protein-stimulus experimental setup is the necessity for IgG opsonisation.

Perhaps, most striking of all the findings is the total loss of freshly-isolated Vδ2 cell Th1-type cytokine production during expansion, concurrent with the acquisition of a phagocytic HLA-DR^{pos} $CD86^{pos}$ phenotype. This observation, in combination with IgG-opsonisation as a pre-requisite of phagocytosis (and other pAPC functions), suggests careful regulation of Vδ2 T cell effector function. It is tempting to speculate that, whenever an early immune response is sufficient to neutralize infection, Vδ2 cells are prevented from posing an unnecessary inflammatory threat by amplifying responses further through downregulation of their early cytokine responsiveness. Noteworthy was the finding that, despite the loss of Th1-type cytokine production, Vδ2 cells retained highly efficient bactericidal capacity. It is unusual to consider that T cells are capable of such potent and direct bacterial killing. While significant further study is warranted to establish the exact mechanisms, the data presented in this chapter highlights the plethora of information yet to be learned. Vδ2 cell CD107a-mediated degranulation was decreased with expansion, but not entirely. It is conceivable that the residual CD107a-mediated degranulation was sufficient to kill *E.coli* in culture. It is also possible that the bactericidal functions of expanded Vδ2 cells are not limited to CD107a-mediated cytotoxicity. Mass

spectrometry of *E.coli*-stimulated Vδ2 cell supernatants would provide fascinating insight into the efficient killing of bacteria observed.

γδT cell direct killing of cellular and/or microbial targets combined with inflammatory cytokine production followed by uptake of the target into acidifying antigen processing compartments raises a novel paradigm. It is tantalizing to hypothesize that the combination of innate-like recognition and killing followed by myeloid cell-like phagocytosis by a lymphocyte-like cell may evolutionarily have preceded the full development of T lymphocyte-mediated adaptive immunity. Such a hypothesis is supported by previous studies showing that γδTCR chain genes may have preceded the development of αβTCR chain genes (205). In addition, the existence in jawless fish of three lymphocyte-like cells expressing variable lymphocyte receptors instead of TCR or BCR chain genes also supports this contention, as they otherwise resemble γδT, αβT and B cells by the expression of other orthologous genes (206).

5.5. Summary

Data examined in this chapter revealed that Vδ2 cell acquisition of a pAPC phenotype during expansion with *E.coli* or zoledronate is accompanied by a loss of a Th1-type pro-inflammatory phenotype, whilst maintaining potent cytotoxicity.

6. Vδ2 γδT CELL EFFECTOR FUNCTION DEPENDENCE ON THE TCR

6.1. Aims

Having documented the evolution of peripheral γδT cell effector function in response to stimulation by both, *E.coli* and zoledronate, I ventured to explore a mechanistic basis for this transition. The data presented in this chapter addresses the above-described range of effector functions in the context of the γδTCR. Blocking with monoclonal antibody was employed to determine γδT cell functional dependence on the TCR.

6.2. Phagocytosis by expanded γδT cells is TCR-dependent

As described in Chapter III, *E.coli* and zoledronate-expanded γδT cells phagocytosed opsonized bacteria with similar dynamics – with a similar level of uptake of beads, and uptake as well as acidification of bacteria. Chapter V, meanwhile, described that *E.coli* and zoledronate-expanded γδT cells express highly homologous TCR repertoires. I, thus, hypothesized that both stimuli may lead to γδT cell engagement of effector functions in a similar manner in the context of the TCR. To examine the importance of the TCR in γδT cell phagocytosis, *E.coli* or zoledronate-expanded γδT cells were cultured with anti-γδTCR mAb (clone: B1), isotype-matched control mAb of known non-*E.coli* specificity (clone: MG1-45) or media alone, prior to co-culture. Both uptake and acidification of opsonized bacteria by *E.coli*-expanded γδT cells were highly sensitive to TCR inhibition (Fig.6.1). Zoledronate-expanded γδT cells too displayed TCR-dependence for both measures of phagocytosis, although the sensitivity to inhibition was less marked than that

seen with *E.coli*-expanded cells (Fig.6.1). It remains unclear as to whether this difference between the expansion conditions is a real one or caused by technical discrepancy, *e.g.* better viability of zoledronate- *versus* *E.coli*-expanded γδT cells. I note that all the phagocytosis data presented herein is only that of expanded PBMC culture, as I was unable to detect substantial, and therefore quantifiable, phagocytosis by freshly-isolated γδT cells. Measurements of expanded γδT cell TCR dependence for phagocytosis are, thus, largely relevant only to the predominant population within expanded γδT cells - Vγ9Vδ2 cells.

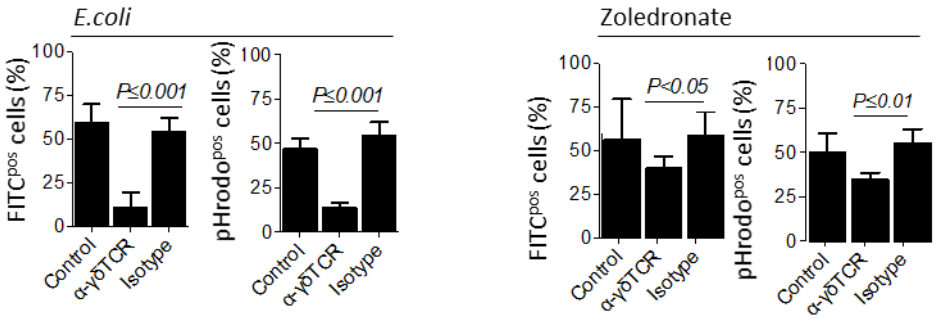

Fig. 6.1. E.coli and zoledronate-expanded γδT cells phagocytose E.coli in a TCR-dependent manner. Freshly-isolated PBMC (n=5) were expanded with UV-irradiated *E.coli* or zoledronate for 14 days, and examined for *E.coli* phagocytosis on day 14. To determine uptake, PBMC were incubated with IgG-opsonized *E.coli* for 60min. PBMC were pre-cultured with normal media (control), anti-γδTCR mAb (clone: B1) or isotype-matched control. The effect of pre-culture with anti-γδTCR mAb on phagocytosis was examined; data is shown for *E.coli*-expanded (left) or zoledronate-expanded (right) γδT cells, incubated with FITC-*E.coli* or pHrodo-*E.coli*. Bar graphs indicate the mean ± standard deviation.

To further investigate the role of the TCR in Vγ9Vδ2 cell phagocytosis, expanded PBMC were incubated with IgG-opsonized green fluorescent beads for 60 minutes, and analyzed via Trypan Blue quenching and flow cytometry, with or without γδTCR blocking (Fig.6.2A). Uptake of opsonized beads by expanded γδT cells was significantly inhibited in the presence of anti-γδTCR mAb (Fig.6.2B). Bead adherence, as measured in non-quenched PBMC-bead co-culture, was not affected by blocking of the TCR.

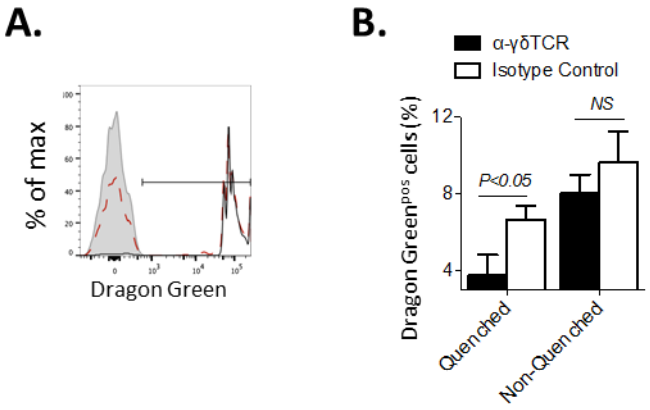

Fig. 6.2. Expanded γδT cells take up 1.0µm beads in a TCR-dependent manner. Freshly-isolated PBMC (n=5) were expanded with UV-irradiated *E.coli* for 14 days, and examined for uptake of fluorescent green beads on day 14. (**A**) PBMC were incubated with opsonized green fluorescent beads and quenched post-culture with Trypan Blue. Shown are representative stains, gated on γδT cells: i) non-quenched co-culture indicating total bead fluorescence (black, solid, unshaded), ii) quenched co-culture indicating intracellular bead fluorescence (red, dotted, unshaded), iii) cells alone (gray, shaded). (**B**) The effect of anti-γδTCR mAb was examined on γδT cell uptake of opsonized beads in quenched and non-quenched samples (n=3). Bar graphs indicate the mean ± standard deviation.

6.3. γδT cell cytokine production and proliferation in response to *E.coli* are TCR-dependent, whereas TCR dependence of cytotoxicity appears only partial

To assess the TCR dependence of freshly-isolated γδT cell cytokine and cytotoxic effector function in response to stimulation with UV-irradiated *E.coli*, freshly-isolated PBMC were stimulated with UV-irradiated *E.coli* at MOI 10 and analyzed after overnight (16-18h) co-culture. Exposure to anti-γδTCR mAb prior to co-culture led to abrogation of CD69 upregulation and cytokine production (Fig.6.3)

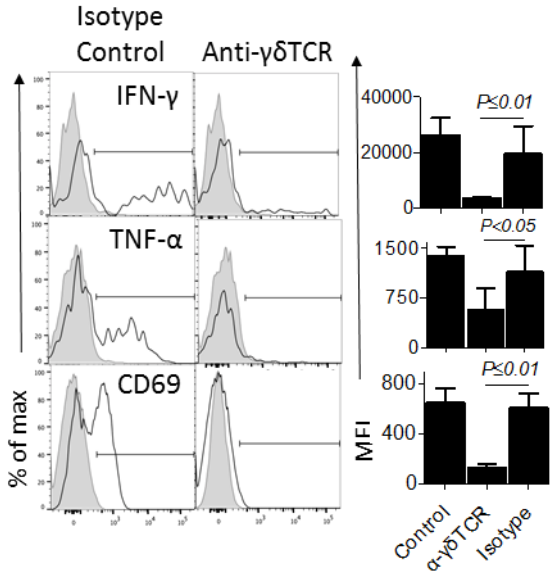

Fig. 6.3. Fresh γδT cell cytokine response to E.coli are TCR-dependent. Prior to stimulation with *E.coli,* freshly-isolated PBMC (n=5) were cultured for 2h with blocking anti-γδTCR mAb or isotype-matched control. PBMC were then stimulated with *E.coli* and analyzed after overnight (16-18h) culture. γδT cell accumulation of intracellular IFN-γ, TNF-α and cell surface CD69 were assessed via FACS analysis after overnight stimulation of freshly-isolated PBMC. Shown left are representative stains from one donor (marker in black, unshaded; isotype control in gray, shaded); shown right are donor parameter compilation MFI. Bar graphs indicate the mean ± standard deviation.

Pre-culture with anti-γδTCR mAb further lead to a decrease in the double positive IFN-γ^{pos} CD107a^{pos} but not in single positive CD107a^{pos} γδT cell populations (Fig.6.4). The decrease in IFN-γ^{pos}CD107a^{pos} γδT cells was subset specific, as only Vδ2 but not Vδ1 γδT cell cytotoxic degranulation was sensitive to a pre-blocked TCR (Fig.6.5).

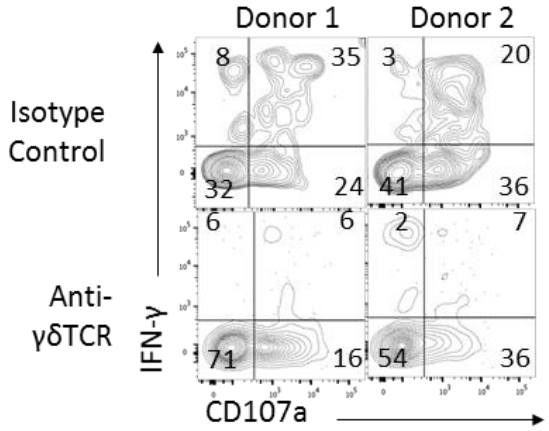

Fig. 6.4. IFN-γ^{pos}CD107a^{pos} and IFN-γ^{neg}CD107a^{pos} γδT cells display different sensitivity to TCR inhibition. Prior to stimulation with *E.coli,* freshly-isolated PBMC (n=5) were cultured for 2h with blocking anti-γδTCR mAb or isotype-matched control. PBMC were then stimulated with *E.coli* and analyzed after overnight (16-18h). Two representative donor samples, gated on γδT cells, are shown correlating intracellular IFN-γ and cell surface CD107a post overnight *E.coli* stimulation of PBMC.

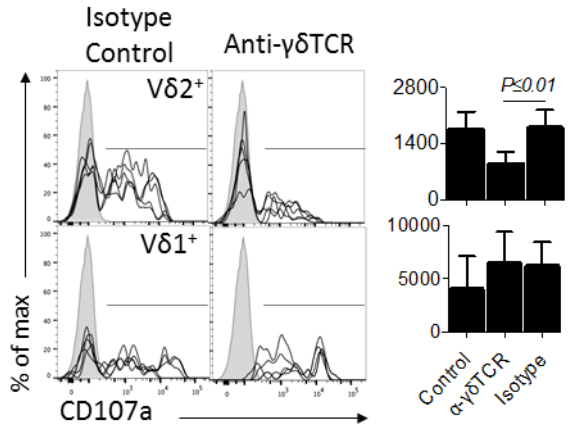

Fig. 6.5. Vδ2 cell, but not Vδ1 cell, CD107a-mediated degranulation is partially TCR-dependent. Prior to stimulation with *E.coli,* freshly-isolated PBMC (n=5) were cultured for 2h with blocking anti-γδTCR mAb or isotype-matched control. PBMC were then stimulated with *E.coli* and analyzed after overnight (16-18h) culture. The effect of anti-γδTCR mAb on fresh γδT cell surface CD107a post-PBMC *E.coli* stimulation is shown for five representative donors, comparing Vδ1 and Vδ2 cells. Bar graphs indicate the mean ± standard deviation.

To evaluate the impact of TCR blocking on direct γδT cell cytotoxicity against *E.coli*, FACS-sorted *E.coli*-expanded γδT cells (predominantly with a Vδ2 TCR) were pre-treated with blocking anti-γδTCR mAb and exposed to live bacteria; cytotoxicity was inferred from residual colony numbers on agar plates. As described in the previous chapter, *E.coli* colony forming unit (CFU) count declined by >90% within 30 minutes of exposure to purified γδT cells. Interestingly, this effect was not dependent upon γδTCR engagement (Fig.6.6). Although γδTCR blocking showed no significant impact on bactericidal function, it completely blocked γδT cell proliferation in response to *E.coli* (Fig.6.7).

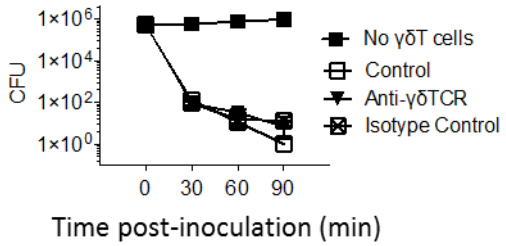

Fig. 6.6. Expanded γδT cell killing of live E.coli is not significantly TCR-dependent. Freshly-isolated PBMC (n=4) were stimulated with *E.coli* and left to expand for 14 days. γδT cells were then purified by twice-repeated positive magnetic selection and rested overnight. Prior to stimulation with live *E.coli,* γδT cells were cultured for 2h with blocking anti-γδTCR mAb or isotype-matched control. γδT cells were then co-cultured with live *E.coli* at MOI 10. At designated time points the co-culture was lysed in H_2O, and plated on agar for overnight growth; resulting *E.coli* CFU were counted.

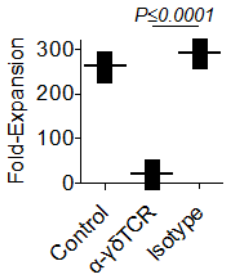

Fig. 6.7. γδT cell proliferation in response to E.coli is TCR-dependent. Prior to stimulation with *E.coli,* freshly-isolated PBMC (n=5) were cultured for 2h with blocking anti-γδTCR mAb or isotype-matched control. PBMC were then stimulated with *E.coli* and left to expand for 14 days. Proliferation in response to *E.coli* stimulation was assessed.

6.4. Discussion

Data presented in this chapter documents freshly-isolated and expanded γδT cell effector function dependency on the TCR. Findings included:

i) *E.coli* and zoledronate-expanded γδT cells take up and acidify IgG-opsonized *E.coli* in a TCR-dependent manner.

ii) *E.coli*-expanded γδT cells take up IgG-opsonized 1.0μm beads in a TCR-dependent manner.

iii) Freshly-isolated γδT cell activation (as measured by CD69 upregulation), IFN-γ and TNF-α production in response to *E.coli* is TCR-dependent.

iv) Freshly-isolated γδT cell CD107a-mediated cytotoxic degranulation is partially TCR-dependent for Vδ2 cells, but not Vδ1 cells.

v) The decrease in viable *E.coli* CFU number after exposure to expanded γδT cells was not affected by γδT cell pre-culture with anti-TCR mAb.

Prior to engaging in discussions of the physiological implications of the TCR blocking data presented in this chapter, I note that all data herein presented hinges on the use of a particular anti-γδTCR mAb, clone B1. There exists some literature on the use of this antibody for the effective blocking of human γδT cell effector function , particularly in the context of recognizing tumour (and virus)-associated antigens (207). As in the present thesis, data indicated that anti-γδTCR mAb, clone B1, inhibited Vδ2 IFN-γ production. Other γδT cell subsets remained unexamined. The question arises, therefore, whether the B1 antibody can be used as a credible, broad blocker of all γδTCRs. There are several ways of assessing this question. Firstly, is fluorescent staining with the B1 mAb inclusive of non-Vδ2 cells? Indeed, a number of commercial suppliers provide pan anti-γδTCR B1 mAb; numerous peer-reviewed publications cite the use of this antibody clone for fluorescent pan-γδT cell staining (49,208–210). Moreover, published protocols describe the use of anti-γδTCR B1 mAb for the non-biased expansion of Vδ1, Vδ2 and non- Vδ1/ Vδ2 γδT cells (211), or the induction of proliferation and IFN-γ of non-Vγ9Vδ2 γδT cells (212). Crucially, the difference between using the anti-γδTCR B1 mAb as a pan-

stimulator or pan-blocker of peripheral human γδT cells appears to lie in the way that the antibody is presented to the cells. Free-floating, monomeric IgG exerts a blocking effect, whereas IgG conjugated to beads or the well of a culture well-plate – a stimulatory one, as resultant from antibody binding to single TCRs or cross-linking multiple TCRs on the same cell, respectively.

Having established the existence of a substantive body of peer-reviewed literature that cites the use of anti-γδTCR B1 mAb, it was fascinating to note that pre-incubation with this antibody substantially decreased *E.coli* as well as zoledronate-expanded γδT cell phagocytosis of opsonized *E.coli.* Previous chapters present my finding that expansion with either stimulus leads to highly homologous Vγ9Vδ2 TCR repertoires. Knowing that zoledronate activates Vγ9Vδ2 cells via accumulation of endogenous IPP, this observation, thus, alludes strongly to a scenario where *E.coli* is not being engaged by the γδTCR directly. It suggests instead that *E.coli* too is inducing accumulation of endogenous pyrophosphates, which is what then drives γδT cell effector function and proliferation – possibly via inducing conformational changes in BTN3A molecules, as laid out in previous chapters. If it is indeed stressed self that is engaged upon γδT cell exposure to *E.coli* and not *E.coli* directly, TCR dependence of *E.coli* phagocytosis suggests a 'licensing'-type reaction occurring. I stipulate that 14 day expanded PBMC exposure to *E.coli* – via HMBPP release, endogenous IPP stimulation or otherwise – induces markers of cell stress. It is conceivable, even, that expanded γδT cells are recognizing these stress markers on each other, given that they constitute the vast majority of live cells remaining in culture. A signal of self-stress may then 'license' HLA-DR^{pos} CD86^{pos} γδT cell phagocytic effector function, which is further regulated by the necessity for target opsonisation with IgG.

To test whether phagocytosis of IgG opsonized targets is dependent on TCR engagement, I examined whether expanded γδT cell uptake of opsonized 1.0μm beads is affected by pre-culture with anti-γδTCR B1 mAb. It is conceivable, for example, that TCR blocking impedes *E.coli* adherence to γδT cells, but not phagocytosis directly. Upon examination of IgG-coated beads I found, however, that bead adherence to expanded γδT cells was not significantly affected, while phagocytosis was. As discussed in Chapter III, I maintain that

further investigation and experimental approaches are necessary to establish the fine details of γδT cell phagocytosis. For example, are receptors other than those for IgG opsonin or (possibly) BTN3A involved? Are γδT cell TLRs triggered by exposure to *E.coli* and do these interactions modulate the effector function observed? The observation, however, that *E.coli* and zoledronate-expanded γδT cell phagocytosis of IgG-opsonized beads or *E.coli* is inhibited by pre-incubation with anti-γδTCR B1 mAb lead us to postulate that expanded HLA-DR^{pos} CD86^{pos} Vγ9Vδ2 cell phagocytosis requires an interactive 'licensing'-type event between the γδTCR and FcγR. It remains unclear whether the interaction between γδTCR and FcγR is direct or indirect.

E.coli exposure led to potent TCR-dependent freshly-isolated γδT cell IFN-γ and TNF-α production, proliferation as well as substantial cytotoxic degranulation and bacterial killing. Interestingly, freshly-isolated Vγ9Vδ2 cell CD107a-mediated cytotoxicity was only partially TCR-dependent, while *E.coli* killing was entirely unaffected by pre-blocking of the γδTCR. The observation that expanded, purified Vδ2 cells exhibited high bactericidal activity despite decreased CD107a-mediated cytotoxic degranulation and blocking of the TCR, may be attributable to the high efficiency of γδT cell CD107a-mediated *E.coli* killing, whereupon low level degranulation is sufficient to significantly decrease bacterial viability, or may allude instead to CD107a-independent bactericidal mechanisms. These data suggest differential control for Vγ9Vδ2 cell Th1 responses, proliferation and pAPC function *versus* cytotoxicity. Such a difference is, perhaps, unsurprising in a physiological context. γδT cells reside largely at epithelial surfaces populated by billions of commensal and pathogenic microorganisms. Encountering non-self via non-TCR PRRs (*e.g.* TLRs) may trigger an immediate, cytotoxic response. Once the target is killed, however, decisions about further inflammatory processes or even the initiation or regulation of an adaptive immune response are tightly regulated by additional layers of control, involving *e.g.* γδTCR and FcγR. I note that freshly-isolated Vδ1 cell CD107-mediated cytotoxic degranulation appeared entirely γδTCR independent. As discussed in the chapter introduction, similar TCR dependence differences have been observed among mouse γδT cell subsets. Significant further experimental probing is necessary to determine the nature and reasons for this difference between human peripheral Vδ1 and Vδ2 γδT cells. It would be interesting to examine further whether

intestinal Vδ1 IELs display a similar TCR-independence to PBMC Vδ1 cells with regard to cytotoxicity.

To our knowledge, we are the first group to document direct bactericidal activity of human Vγ9Vδ2 cells. While I've also demonstrated simultaneous CD107a-mediated cytotoxic degranulation, it is unclear whether this is the mechanism by which the remarkably efficient killing took place. Significant further investigation of Vγ9Vδ2 cell direct bactericidal activity is necessary, spanning different microorganisms, strains, from Gram-positive to Gram-negative and intracellular bacteria, plasmodia, fungi, etc. It would be interesting to determine, for example, whether γδT cells can distinguish, in the context of bacterial killing, commensal *E.coli* from enteropathogenic *E.coli.* In terms of mechanisms of killing, reproducible mass spectrometry of *E.coli*-Vγ9Vδ2 cell co-culture supernatants would doubtless provide fascinating insight into what are likely multiple modes of bactericidal attack that Vγ9Vδ2 cells engage in.

TCR-engagement as a pre-requisite of Vγ9Vδ2 cell Th1 cytokine production, proliferation and pAPC function suggests careful regulation of the Vγ9Vδ2 T cell compartment. Whenever an early immune response is sufficient to neutralize infection, MHC class $II^{pos}CD86^{pos}$ Vγ9Vδ2 cells expanded in response to encountering self-stress markers may be prevented from posing an unnecessary inflammatory threat by amplifying responses further. This then would be achieved by downregulation of their initial Th1 cytokine responsiveness, as well as imposing a necessity for TCR triggering by stressed-self to exert pAPC function. The requirement for opsonization of a target may be a further safety feature. This could be operative at two levels: i) as herein, when opsonizing with switched target-specific IgG, and ii) in a 'naïve' non-immune situation, where natural antibodies (NAb) of different isotype, including IgG, may be involved.

Further study of the engagement of the Vγ9Vδ2 TCR, possibly with BTN3A targets, is likely to carry significant implications for γδT cell anti-tumor immunity by supporting the notion that stress recognition, particularly in combination with Ab-opsonisation, may be sufficient not only for killing of a tumor cell but also for uptake, processing and presentation of tumor-associated antigens (49). Documenting the significance of the TCR

for the engagement of γδT cell pAPC function, I set out to determine in the next chapter what relationship this bears to endogenous BTN3A targets. Indeed, is the observed Vγ9Vδ2 cell TCR dependence linked intimately to recognition of self-stress, even upon exposure to such a target-rich stimulus as *E.coli*?

6.5. Summary

Data laid out in this chapter documents that Th1-type cytokine production, proliferation and phagocytic capacity of Vγ9Vδ2 cells is dependent on the TCR. CD107a-mediated cytotoxic degranulation displayed only partial TCR dependency, while Vγ9Vδ2 cell bactericidal activity was unaffected by blocking of the γδTCR.

7. Vγ9δ2 γδT CELL EFFECTOR FUNCTION DEPENDENCE ON BTN3A

7.1. Aims

Finding that freshly-isolated and expanded Vδ2 γδT cell activation, cytokine production and pAPC phenotype in response to stimulation with *E.coli* are TCR-dependent, the question arose of what the TCR may be recognizing. The following chapter explores whether the Vγ9Vδ2 TCR ligand in my model is indeed the BTN3A molecule. γδT cell responses in the presence or absence of TCR / BTN3A blocking were explored in the context of various 'self' and 'non-self' stimuli.

7.2. BTN3A is ubiquitously expressed on PBMC

In order to decipher the role of BTN3A (CD277) in γδT cell recognition of target cells, I first set out to determine the expression pattern of BTN3A in freshly-isolated and differentially expanded PBMC. FACS-staining revealed that nearly all live, freshly-isolated PBMC were positive for BTN3A, with expressioin levels largely equivalent among $CD3^{pos}$ and $CD3^{neg}$ cells (Fig.7.1A). Overnight stimulation with irradiated *E.coli* lead to a modest downregulation of BTN3A on all PBMC, although predominant positivity was maintained, with expression levels recovering during expansion. PBMC re-stimulation with *E.coli* on day 14 of the expansion resulted in a less marked downregulation of BTN3A.

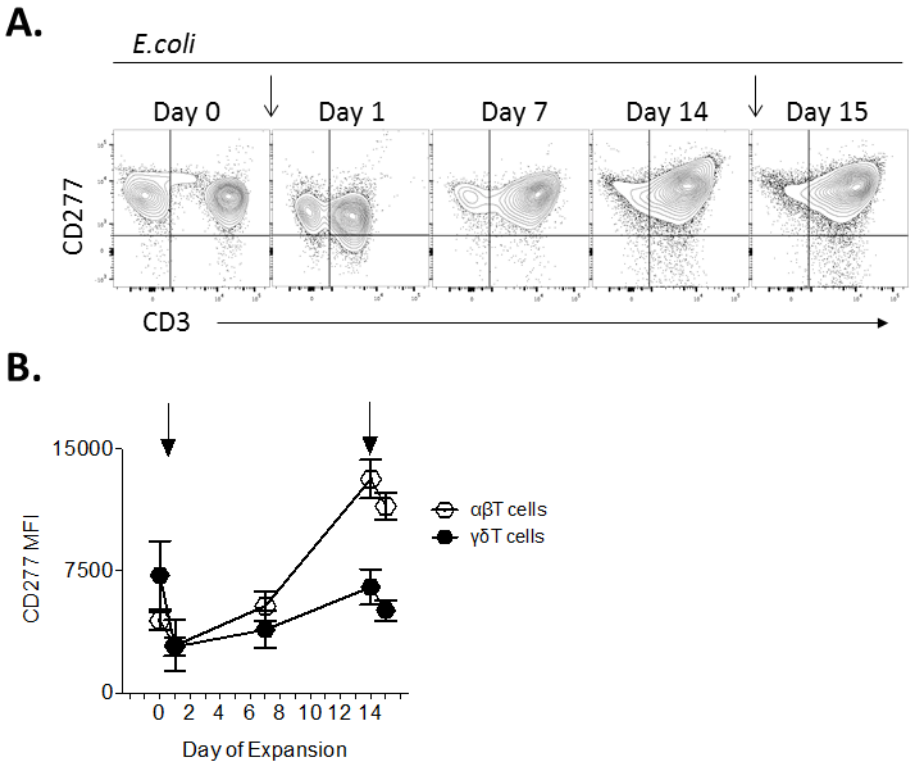

Fig. 7.1. PBMC express high levels of CD277 throughout expansion with E.coli. Freshly-isolated PBMC (n=5) were expanded with UV-irradiated *E.coli* for 14 days. Cell surface CD277 levels were monitored throughout. (**A**) Representative stains of one donor *E.coli*-stimulated PBMC is shown, comparing CD277 levels on $CD3^{pos}$ and $CD3^{neg}$ cells. Points of *E.coli* stimulation are indicated with black arrows. (**B**) Donor CD277 levels in response to *E.coli* were compiled and compared in γδT and αβT cells.

Interestingly, *E.coli* stimulation resulted in significantly higher BTN3A levels on αβT cells than γδT cells as the expansion progressed (Fig.7.1B). Similar results were obtained upon freshly-isolated PBMC stimulation with zoledronate (Fig.7.2.).

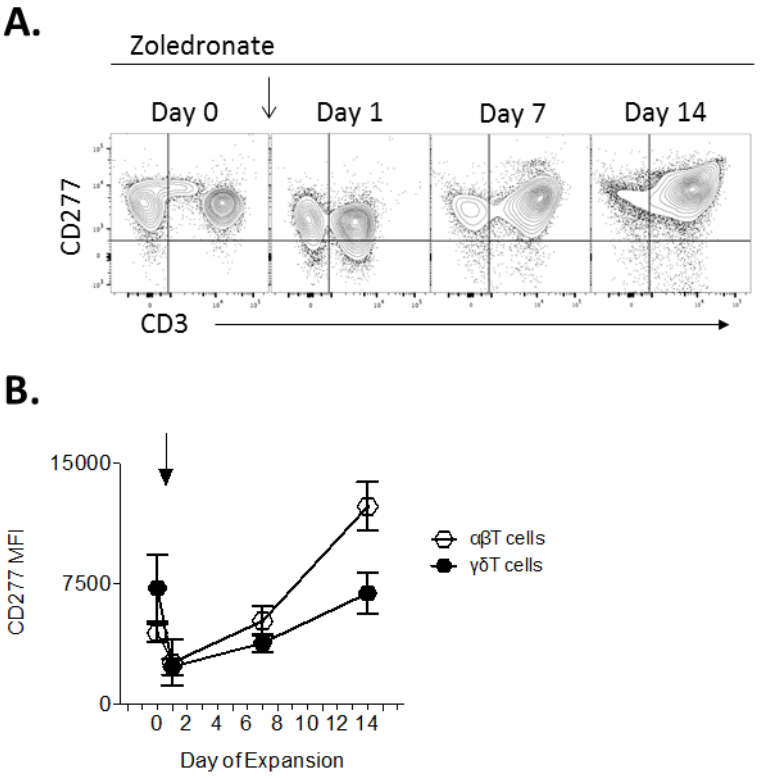

Fig. 7.2. PBMC express high levels of CD277 throughout expansion with E.coli and zoledronate. Freshly-isolated PBMC (n=4) were expanded with zoledronate for 14 days. Cell surface CD277 levels were monitored throughout. (**A**) Representative stains of one donor zoledronate-stimulated PBMC is shown, comparing CD277 levels on $CD3^{pos}$ and $CD3^{neg}$ cells. Points of zoledronate stimulation are indicated with black arrows. (**B**) Donor CD277 levels in response to zoledronate were compiled and compared in γδT and αβT cells.

7.3. γδT cells are activated by autologous pAg-stimulated PBMC

To determine the role of BTN3A in γδT cell antigen recognition, an autologous stimulation experimental system was set up (illustrated in the materials and methods section). Briefly, γδT cells were stimulated with autologous PBMC, which in turn had previously been co-cultured overnight with various pAg-inducing and/or –containing stimuli, *e.g.* zoledronate and irradiated *E.coli.* To test whether such a system may be a viable experimental probe for γδT cellactivation, γδT 'responder' cells were stained for various activation markers after overnight co-culture with differentially-stimulated 'stimulator' PBMC.

Indeed, overnight co-culture resulted in responder γδT cell upregulation of CD69 in response to *E.coli*- and zoledronate- but not mock (medium only)-exposed stimulator PBMC (Fig.7.3).

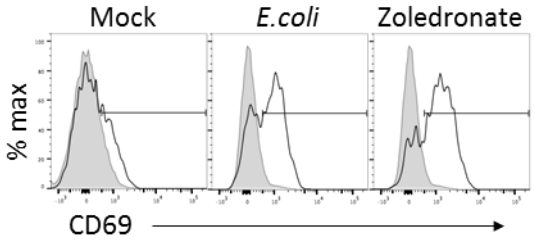

Fig. 7.3. γδT cells are activated by autologous zoledronate or E.coli-stimulated PBMC. Freshly-isolated PBMC were co-cultured overnight with autologous, freshly-isolated, γδT cell-depleted and overnight *E.coli* or zoledronate-stimulated PBMC. γδT cell upregulation of cell surface CD69 in response to differentially-stimulated, autologous PBMC was recorded. PBMC were analyzed via FACS. γδT cell surface CD69 was compared post-PBMC O/N co-culture with mock-, *E.coli*- or zoledronate-exposed stimulator cells. Shown are representative stains from one donor (marker in black, unshaded; isotype control in gray, shaded).

A potent IFN-γ response was observed after responder γδT cell stimulation with both, *E.coli* and zoledronate-exposed, stimulator cells (Fig.7.4A). As seen with direct PBMC stimulation with *E.coli* or zoledronate (discussed in Chapter V), the IFN-γ response was Vδ2 cell specific (Fig.7.4B).

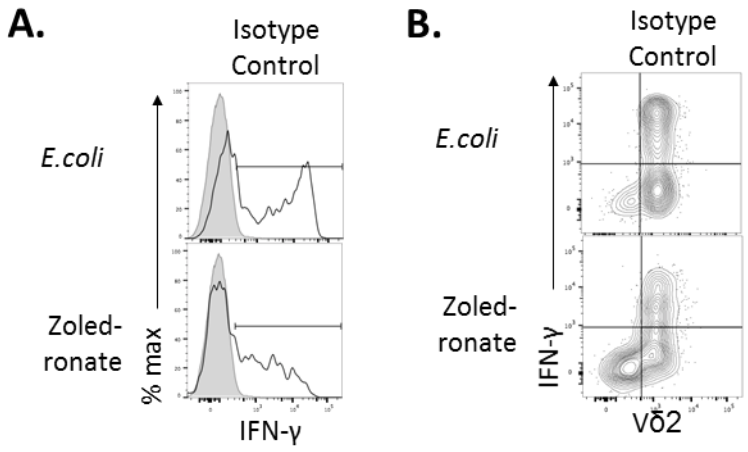

Fig. 7.4. Vδ2 γδT cells produce IFN-γ in response to autologous E.coli or zoledronate-stimulated PBMC. Freshly-isolated PBMC (n=4) were co-cultured overnight with autologous, freshly-isolated, γδT cell-depleted and overnight *E.coli* or zoledronate-stimulated PBMC. γδT cell responses to differentially-stimulated, autologous PBMC were recorded. PBMC were analyzed via FACS; representative single donor stains are shown (marker in black, unshaded; staining isotype control in gray, shaded). (**A**) γδT cell intracellular IFN-γ was compared post-PBMC O/N co-culture with *E.coli*- or zoledronate-stimulated targets. (**B**) Intracellular IFN-γ was correlated to γδT cell expression of the Vδ2 TCR chain.

γδT cell stimulation with stimulator PBMC further elicited potent CD107a-mediated cytotoxic degranulation. Strikingly, the level of cytotoxicity elicited by direct γδT cell stimulation with *E.coli* within whole PBMC was statistically indistinguishable from that elicited by γδT cell stimulation with *E.coli*-exposed autologous stimulator PBMC (Fig.7.5).

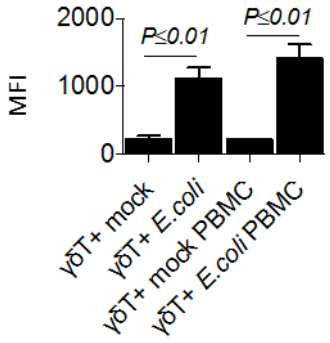

Fig. 7.5. γδT cells display equivalent CD107a-mediated cytotoxic degranulation in response to direct E.coli stimulation or stimulation with E.coli-exposed stimulator PBMC. E.coli-expanded PBMC (n=3) were co-cultured overnight with UV-irradiated *E.coli* at MOI 10 or autologous, γδT cell-depleted and overnight *E.coli*-stimulated PBMC, or mock-stimulated (media only). Cell surface CD107a was compared between the various stimulated groups. Donor MFI were compiled and compared**.**

7.4. Vγ9Vδ2 cell effector responses to autologous stimulator PBMC are differentially sensitive to blocking of the γδTCR and BTN3A

Having established that γδT cells are indeed responsive to differentially stimulated autologous PBMC, I proceeded to examine the sensitivity of this responsiveness to blocking of BTN3A on stimulator PBMC and blocking of the γδTCR on responder γδT cells (as illustrated in the materials and methods section). Given that BTN3A dependency has been ascribed to Vγ9Vδ2 cells specifically, I first examined Vγ9 chain expression within freshly-isolated peripheral Vδ2 cells. In all donors examined, all freshly isolated Vδ2 also expressed a Vγ9 TCR chain (Fig.7.6).

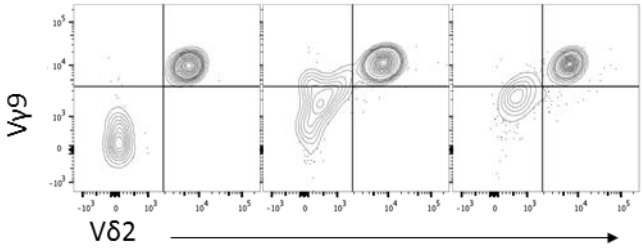

Fig. 7.6. Peripheral Vδ2 cells express a predominantly Vγ9Vδ2 TCR. Freshly-isolated PBMC were stained for CD3, αβTCR and the TCR chains Vδ2 and Vγ9. Shown are three representative donor samples, gated on live $CD3^{pos}$ $\alpha\beta TCR^{neg}$ cells.

Vγ9Vδ2 cell responses to various stimuli were then examined in the presence or absence of pre-culture with blocking antibody. Freshly-isolated stimulator PBMC were cultured with medium only (mock), irradiated *E.coli*, HMBPP, IPP or zoledronate. Such a panel was designed to include i) a complex, pAg-expressing non-self target that further includes non-TCR inflammatory stimuli for Vγ9Vδ2 cells, *e.g.* LPS via TLR-4 (*E.coli*), ii) exogenous non-self pAg (HMBPP), iii) exogenous self pAg (IPP) and iv) a pharmacological agent that induces accumulation endogenous pAg, including IPP (zoledronate).

Vγ9Vδ2 cell CD69 upregulation in response to overnight co-culture with these differentially stimulated autologous PBMC was examined (Fig.7.7). Significant CD69 upregulation was seen in response to all stimulator conditions (Fig.7.7A). Donor responses to the various conditions were, however, heterogeneous in potency. Data on the effects of blocking antibodies were, therefore, standardized against each donor's CD69 MFI in the absence of any blocking mAb (i.e. R(iso)+S(iso)), and expressed instead as a percentage change in marker MFI after the addition of blocking antibody (Fig.8.13B). Unexpectedly, it was apparent that responder pre-blocking with anti-γδTCR or stimulator pre-blocking with anti-BTN3A exerted no significant effect on Vγ9Vδ2 upregulation of CD69 (Fig.7.7B).

The same assay was repeated with five donors, and measured CD107a-mediated cytotoxic degranulation as well as accumulation of intracellular IFN-γ. Once again, whilst all stimuli elicited Vγ9Vδ2 cell cytotoxicity and IFN-γ production, significant donor-dependent variation could be observed (Fig.7.8A and Fig.7.9A). Standardisation against each condition's cell surface CD107a in the absence of blocking mAb revealed that cytotoxic degranulation too was unaffected by responder or stimulator pre-culture with anti- γδTCR or anti-BTN3A mAb, respectively (Fig.7.9A and B). Vγ9Vδ2 intracellular IFN-γ levels, meanwhile, were sensitive to blocking γδTCR or anti-BTN3A mAb in response to every stimulus examined (Fig.7.9B). It must be noted that an exception was the IFN-γ response to IPP-stimulated PBMC in the presence of clone B1 mAb, which decreased upon addition of blocking antibody, but failed to acquire meaningful statistical significance at a *p* value of 0.08 (Fig.7.9B). It is possible that statistical significance could have been acquired with an increased donor number. A vast difference was, thus, observed between CD69 upregulation and CD107a-mediated cytotoxic degranulation *versus* IFN-γ production in terms of sensitivity to the blocking mAbs B1 and 103.2.

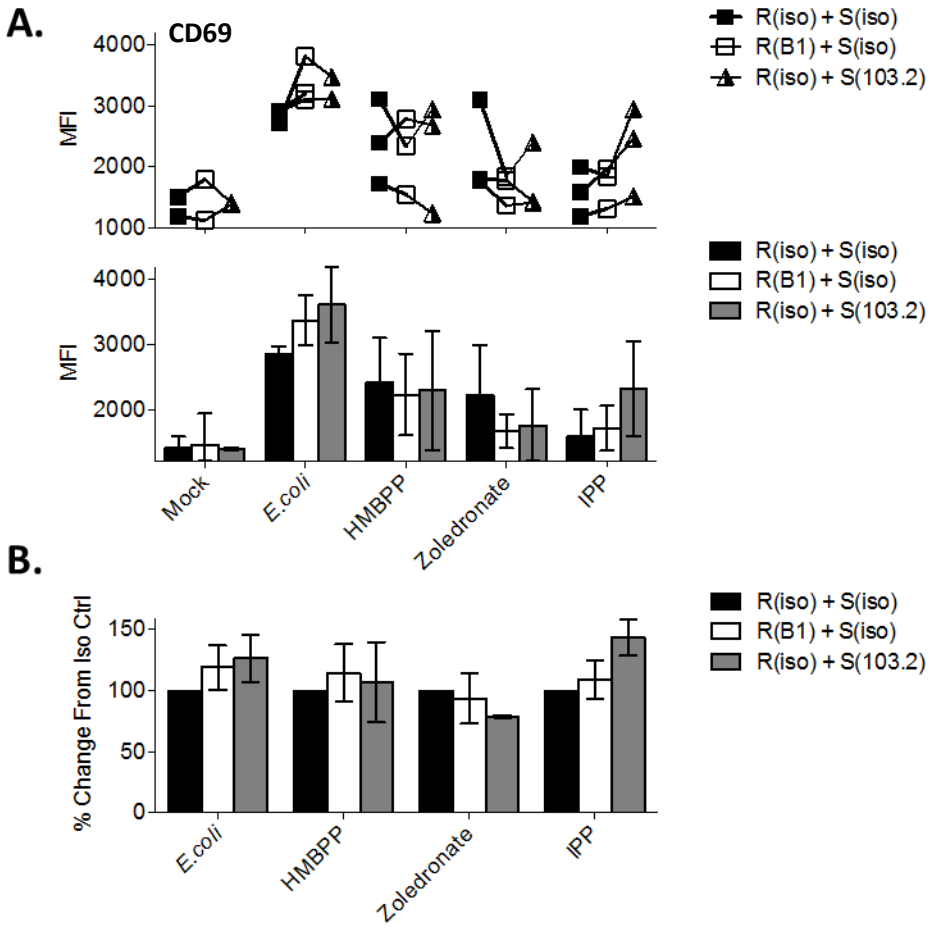

Fig. 7.7. Peripheral Vγ9Vδ2 responder cell CD69 upregulation in response to autologous stimulator cells is not sensitive to blocking of BTN3A or γδTCR. Freshly-isolated PBMC (n=3) responder cells were stimulated with autologous stimulator PBMC. Stimulator PBMC were prepared by culturing γδT cell-depleted, freshly-isolated PBMC overnight with irradiated *E.coli,* HMBPP, zoledronate or IPP. Stimulator PBMC were then pre-blocked with either antagonistic anti-BTN3A mAb (clone: 103.2) or isotype-matched control. Freshly-isolated responder PBMC were, meanwhile, rested overnight, and pre-blocked with anti-γδTCR mAb (clone: B1) or isotype-matched control. Responder Vγ9Vδ2 cell upregulation of CD69 in response to co-culture with stimulator PBMC was recorded via FACS. "R(iso)" indicates responder cells pre-incubated with isotype control mAb; "R(B1)" indicates responder cells pre-incubated with B1 mAb; "S(iso)" indicates stimulator cells pre-incubated with isotype control mAb; "S(103.2)" indicates stimulator cells pre-incubated with 103.2 mAb. (**A**) Shown are donor Vγ9Vδ2 cell CD69 MFI after O/N co-culture in the presence of various blocking conditions. The upper panel shows individual donor points and the lower panel shows a mean and standard deviation of the data compiled. (**B**) Shown is the percentage change in response to blocking mAb for each donor data point. Each donor's CD69 upregulation in the presence of blocking mAb was compared to that seen with "R(iso)+S(iso)", which represents 100%. Bar graphs indicate the mean ± standard deviation.

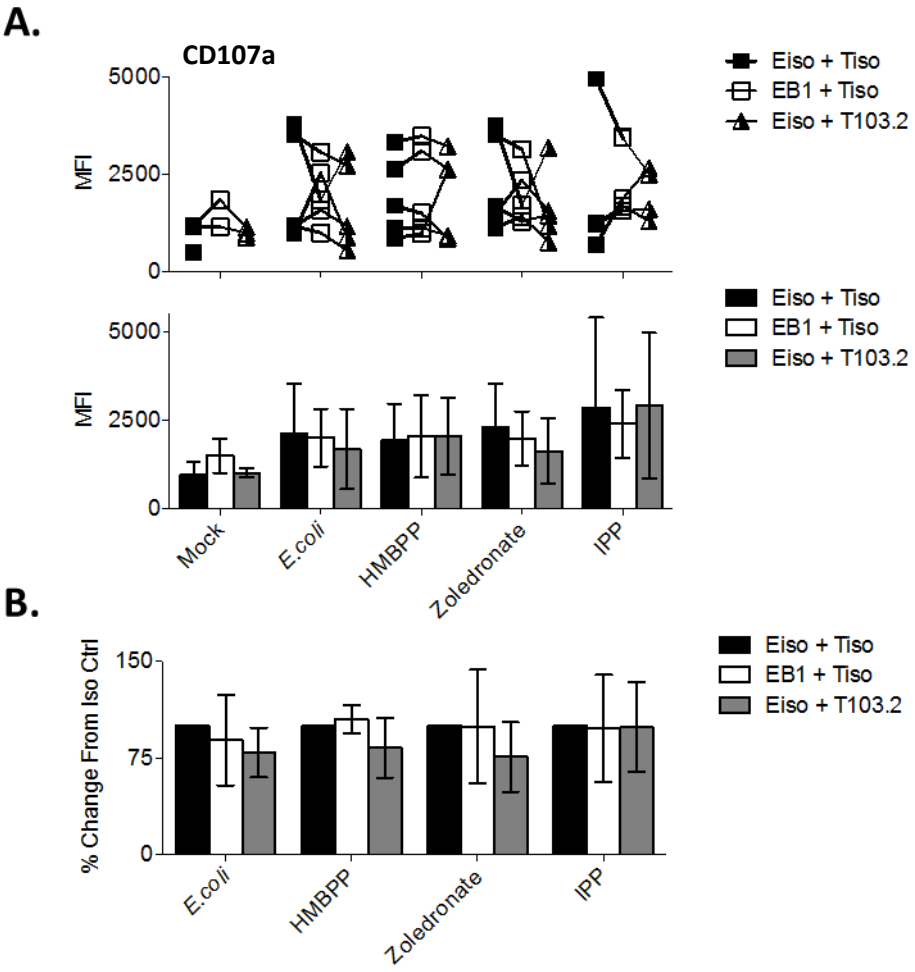

Fig. 7.8. Peripheral Vγ9Vδ2 responder cell CD107a upregulation in response to autologous stimulator cells is not sensitive to blocking of BTN3A or γδTCR. Freshly-isolated PBMC (n=5) responder cells were stimulated with autologous stimulator PBMC. Stimulator PBMC were prepared by culturing γδT cell-depleted, freshly-isolated PBMC overnight with irradiated *E.coli,* HMBPP, zoledronate or IPP. Stimulator PBMC were then pre-blocked with either antagonistic anti-BTN3A mAb (clone: 103.2) or isotype-matched control. Freshly-isolated responder PBMC were, meanwhile, rested overnight, and pre-blocked with anti-γδTCR mAb (clone: B1) or isotype-matched control. Responder Vγ9Vδ2 cell upregulation of CD107a in response to co-culture with stimulator PBMC was recorded via FACS. "R(iso)" indicates responder cells pre-incubated with isotype control mAb; "R(B1)" indicates responder cells pre-incubated with B1 mAb; "S(iso)" indicates stimulator cells pre-incubated with isotype control mAb; "S(103.2)" indicates stimulator cells pre-incubated with 103.2 mAb. (**A**) Shown are donor Vγ9Vδ2 cell CD107a MFI after O/N co-culture in the presence of various blocking conditions. The upper panel shows individual donor points and the lower panel shows a mean and standard deviation of the data compiled. (**B**) Shown is the percentage change in response to blocking mAb for each donor data point. Each donor's CD107a upregulation in the presence of blocking mAb was compared to that seen with "R(iso)+S(iso)", which represents 100%. Bar graphs indicate the mean ± standard deviation.

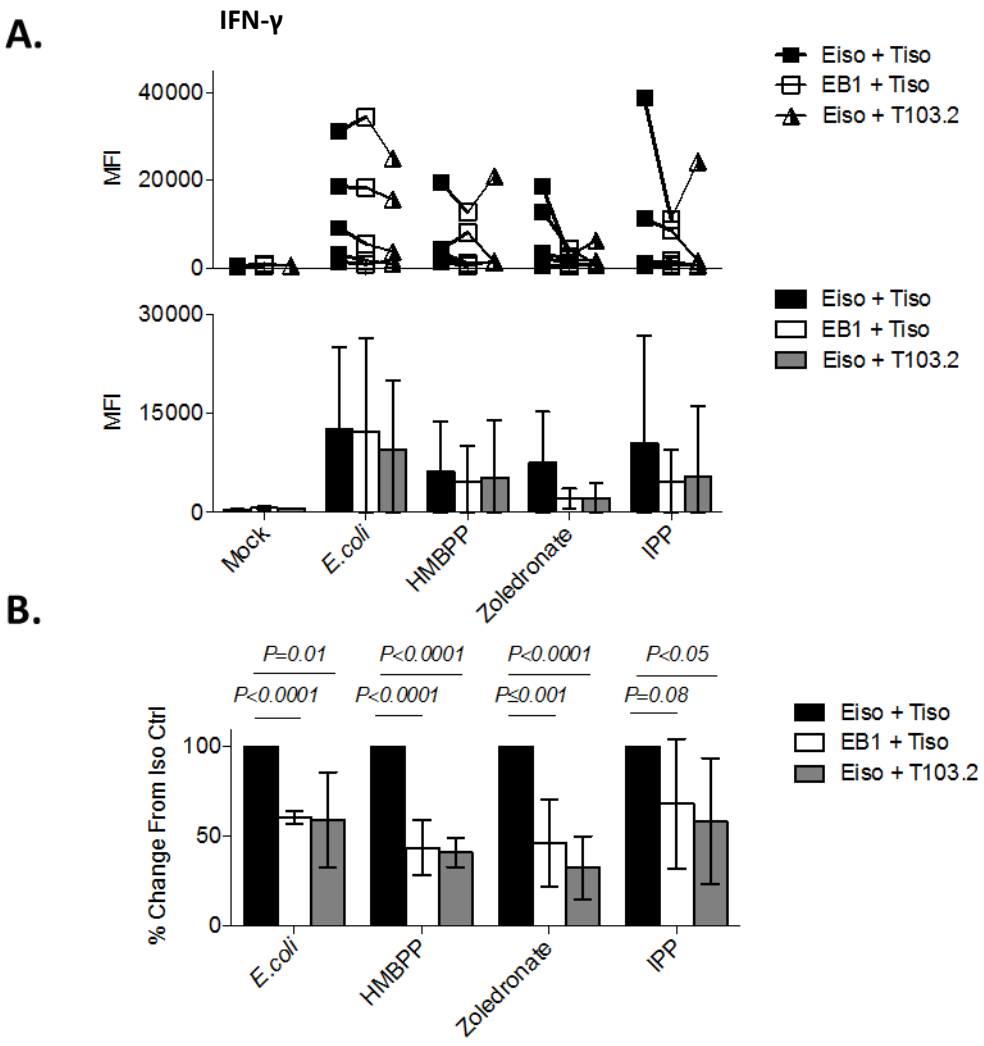

Fig. 7.9. Peripheral Vγ9Vδ2 responder cell IFN-γ production in response to autologous stimulator cells is sensitive to blocking of BTN3A as well as γδTCR. Freshly-isolated PBMC (n=5) responder cells were stimulated with autologous stimulator PBMC. Stimulator PBMC were prepared by culturing γδT cell-depleted, freshly-isolated PBMC overnight with irradiated *E.coli,* HMBPP, zoledronate or IPP. Stimulator PBMC were then pre-blocked with either antagonistic anti-BTN3A mAb (clone: 103.2) or isotype-matched control. Freshly-isolated responder PBMC were, meanwhile, rested overnight, and pre-blocked with anti-γδTCR mAb (clone: B1) or isotype-matched control. Responder Vγ9Vδ2 cell upregulation of IFN-γ in response to co-culture with stimulator PBMC was recorded via FACS. "R(iso)" indicates responder cells pre-incubated with isotype control mAb; "R(B1)" indicates responder cells pre-incubated with B1 mAb; "S(iso)" indicates stimulator cells pre-incubated with isotype control mAb; "S(103.2)" indicates stimulator cells pre-incubated with 103.2 mAb. (**A**) Shown are donor Vγ9Vδ2 cell intracellular IFN-γ MFI after O/N co-culture in the presence of various blocking conditions. The upper panel shows individual donor points and the lower panel shows a mean and standard deviation of the data compiled. (**B**) Shown is the percentage change in response to blocking mAb for each donor data point. Each donor's intracellular IFN-γ in the presence of blocking mAb was compared to that seen with "R(iso)+S(iso)", which represents 100%. Bar graphs indicate the mean ± standard deviation.

The majority of published data addressing the interaction between Vγ9Vδ2 cells and BTN3A on target cells (numerous seminal papers included) has been generated using cell lines rather than autologous cells as stimulators (101,152,161,164,213). A likely contributing factor to these experimental setups is the practical difficulty of obtaining and working with large numbers of primary human cells, exacerbated particularly by the usual scarcity of γδT cells in human peripheral blood. An additional hurdle of working with primary cells is introduced by the inherent magnitude heterogeneity of γδT cell responses. It has been speculated, for example, that γδT cell constitution and responsiveness to various stimuli may be significantly impacted by infectious exposure in early life (7). The use of cell line stimulators is a way of standardizing immunogenic targets for all donors within a given study. A number of important γδT cell-BTN3A publications have relied on the use of THP-1 cells, specifically (101,164,214).

7.5. Vγ9Vδ2 cell effector responses to stimulator THP-1 cells are differentially sensitive to blocking of the γδTCR and BTN3A

In a setup that differed from the autologous stimulator-responder system with the use of THP-1 cell line stimulator cells (illustrated in Fig.7.6), Vγ9Vδ2 cell responses were tested. In order to validate initial experimental reservations on my part regarding the use of allogeneic 'non-self', transformed stimulator cells, I tested Vγ9Vδ2 cell activation upon stimulation with THP-1 cells only in the absence of further pAg stimulus. Indeed, Vγ9Vδ2 cells upregulated CD69, engaged in CD107a-mediated cytotoxic degranulation and produced IFN-γ in response to overnight co-culture with otherwise unmodified THP-1 cells (Fig.7.10).

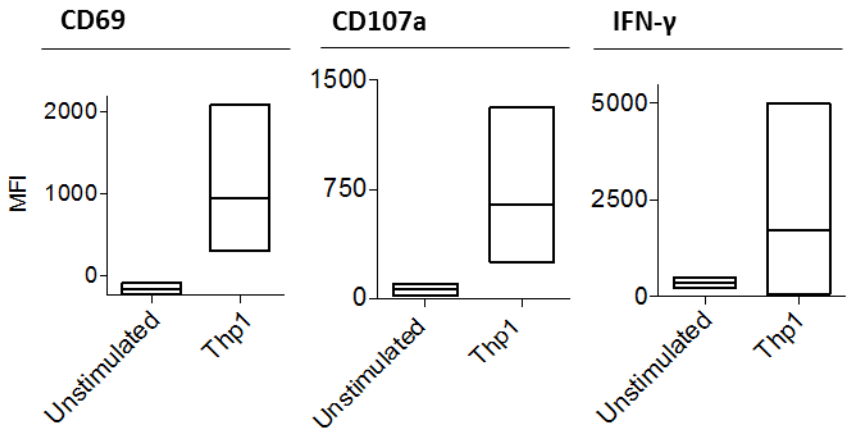

Fig. 7.10. Vγ9δ2 cells are potently activated by co-culture with THP-1 cells. Freshly-isolated PBMC (n=3) responder cells were stimulated with THP-1 cells. Shown are compilations of donor Vγ9Vδ2 cell surface CD69, CD107a and intracellular IFN-γ MFI. The graphs indicate mean and range of the data data. Effector responses were compared between unstimulated Vγ9Vδ2 cells (unstimulated) and Vγ9Vδ2 cells that had undergone O/N co-culture with THP-1 cells.

Having documented a baseline reactivity of freshly-isolated Vγ9Vδ2 cells to unstimulated THP-1 cells, I set out to clarify whether this reactivity can be modified by exposing stimulator THP-1 cells to pAg in the form of *E.coli*, HMBPP, IPP or zoledronate. Vγ9Vδ2 cell CD69 upregulation in response to overnight co-culture with differentially stimulated THP-1 cells was examined (Fig.7.11).

Significant CD69 upregulation upon co-culture with pAg-exposed *versus* pAg-non-exposed (mock) THP-1 cells could be seen with most donor Vγ9Vδ2 cells (Fig.7.11A). Notably, in one of the three examined donors, extremely high CD69 upregulation could be seen even with the addition of only mock-stimulated THP-1 cells. As was the case in the autologous donor PBMC stimulator system, significant donor variation could be observed, if to a lesser extent. As before, the effect of blocking antibodies was calculated as a percentage change set against each donor's CD69 MFI in the absence of any blocking mAb (Fig.7.11B). In contrast to observations from the autologous PBMC system, stimulator pre-culture with anti-BTN3A mAb 103.2 significantly inhibited Vγ9Vδ2 cell CD69 upregulation. Borderline statistical significance, which may have been improved upon increased donor number, was observed for *E.coli*-stimulated (p=0.06) as well as mock-stimulated (p=0.08) THP-1 cell stimulators. Responder pre-culture with anti-γδTCR mAb B1, meanwhile, appeared to exert little to no effect on most Vγ9Vδ2 cell THP-1 responses, save for, interestingly, in the case of IPP-stimulation.

Similar results were obtained when examining CD107a-mediated cytotoxic degranulation (Fig.7.12). Vγ9Vδ2 cell degranulation was inhibited by stimulator pre-culture with anti-BTN3A mAb for every pAg THP-1 stimulus examined, but not mock-stimulated THP-1. Borderline statistical significance could be obtained also for inhibition by responder pre-culture with anti-γδTCR mAb (Fig.7.12B). Perhaps the most striking sensitivity to blocking mAbs was obtained upon examining the Vγ9Vδ2 cell IFN-γ response (Fig.7.13). As with cytotoxicity, all responses to pAg-exposed, but not mock, THP-1 stimulators were sensitive to anti-BTN3A mAb (Fig.7.13A). It appeared that anti-BTN3A mAb pre-culture abrogated Vγ9Vδ2 cell IFN-γ production entirely. Significant inhibition could also be achieved with anti-γδTCR mAb, when THP-1 stimulators were specifically HMBPP or IPP-exposed. Vγ9Vδ2 cell responses to pAg-stimulated THP-1 cells, thus, appeared

largely sensitive, in terms of activation, cytotoxicity and IFN-γ production, to blocking by the 103.2 and B1 mAbs. It must be noted, however, that the vast inter-donor differences necessitate repeated experiments with increased donor numbers. It is possible that greater statistical significance could be achieved for all pAg stimulation conditions, blocking of the γδTCR and even Vγ9Vδ2 cell responses to unmodified THP-1 cells.

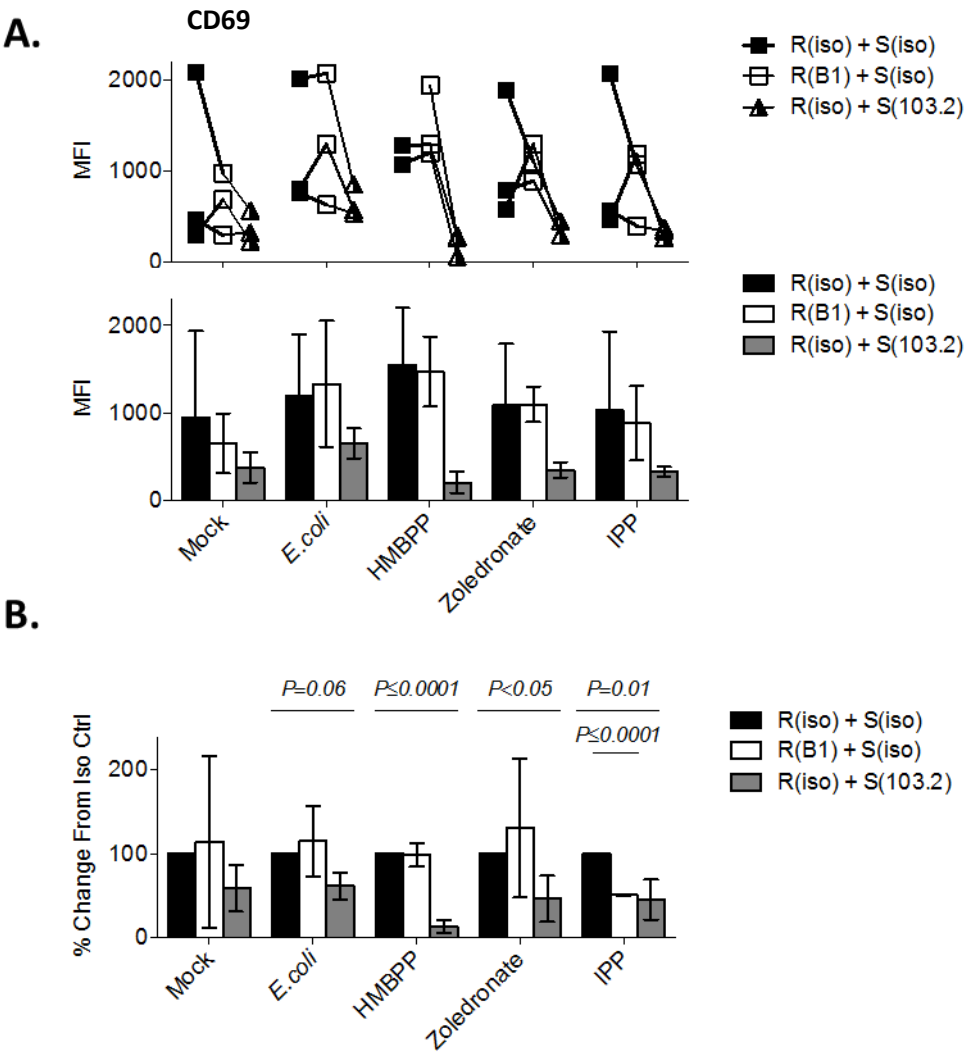

Fig. 7.11. Peripheral Vγ9Vδ2 responder cell surface CD69 in response to THP-1 stimulator cells is sensitive to blocking of BTN3A. Freshly-isolated PBMC (n=3) responder cells were stimulated with THP-1 stimulator cells. Stimulator THP-1 were prepared by culturing THP-1 cells overnight with irradiated *E.coli,* HMBPP, zoledronate or IPP. Stimulator THP-1 were then pre-blocked with either antagonistic anti-BTN3A mAb (clone: 103.2) or isotype-matched control. Freshly-isolated responder PBMC were, meanwhile, rested overnight, and pre-blocked with anti-γδTCR mAb (clone: B1) or isotype-matched control. Responder Vγ9Vδ2 cell upregulation of CD69 in response to co-culture with stimulator THP-1 was recorded via FACS. "R(iso)" indicates responder cells pre-incubated with isotype control mAb; "R(B1)" indicates responder cells pre-incubated with B1 mAb; "S(iso)" indicates stimulator cells pre-incubated with isotype control mAb; "S(103.2)" indicates stimulator cells pre-incubated with 103.2 mAb. (**A**) Shown are donor Vγ9Vδ2 cell surface CD69 after O/N co-culture in the presence of various blocking conditions. The upper panel shows individual donor points and the lower panel shows a mean and standard deviation of the data compiled. (**B**) Shown is the percentage change in response to blocking mAb for each donor data point. Each donor's cell surface CD69 in the presence of blocking mAb was compared to that seen with "R(iso)+S(iso)", which represents 100%. Bar graphs indicate the mean ± standard deviation.

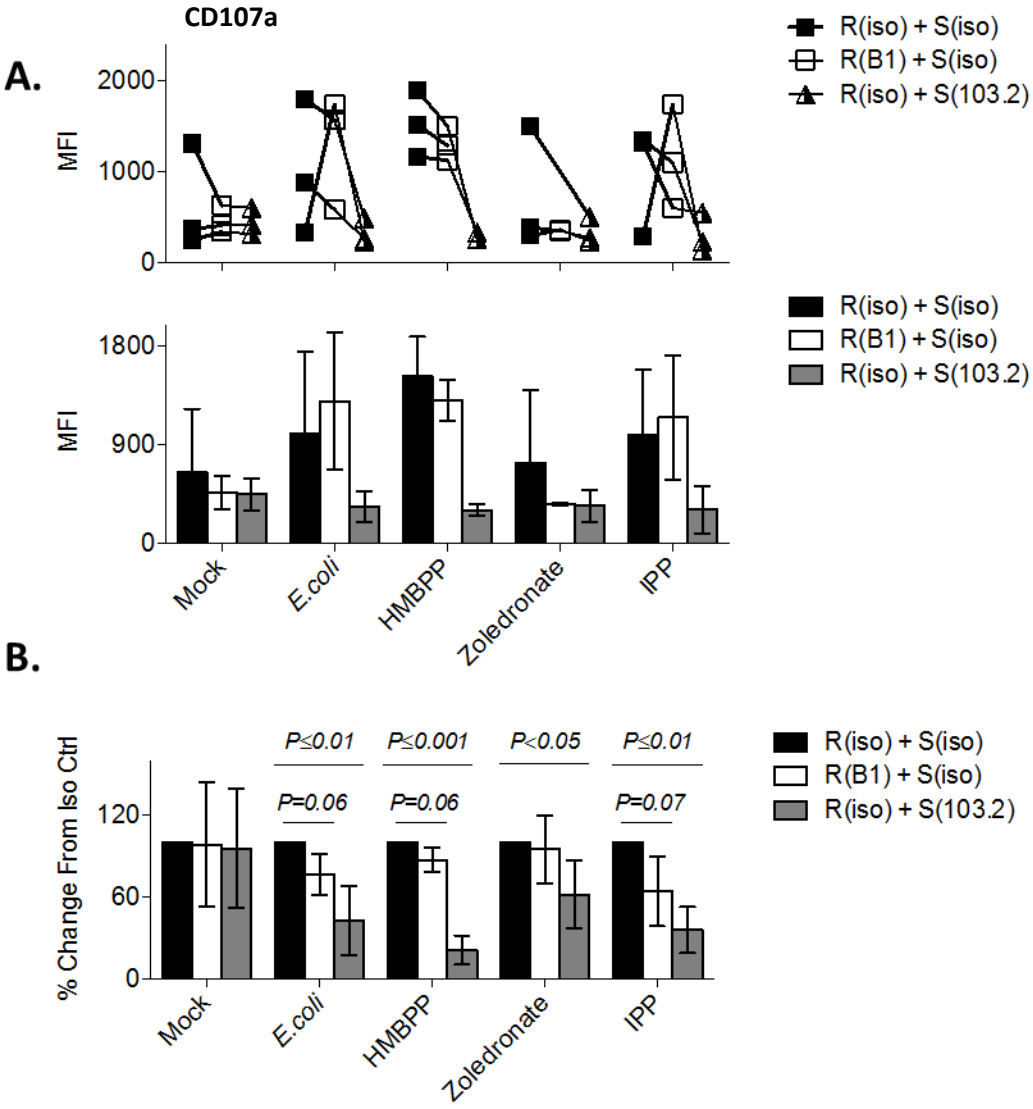

Fig. 7.12. Peripheral Vγ9Vδ2 responder cell surface CD107a in response to THP-1 stimulator cells is sensitive to blocking of BTN3A. Freshly-isolated PBMC (n=3) responder cells were stimulated with THP-1 stimulator cells. Stimulator THP-1 were prepared by culturing THP-1 cells overnight with irradiated *E.coli,* HMBPP, zoledronate or IPP. Stimulator THP-1 were then pre-blocked with either antagonistic anti-BTN3A mAb (clone: 103.2) or isotype-matched control. Freshly-isolated responder PBMC were, meanwhile, rested overnight, and pre-blocked with anti-γδTCR mAb (clone: B1) or isotype-matched control. Responder Vγ9Vδ2 cell upregulation of cell surface CD107a in response to co-culture with stimulator THP-1 was recorded via FACS. "R(iso)" indicates responder cells pre-incubated with isotype control mAb; "R(B1)" indicates responder cells pre-incubated with B1 mAb; "S(iso)" indicates stimulator cells pre-incubated with isotype control mAb; "S(103.2)" indicates stimulator cells pre-incubated with 103.2 mAb. (**A**) Shown are donor Vγ9Vδ2 cell surface CD107a after O/N co-culture in the presence of various blocking conditions. The upper panel shows individual donor points and the lower panel shows a mean and standard deviation of the data compiled. (**B**) Shown is the percentage change in response to blocking mAb for each donor data point. Each donor's cell surface CD107a in the presence of blocking mAb was compared to that seen with "R(iso)+S(iso)", which represents 100%. Bar graphs indicate the mean ± standard deviation.

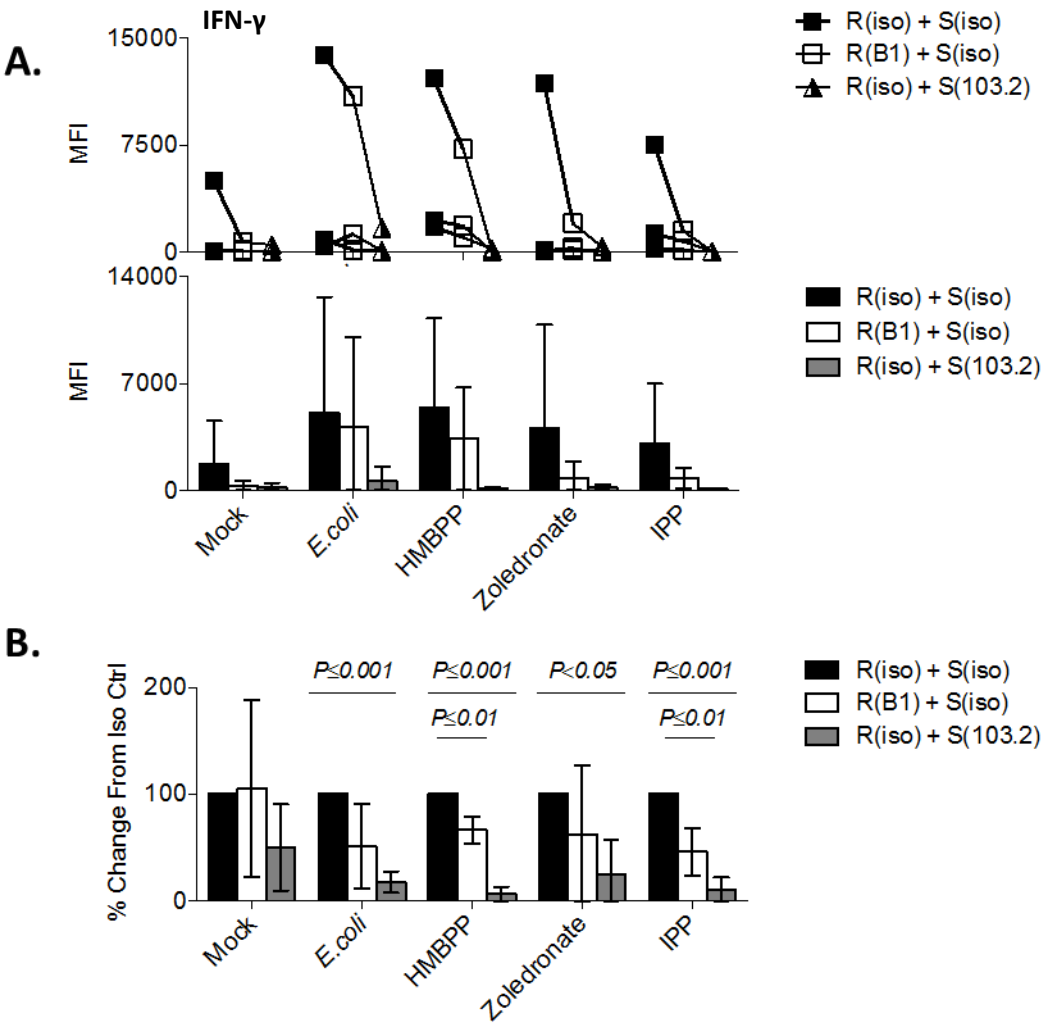

Fig. 7.13. Peripheral Vγ9Vδ2 responder IFN-γ production in response to THP-1 stimulator cells is sensitive to blocking of BTN3A. Freshly-isolated PBMC (n=3) responder cells were stimulated with THP-1 stimulator cells. Stimulator THP-1 were prepared by culturing THP-1 cells overnight with irradiated *E.coli,* HMBPP, zoledronate or IPP. Stimulator THP-1 were then pre-blocked with either antagonistic anti-BTN3A mAb (clone: 103.2) or isotype-matched control. Freshly-isolated responder PBMC were, meanwhile, rested overnight, and pre-blocked with anti-γδTCR mAb (clone: B1) or isotype-matched control. Responder Vγ9Vδ2 cell intracellular IFN-γ in response to co-culture with stimulator THP-1 was recorded via FACS. "R(iso)" indicates responder cells pre-incubated with isotype control mAb; "R(B1)" indicates responder cells pre-incubated with B1 mAb; "S(iso)" indicates stimulator cells pre-incubated with isotype control mAb; "S(103.2)" indicates stimulator cells pre-incubated with 103.2 mAb. (**A**) Shown are donor Vγ9Vδ2 intracellular IFN-γ after O/N co-culture in the presence of various blocking conditions. The upper panel shows individual donor points and the lower panel shows a mean and standard deviation of the data compiled. (**B**) Shown is the percentage change in response to blocking mAb for each donor data point. Each donor's intracellular IFN-γ in the presence of blocking mAb was compared to that seen with "R(iso)+S(iso)", which represents 100%. Bar graphs indicate the mean ± standard deviation.

7.6. Discussion

Data laid out in this chapter explores freshly-isolated Vγ9Vδ2 cell effector function dependency on the TCR and BTN3A in the context of an autologous and an allogeneic stimulation model. Findings included:

i) Nearly all freshly-isolated PBMC express BTN3A.

ii) *E.coli* and zoledronate stimulation of whole PBMC causes mild downregulation of BTN3A, but overall levels are restored throughout expansion with both stimuli.

iii) Freshly-isolated Vγ9Vδ2 cells upregulate CD69, engage in CD107a-mediated cytotoxicity and produce IFN-γ in response to stimulation with *E.coli*, HMBPP, IPP or zoledronate-exposed autologous PBMC.

iv) Vγ9Vδ2 cell IFN-γ production, but not CD69 upregulation and CD107a-mediated cytotoxic degranulation, in this autologous stimulation model were sensitive to blocking by both anti-BTN3A mAb (clone: 103.2) and anti-γδTCR mAb (clone: B1).

v) Freshly-isolated Vγ9Vδ2 cells upregulate CD69, engage in CD107a-mediated cytotoxicity and produce IFN-γ in response to stimulation with the otherwise unstimulated, allogeneic THP-1 cell-line, exhibiting baseline reactivity.

vi) Baseline THP-1-reactivity of Vγ9Vδ2 cells is highly donor-dependent.

vii) This baseline Vγ9Vδ2 cell activation by THP-1 cells is enhanced by THP-1 cell pre-stimulation with *E.coli*, HMBPP, IPP or zoledronate.

viii) Vγ9Vδ2 cell IFN-γ production as well as CD69 upregulation and CD107a-mediated cytotoxic degranulation in response to THP-1 stimulators were sensitive to blocking by anti-BTN3A mAb (clone: 103.2). Sensitivity to anti-γδTCR mAb (clone: B1) was effector function and stimulus-dependent. As with the autologous stimulator experimental system, IFN-γ production exhibited the highest sensitivity to blocking mAb.

Data laid out in this chapter indicates that freshly-isolated peripheral Vγ9Vδ2 cells respond to pAg-stimulated targets in a BTN3A (and, partially, TCR)-dependent manner. This finding expands and further develops data presented in previous chapters, which outlined that apparently similar factors govern γδT cell responses to zoledronate and *E.coli*-stimulated autologous cells. The findings of this chapter are further consistent with the role of BTN3A in Vγ9Vδ2 cell recognition of targets that is described in the literature (152,157,161,213,215).

Despite continuity with previous chapters, I note that the effect of γδTCR blocking was far less pronounced in the autologous or THP-1 stimulator systems described in the present chapter than what was observed with direct addition of anti-γδTCR mAb to whole PBMC, as described in Chapter VII. The efficacy of blocking mAb pre-culture was, furthermore, dependent on the pAg in use (*e.g.* IPP *versus* HMBPP), the type of stimulator cell (autologous PBMC *versus* allogeneic THP-1) and the donor in consideration. Thus, while general trends could be observed, I emphasize that significant further work is necessary to enhance the reliability and reproducibility of the conclusions drawn.

Foremost, noting the donor-dependence of observed Vγ9Vδ2 cell responses, the number of donors tested needs to be increased substantially. Given the differences between blocking potency in whole PBMC *versus* autologous PBMC stimulator *versus* allogeneic THP-1 stimulator systems, testing the blocking capacity of anti-BTN3A and anti-γδTCR mAbs needs to be examined in additional experimental setups. These experimental setups will require careful standardization for factors such as number of washes and culture times. It is possible, for example, that the lower impact of anti-γδTCR mAb in the stimulator systems compared to direct PBMC stimulation (as described in Chapter VI) is due to the higher number of washes and extended experimental protocol used in the BTN3A blocking experiments. It is not inconceivable that the anti-γδTCR mAb simply detaches from the TCR after a given period of time or is instead removed from the cell surface upon re-cycling of the TCR, as part of normal T cell physiology. A way to potentially mitigate these effects would be to leave the anti-γδTCR mAb in culture at a saturating concentration with responder and stimulator cells, so as to permit continuous binding of blocking antibody. A novel danger may be introduced, however, if anti-γδTCR mAb is

bound via its Fc portions to cells in culture (*e.g.* THP-1 stimulator cells) and therefore acts instead as an activatory signal by cross-linking the TCR. Anti-γδTCR clone B1 is a well-established stimulator to γδT cells, if adhered to the surface of beads, well-plates or stimulator cells (211). A troubling recent report that needs to be considered in the context γδTCR blocking mAbs has been compiled by Dutta *et al.* (216). They speculate and present supporting data to the notion that decreased effector function (cytotoxicity in this instance) upon pre-culture with anti-γδTCR is not necessarily or solely the consequence of functional blocking of the TCR, but alternatively or additionally of mAb-induced cell apoptosis. Lowered functional capacity was, thus, merely a reflection of mAb-related dramatically reduced γδT cell viability. While a limited number of experimental setups and readouts were investigated, Dutta *et al.* make the recommendation that B1 and 11F2 anti-γδTCR clones be avoided, and replaced instead with the less viability-damaging 5A6.E9 clone. Reasons for the dramatic effect that mAb binding alone can exert on γδT cell viability and function remain unclear, but are critically important in evaluating the credibility of a vast number of earlier anti-γδTCR mAb-utilizing publications. In the context of the present thesis, I am confident that at a considerable degree of cell viability remained, as continued γδT cell proliferation could be observed even post clone B1 mAb treatment. In many cases, as seen in Figures 7.13 and 7.14, for example, anti-γδTCR mAb failed to exert any functional effect, even in the context of cytotoxicity. Nonetheless, these experiments need to be repeated with the anti-γδTCR clones 11F2 and 5A6.E9, with cell viability tracked closely and carefully.

Similar allowances need to be given to the data presented using anti-BTN3A mAb clone 103.2. As described in the chapter introduction, 103.2 is only one of a number of clones available for agonistic as well as antagonistic BTN3A assays. 103.2 is the only clone documented to act antagonistically on BTN3A, and is stipulated to exert its inhibitory function by 'holding' BTN3A in its inactive, closed conformation (157). Other anti-BTN3A mAb are agonistic and instead hold BTN3A in its active, open conformation, including the clones 19.5, 20.1 and 232.5. Despite a similar effect on BTN3A extracellularly, the functional effect of these clones are vastly different, ranging from strongly activatory to T cells, inducing proliferation, cytokine production and enhancing survival with clone 20.1 and T cell inhibition with clone 232.5. The specific reasons for

the different effects of these mAbs on T cell activation are unclear, but appear to result from epitope location on the extracellular portion of BTN3A, antibody binding affinity, avidity and valency (155–158). In addition to uncertainty of the specific mechanisms of action and physiological relevance of blocking mAbs, also binding of FACS staining mAbs warrants investigation. BTN3A.1 is known to change conformation upon exposure to pAg. It is possible that the decreased positivity of BTN3A seen after PBMC stimulation with *E.coli* and zoledronate (Figures 7.1 and 7.2) was simply reflective of impaired binding of the staining mAb (clone BT3.1) to a conformationally-altered BTN.

Another important yet ambiguous avenue of the data presented in this chapter is the activatory potency of the various target stimuli used, i.e. *E.coli*, HMBPP, IPP and zoledronate. While some trends emerged, overall, all differentially-stimulated stimulator cells (autologous or THP-1) appeared to induce similar Vγ9Vδ2 cell activation, albeit in a strongly donor-dependent manner. It didn't appear to be the case, for example, that *E.coli*-stimulated target cells elicited vastly more CD69 upregulation, cytotoxicity or IFN-γ in Vγ9Vδ2 cells than IPP-stimulated target cells. Such an observation is surprising given that – in contrast to IPP - *E.coli* contain a multitude of immunogenic signals, far in excess of pAg content. Moreover, HMBPP (also found in *E.coli*) has been reported to be a ~10 000 more potent Vγ9Vδ2 cell activator than IPP – a phenoment that is likely due to its binding affinity to the intracellular B30.2 domain of BTN3A.1 (162). Such an observation appears logical in a physiological context, whereupon endogenous stress-induced IPP has a higher threshold towards activating Vγ9Vδ2 cells than *de facto* non-self HMBPP, which is of obligate microbial origin. Nonetheless, my findings of roughly similar activation levels would be consistent with the supposition that, given saturation of pAg stimulus, the central (if not only) activatory signal Vγ9Vδ2 cells perceive is BTN3A conformational changes. It may be the case that, upon direct exposure to *E.coli,* Vγ9Vδ2 cell activation is further modulated by *e.g.* TLR engagement, but it is clearly not necessary for induction of effector function. Indeed, work by De Libero *et al.* suggests that even HMBPP-expressing *E.coli*-induced activation of Vγ9Vδ2 cells is, in fact, mediated by *E.coli* induction of endogenous stress markers and pyrophosphates (including IPP) (101). Examining such questions in the experimental setups described in this chapter, once again, requires an increased donor number, and, importantly, a titration of pAg stimulus applied to target

cells. An interesting experiment would be autologous (or THP-1) target cell stimulation with a pAg-negative bacterium, *e.g. S.aureus*, and observing how that response compares to that of pAg-positive bacteria, such as *E.coli*, and those, in turn, to the addition of IPP.

Interesting differences in response could be observed between THP-1 *versus* autologous PBMC-stimulated Vγ9Vδ2 cells. Unexpectedly, CD69 upregulation, CD107a-mediated degranulation and IFN-γ were far more sensitive to anti-BTN3A and anti-γδTCR blocking in the context of THP-1 cells. THP-1 cells are allogeneic and, therefore, inevitably contain immunogenic signals other than just BTN3A; signals, which include NK cell receptor incompatibilities that are likely to be fortuitously different between donors. Vγ9Vδ2 cells are known to express a number of NK cell receptors, which one would expect to be triggered by allogeneic cells – particularly transformed allogeneic cells, as is the case with THP-1 cells (44,211). It is possible, however, that the standardization of stimulus cell type (i.e. all donor γδT cells being treated with the same THP-1 cells and not with corresponding autologous PBMC) improved reproducibility of data and, thus, improved the statistical significance of inhibitor effects observed. It is difficult to compare results from the two experimental systems, howerver, due to the earlier mentioned protocol differences and technical considerations of working with blocking antibodies. For example, it may be the case that BTN3A cycles from the cell surface more readily in freshly-isolated PBMC than THP-1 cells, therefore, making it appear that anti-BTN3A mAb exerts a greater effect on THP-1 cell recognition than recognition of autologous PBMC. As mentioned earlier, extensive standardization of various experimental setups with titration of antibody as well as pAg is imperative to determine whether such conclusions can indeed be drawn.

It was interesting to note, nevertheless, that the effector function consistently sensitive to blocking mAb across stimulator systems was production of IFN-γ. Unlike CD69 upregulation, induction of IFN-γ production requires overcoming a relatively high activation threshold (217). It may be the case, for example, that cell surface receptor cycling has removed a portion of the blocking anti-BTN3A or anti-γδTCR mAbs exposing sufficient 'active' BTN3A and 'free' γδTCR for the induction of CD69 but not IFN-γ. Cell surface CD107a, meanwhile, presents a more perplexing case. Even in previous chapters

that described a near total loss of Vδ2 cell IFN-γ production upon whole PBMC pre-culture with B1 mAb, these same cells maintained a degree of CD107a-mediated cytotoxic degranulation. The factors that regulate Vγ9Vδ2 cell cytotoxicity appear to include the TCR, FcR and NK-like receptors (*e.g.* killer-immunoglobulin-like receptors and NKG2D) as well as TLRs (44,211,218), but remain to be elucidated completely. Determinants of non-Vδ2 γδT cell cytotoxicity and activation remain even more poorly understood.

One Vγ9Vδ2 cell effector function that would be interesting to explore in the context of BTN3A-dependency is phagocytosis and pAPC function. In Chapter VII, I documented that expanded Vδ2 cell phagocytosis of IgG opsonized *E.coli* is TCR-dependent. Such TCR and IgG-dependence was observed for both *E.coli* and zoledronate-expanded Vδ2 cells, which were subsequently found to express almost identical Vγ9Vδ2 TCR repertoires. The conclusion drawn from this data was that the Vγ9Vδ2 TCR is unlikely to engage *E.coli* directly, but recognize instead a self-molecule that is present also on zoledronate-stimulated PBMC. Most likely this molecule is BTN3A and its conformational changes. I speculate, therefore, that Vγ9Vδ2 cell phagocytosis is regulated by a 'licensing'-type interaction between the TCR and BTN3A, as well as FcγR. It cannot be ruled out that further undetected interactions between Vγ9Vδ2 cells and neighboring PBMC are taking place, required for the successful engagement of phagocytic effector function. If indeed phagocytosis of target material is TCR and BTN3A-dependent, Vγ9Vδ2 cell pAPC function is likely to be as well.

Significant insight could be gained from a repeat of the above-mentioned *E.coli* phagocytosis assays in the presence of BTN3A blocking mAb, clone 103.2. To address specific mechanistic questions, however, perhaps the most valuable source of data may be bead-uptake experiments. Does Vγ9Vδ2 cell pre-culture with mAb 103.2-coated stimulator cells prevent the uptake of IgG-opsonized beads? Is this uptake in the presence of 103.2-blocked cells restored if the beads further contain stimulatory anti-γδTCR mAb? Does pre-culture with the BTN3A agonistic mAb clone 20.1 enhance Vγ9Vδ2 cell phagocytosis of beads? Such a reductionist experimental setup may have questionable direct *in vivo* relevance, but may provide insight into some of the basic questions regarding the mode of action BTN3A, and its role in Vγ9Vδ2 cell. As laid out in the chapter

introduction and Figure 7.3, it is unclear, as of yet, how exactly BTN3A enhances Vγ9Vδ2 cell activation. Is it engaged directly by the TCR? Does it act more as a B7 molecule instead, and bind a homotypic, unknown CTLA-4-like or CD28-like molecule on Vγ9Vδ2 cells? Is 'active' BTN3A instead a crucial adhesion molecule, which is not directly engaged by the TCR, but is necessary to stablizie TCR engagement with another target? Simplistic bead experiments in the presence of stimulator cells, anti-γδTCR mAb and agonistic, as well as antagonistic BTN3A mAbs may shead some light on these questions.

7.7. Summary

Data compiled in this chapter documents that Vγ9Vδ2 cell activation, cytokine production and cytotoxicity in response to pAg-exposed stimulator cells is BTN3A-dependent, in a manner that appears to be linked to TCR-dependency. Such dependency is, however, experimental setup, pAg stimulus and donor-dependent, and requires detailed further investigation.

8. DISCUSSION

The primary driver of scientific interest in human γδT cells derives from their potential as effectors in cancer and infectious disease immunotherapy (7,9,10,219,220). As the literature suggests, and indeed my data supports, peripheral γδT cells are highly effective cytotoxic killers and producers of pro-inflammatory cytokines, yet exhibit high, lymphoid-like specificity for targets, and appear tightly regulated by the need for detection of cellular stress and IgG opsonisation. Moreover, γδT cells appear capable of significant immune regulation and perhaps even initiation of αβT cell responses in a manner reminiscent of DCs. Unlike DCs, however, Vγ9Vδ2 cells can be accessed in the human periphery with relative ease, and amplified exponentially *in vitro* in an antigen-specific context. Strikingly, infiltrating γδT cells have been identified as the single most positive predictive factor in human cancer survival in both pan-cancer and solid-tumour analysis (221).

As approach to the clinic of γδT cell-based therapies for cancer and infectious disease gathers momentum, crucial basic biology questions about the nature of these enigmatic lymphocytes remain. How do γδT cells recognize self- and non-self antigen en masse in an apparently similar manner? How, if at all, do γδT cells differentiate between self- and non-self targets? How are γδT cell responses generated so rapidly? How exactly do γδT cells kill virally infected and transformed self-cells, and how does this compare to their direct killing of microorganisms? Are γδT cells truly capable of DC-like professional antigen presentation? Is their pAPC function important in the initiation of an immune response, or, rather, in the regulation of an existing response? How is γδT cell phagocytosis carried out? What are the molecular interactions that govern γδT cell effector function and proliferation in response to stimulation? Do the same γδT cells engage in cytotoxicity and pro-inflammatory cytokine production, as well as antigen presentation

and immune regulation? How do these disparate functional phenotypes relate to one another during acute immune activation events, such as sepsis?

The examination of these questions is hugely complicated by the physiological differences between human and rodent γδT cells. A lack of cheap and easily modifiable animal models has presented a significant hurdle to the dissection of γδT cell function and clinical potential. Given the near-necessity for work with human γδT cells when attempting to decipher γδT cell clinical potential, an important consideration emerges: Are *in vitro* observations regarding γδT cell functionality, including pAPC function, relevant *in vivo*? While this question remains acutely present in my evaluation of data output from the γδT cell field, an increasing body of clinical data is adding credibility to the notion that conclusions from *in vitro* study of human γδT cells bear resemblance to what is happening *in vivo* during the early stages of infection. Significant increases in systemic (and mucosal) γδT cell numbers are seen during acute infection with a wide variety of viral, parasitic and bacterial infections, ranging from HCMV and *Escherichia* to *Plasmodium* and *Mycobacterium* (19,62–64,102,134,208,222,223). Data indicates that these γδT cells display an activated phenotype (as evidenced by cell surface CD69), are positive for MHC class II and CD86, and correlate negatively with mortality during sepsis and acute systemic inflammatory response syndrome (21,22,24–26,28). Furthermore, natural or experimental elevation of γδT cell numbers in human, non-human primate and murine models has correlated with improved prognosis or even protection from a range of infections, including *M. tuberculosis,* HCMV, *T. gondii* and *L. monocytogenes* (28–36).

The present thesis describes an attempt at *ex vivo* modelling of peripheral human γδT cell behavior during acute *in vivo* microbial exposure as is seen during *e.g.* sepsis. Cell responses were examined and tracked after whole freshly isolated PBMC stimulation with irradiated *E. coli.* While the data describes γδT cell phenotypic and functional shift in response to *in vitro* stimulation with *E. coli,* ultimately, the themes and findings of the work extended beyond a description of γδT cell interaction with *E.coli*. At its core, the experimental setup is an exploration of the factors that govern peripheral γδT cell

(Vγ9Vδ2 cell, specifically) recognition of target, behaviour after encounter with target and subsequent functional evolution – whether the inflammatory context is of non-self (*e.g. E.coli*) or self (*e.g.* IPP) origin. The main experimental findings of the present thesis were as follows:

i. *E.coli* as well as zoledronate-expanded γδT cells are capable of effective phagocytosis of IgG-opsonized targets, including *E.coli*, while freshly-isolated γδT cells are not.
ii. *E.coli* and zoledronate-expanded γδT cells bear a close resemblance to one another in terms of functional cell surface marker expression, with both populations presenting with a predominantly Vγ9Vδ2 TCR^{pos} HLA-DR^{pos} $CD86^{pos}$ $CCR7^{neg}$ $CD62L^{low}$ $CD4^{neg}$ $CD8^{neg}$ $CD16^{pos}$ $CD56^{pos}$ phenotype.
iii. Further homology in the differentially expanded cells is seen with TCR CDR3 sequences, which are similar to one another, but different to freshly-isolated TCR CDR3s. This suggests that PBMC stimulation with *E.coli* and zoledronate leads to Vγ9Vδ2 CDR3 focusing on a similar set of ligands.
iv. Vγ9Vδ2 cell acquisition of a pAPC phenotype during expansion with *E.coli* or zoledronate is accompanied by a loss of a Th1-type pro-inflammatory phenotype, whilst maintaining potent cytotoxicity.
v. Expansion does not alter γδT cell memory phenotype, which remains effector memory ($CD27^{neg}$ $CD45RA^{neg}$) prior to, during and after 14 day expansion. Almost no naïve Vγ9Vδ2 cell can be detected at any time.
vi. Th1-type cytokine production, proliferation and phagocytic capacity of Vγ9Vδ2 cells are all dependent on the TCR. CD107a-mediated cytotoxic degranulation appears only partially TCR dependent, while bactericidal activity appears unaffected by blocking of the γδTCR.
vii. Vγ9Vδ2 cell activation, cytokine production and cytotoxicity in response to pAg-exposed stimulator cells are BTN3A-dependent, in a manner that appears linked to TCR-dependency. Whether this dependency is indeed consistent among various pAg stimuli and donors remains to be determined.

Altogether, the data permit development of an intriguing hypothesis as to the unique role of γδT cells (with a Vγ9Vδ2 TCR, specifically) in inflammation related to acute peripheral infection and/or cellular stress. I form the following narrative with published γδT cell literature in mind, expanded by the laboratory findings described in this thesis. A summary of my hypothesis of Vγ9Vδ2 cell functional evolution after exposure to antigen can be viewed in figure 8.1.

I postulate that circulating Vγ9Vδ2 cells, of an effector memory phenotype, surveil peripheral blood (and possibly mucosal epithelial sites) for evidence of cellular stress. This stress, manifesting itself as BTN3A conformational changes, can be induced by a wide variety of stimuli, all of which result in the rapid intracellular accumulation of pAg. Such stimuli include abnormally rapid cell proliferation, mutagenic transformation, infection with virus, intracellular bacteria, extracellular bacteria or parasites (64,135,164,165,179,195,224). While it may be the case that some microorganisms, such as *M. tuberculosis, P. falciparum* and *E.coli,* express pAgs that are exclusively microbial in origin (*e.g.* HMBPP), it remains unclear whether these induce BTN3A conformational changes directly or act instead by inducing accumulation of endogenous pAg, or both (64,101). Upon encounter with BTN3A in its 'active' conformation, effector memory Vγ9Vδ2 cells are activated in a TCR-dependent manner, and respond with production of Th1-type cytokines (*e.g.* IFN-γ, TNF-α) as well as potent cytotoxicity. Given the apparent broad specificity of the Vγ9Vδ2 TCR for BTN3A (or cells that express 'active' BTN3A, rather), γδT cells are activated en masse, thus, mounting a rapid and vigorous pro-inflammatory response.

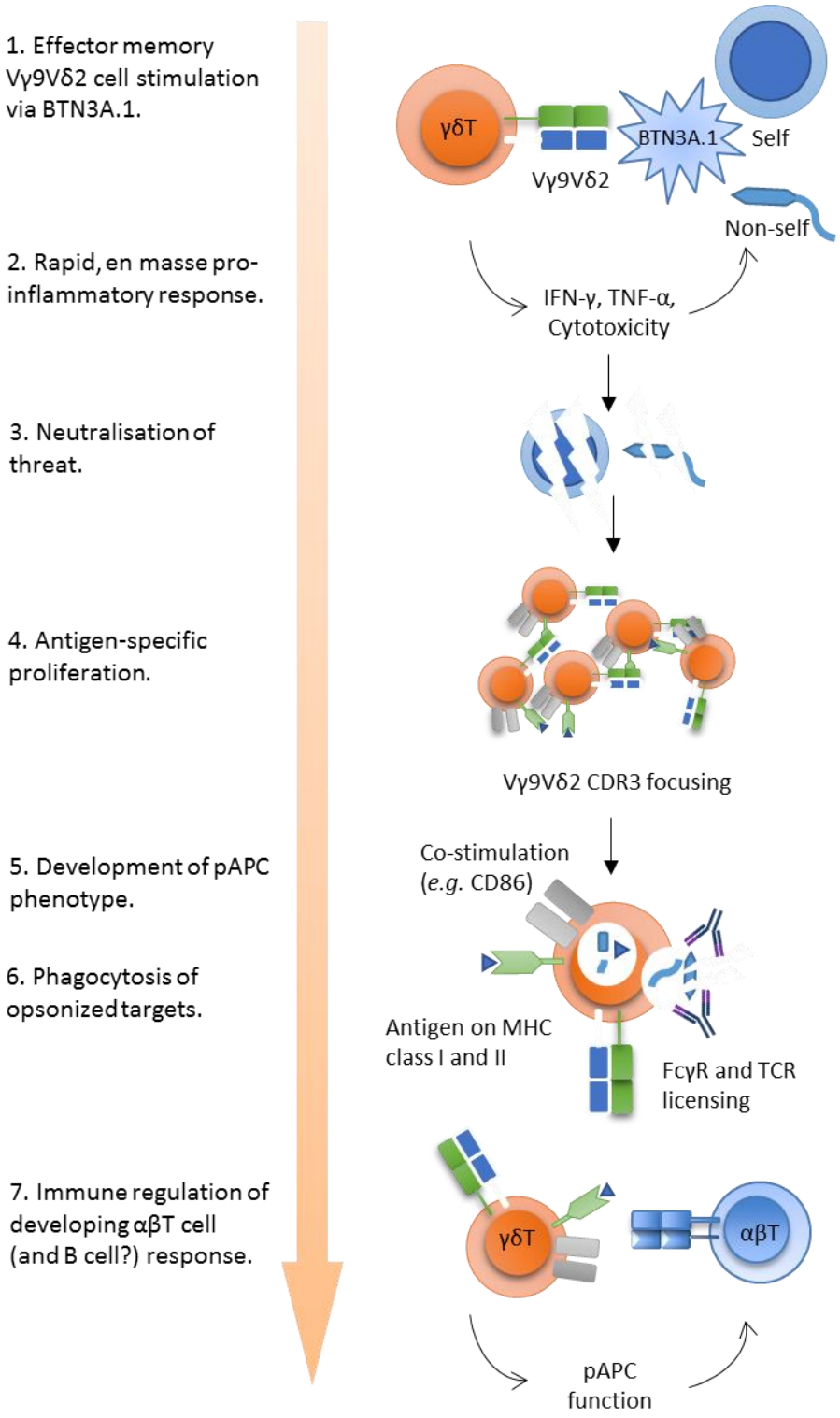

Fig. 8.1. A model of peripheral Vγ9Vδ2 T cell functional evolution following antigenic challenge. Informed by the laboratory data gathered throughout the progression of this thesis, and placed within a context of existing literature on γδT cell physiology, I postulate that, during acute infection-driven cellular stress, Vγ9Vδ2 cell effector phenotype is plastic, and transitions from a Th1-type to a pAPC phenotype following exposure to target antigen.

Through direct, en masse target lysis, as well as activation of downstream immune effectors, Vγ9Vδ2 cells form a widely-acting bulwark against a variety of environmental, infectious or mutagenic threats. Upon neutralizing the immediate threat, activated Vγ9Vδ2 cells proliferate, and undergo a profound functional evolution. Shedding their initial Th1-type pro-inflammatory phenotype, expanding Vγ9Vδ2 cells acquire in its stead a regulatory pAPC phenotype. The loss of canonical T cell capacities, such as IFN-γ production, is paralleled by acquisition of phagocytic capacity, expression of MHC class II and co-stimulatory molecules, necessary for the initiation and/or regulation of the developing adaptive immune response. While my data and data published by others indicates that Vγ9Vδ2 cells can act as pAPCs to αβT cells (60,178,225), γδT cells have also been implicated in regulation of humoral immunity (225). Such a narrative places peripheral Vγ9Vδ2 cells squarely at the interface between the innate and adaptive immune systems. An almost PRR-like TCR with apparently limited specificity, rapid, en masse reactivity, phagocytic capacity and antigen presenting properties allude to the many myeloid-like characteristics of these cells. The maintenance, nonetheless, of an oligoclonal TCR repertoire, expansion and early Th1-like reactivity place Vγ9Vδ2 cells in the lymphoid branch of the immune system.

One functional hallmark that my data indicates as sustained throughout γδT cell functional transition is potent cytotoxicity. While the rapid and efficient killing of *E. coli* by expanded MHC class II^{pos} CD86^{pos} Vγ9Vδ2 cells I report is atypical of most classic pAPCs, such as DCs, it may allude to the unique effector phenotype of activated γδT cells. It appears that even Vγ9Vδ2 cell pAPCs retain a highly cytotoxic phenotype. Such potent cytotoxic capacity is well beyond what is required for the killing of a phagocytosis or antigen presentation target. It is known, for example, that DCs can effectively present antigens of internalized cell debris, revealing that killing of targets is not a pre-requisite for professional antigen presentation (226). It is unlikely, therefore, that activated, expanding Vγ9Vδ2 cells retain cytotoxicity in service of pAPC function. The purpose of γδT pAPC cytotoxicity remains to be determined. It is, moreover, an important consideration in the design of γδT cell-based immunotherapies. Almost all current immunotherapeutic strategies involve *ex vivo* expansion of Vγ9Vδ2 cells, and is, thus,

likely to induce the above-described functional shift, which is accompanied throughout by potent cytotoxic capacity.

Given the plethora of effector functions at the disposal of peripheral Vγ9Vδ2 cells and their plasticity in employing these functions, careful control of the γδT cell compartment appears necessary. TCR-engagement as a pre-requisite for activation, proliferation, cytokine production, phagocytosis (and other pAPC functions) paired with a dependence on stress-induced BTN3A conformational changes suggests an elegant mode of regulation. Potentially dangerous pro-inflammatory effector functions of Vγ9Vδ2 cells are prevented from occurring in the absence of cellular stress through the dependence on 'active' BTN3A. Moreover, whenever an early immune response is sufficient to neutralize infection, MHC class II^{pos} $CD86^{pos}$ Vγ9Vδ2 cells may be prevented from posing an unnecessary inflammatory threat through downregulation of their early cytokine (*e.g.* IFN-γ, TNF-α) responsiveness. Even in the event that the infectious ('stressful') threat is sustained throughout Vγ9Vδ2 cell functional transit after encounter with antigen, IgG opsonisation of target is required for such effector functions as phagocytosis and consequent antigen presentation. This could be operative at two levels: i) as herein, when opsonizing with isotype-switched target-specific IgG, ii) in a 'naïve' non-immune situation, where natural antibodies (NAb) of different isotype, including IgG, may be involved. It has previously been demonstrated that NAb can enhance DC uptake and antigen presentation of viruses (227). Numerous, inter-connected layers of regulation, thus, exist to guide Vγ9Vδ2 cell activity. In addition, while dependence on stress-induced BTN3A conformational changes and IgG opsonin restricts their functionality, it permits broad and rapid en masse activation of Vγ9Vδ2 cells if the appropriate conditions are met.

A curious issue with this narrative, nonetheless, persists. Namely, if Vγ9Vδ2 cells do indeed specialize in the recognition of stress-induced BTN3A conformational changes, then why is an oligoclonal (or even polyclonal) Vγ9Vδ2 TCR repertoire maintained? Data presented in this thesis suggests that expansion in response to *E. coli* or zoledronate lead to a focusing of Vγ9Vδ2 CDR3 sequences. Even though the freshly-isolated Vγ9Vδ2

repertoire was dominated by several, highly numerous clones, almost no overlap could be seen between the freshly-isolated *versus E.coli* or zoledronate-expanded repertoire delta chain CDR3 (Fig.4.17). The suggestion is, therefore, that Vγ9Vδ2 cells do indeed have a baseline oligoclonal repertoire, but that it is *not*, in fact, finely-tuned to recognize *E.coli* or zoledronate-induced antigens, which are, presumably, BTN3A conformational changes. Perhaps an even more obvious question emerges upon considering why Vγ9Vδ2 cells need have a varied TCR repertoire at all. If Vγ9Vδ2 cells act primarily as surveyors of cellular stress that manifests itself via pre-determined BTN3A conformational changes, then why maintain such TCR CDR3 diversity? While an argument could be made for physiological redundancy, TCR re-arrangements are, nevertheless, a life-long energy expenditure. Moreover, if γδT cells are indeed as evolutionarily ancient as has been suggested by the study of lampreys (206), then their maintenance of a resource-intensive but unnecessary feature over millions of generations seems unlikely. It is known, for example, that mouse dendritic epidermal γδT cells bind a murine B7-homologue on keratynocytes, SKINT1, that is crucial for their organ-specificity and survival, in a manner that may parallel human Vγ9Vδ2 cell-BTN3A interactions (141,228,229). These mouse dendritic epidermal γδT cells do, however, express an almost entirely homogeneous, invariant TCR repertoire, consistent with their TCR-dependent recognition of a totally restricted set of target antigens focused on the SKINT1 molecule (69,141,229,230). As the literature and my own data indicates, such baseline TCR focus is not seen with peripheral human Vγ9Vδ2 cells (6,32,210).

I note that none of the data presented in this thesis sheds light on whether the Vγ9Vδ2 TCR engages BTN3A directly or whether BTN3A leads to TCR-mediated activation via other means, *e.g.* by binding to a co-stimulatory molecule on Vγ9Vδ2 cells. Perhaps, the maintenance of an oligoclonal (or even polyclonal) Vγ9Vδ2 repertoire indicates that the TCR binds a diverse set of target antigens that are not merely BTN3A conformational changes, even if it is done so in a BTN3A-dependent manner. Significant ambiguity and controversy on the details of Vγ9Vδ2 cell antigen recognition persists. A recent publication by Starick *et al.* suggests that Vγ9Vδ2 cells exhibit differential pAg *versus* agonistic anti-BTN3A mAb reactivity depending on fine CDR3 differences, implicating

the TCR in direct recognition of the conformational changes therein (231). They further postulate that Vγ9Vδ2 cells may also recognize pAg in a BTN3A-independent manner, given particular TCR specificity. These postulates were, however, formed using reductionist animal transductant models, inter-species hybrid cell lines and anti-BTN3A mAbs. The *in vivo* relevance of these findings is yet to be confirmed experimentally. In this respect, for now, Vγ9Vδ2 cells remain a quandary.

Despite a lack of clarity regarding the exact mode of antigen recognition and engagement, clues as to the physiological role of peripheral Vγ9Vδ2 cells can be drawn from their species specificity. Absent in rodents, Vγ9Vδ2 cells are present in such animals as humans and non-human primates. They have been ascribed a particularly important role in the life of young animals, with a disproportionate reactivity to infectious challenge by pAg-expressing pathogens, *M. tuberculosis* and *P. falciparum*, that are further pathogens of high significance in humans as well as non-human primates, but not rodents (7). Indeed, animal models and clinical human data have implicated Vγ9Vδ2 cells in anti-malarial and tuberculosis responses (19,31,34,62,134,223,224,232). Both *M. tuberculosis* and *P. falciparum* are a frequent cause of serious and chronic infection in humans that is often exacerbated by inappropriate immune responses. It is, perhaps, the ever-present threat of *M. tuberculosis* and *P. falciparum* infection that has shaped the human peripheral γδT cell compartment. It is, perhaps, a co-evolutionary context in which Vγ9Vδ2 cells developed a plastic and transitory effector phenotype. Potent, Th1-type pro-inflammatory reactions upon encounter with *M. tuberculosis* and *P. falciparum* may be mounted in an attempt to prevent productive infection. These are then followed by the development of a regulatory phenotype upon triggering of the adaptive response, and when the disease enters its chronic stages. Importantly, such a physiological role would account for the highly potent cytotoxicity that Vγ9Vδ2 cells retain even upon acquiring an apparently regulatory phenotype. Cytotoxic killing of *M. tuberculosis* or *P. falciparum*-infected cells by immune effectors is likely crucial during the acute as well as chronic stages of disease.

Further study of the engagement of the Vγ9Vδ2 TCR, possibly with BTN3A targets or co-receptors, will carry significant implications for γδT cell anti-tumor immunity. Data is increasingly supportive of the notion that stress recognition, particularly in combination with Ab-opsonisation, may be sufficient not only for killing of a tumor cell but also for uptake, processing and presentation of tumor-associated antigens (49). γδT cell direct killing of cellular and/or microbial targets combined with inflammatory cytokine production followed by uptake of the target into acidifying antigen processing compartments raises a novel paradigm. It is tempting to hypothesize that the combination of innate-like recognition and killing followed with myeloid-like phagocytosis by a lymphocyte-like cell may evolutionarily have preceded the full development of T lymphocyte-mediated adaptive immunity. Interestingly, the raised hypothesis is supported by previous studies showing that γδTCR chain genes may have preceded the development of αβTCR chain genes (205). In addition, the existence in jawless fish of three lymphocyte-like cells expressing variable lymphocyte receptors instead of TCR or BCR chain genes also supports this contention, since they otherwise resemble αβT, γδT and B cells by the expression of other orthologous genes (206).

Detailed further study of γδT cell antigen recognition, engagement and responses in human and humanized animal models is key to unlocking the vast immunotherapeutic potential of these enigmatic lymphocytes. If current suppositions on the physiology of human γδT cells are confirmed and clarified in the laboratory, their clinical application is likely to extend well beyond treatment for malignancy, and expand into therapy development for infectious and even immunopathological diseases.

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SUPLLEMENTARY MEDIA MATERIAL

Fig. 5.4. Vδ2 cell expansion leads to their acquisition of a phagocyte-like morphology and probing behaviour.

Panel (**C**). Day 14 *E.coli*-expanded PBMC were stained with Vδ2-PE mAb and examined via confocal microscopy. Shown is a representative field of vision, with PBMC in light phase contrast. Light phase contrast images from the same field of vision as shown in Fig.5.4 panel (A) were collected every 2 seconds for 5 minutes. Shown is a video sequence of the collected images. Vδ2 PBMC are indicated with white arrows.

APPENDIX

E.coli promotes human Vγ9δ2 T cell transition from cytokine-producing bactericidal effectors to professional phagocytic killers in a TCR-dependent manner

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