Investigating the anti-microbial capabilities of gamma delta T cells

PhD doctoral thesis: Infection & Immunity

Ayesha Khan [redacted]

UCL GOS Institute of Child Health

Declaration

I, Ayesha Khan, hereby declare the submission of this doctoral dissertation as results of my own work and findings. The outcomes of this thesis were carried out within the Great Ormond Street Institute of Child Health and the Department of Biochemical Engineering, University College London, with use of the flow cytometry core facility at the Zayed Centre of Research. Any contributions made have been clearly stated within the appropriate sections.

The research of this thesis has not been submitted for a degree, diploma, or any other qualification elsewhere. However, some of the work in this thesis has been presented in the following conferences:

- Khan, A *et al* (2018). 8th International γδT cell conference, Bordeaux
- Khan, A *et al* (2021). 9th International γδT cell conference, Beijing

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Abstract

Gamma delta T (γδT) cells represent a subset of T lymphocytes that exhibit a variety of functions, from killing capacity in response to stress ligands to the ability to kill bacteria such as *Escherichia coli* (*E. coli)* and behaviour similar to professional antigen-presenting cells (pAPCs) upon expansion, as shown previously by our group. As γδT cells are not restricted by the requirement of antigen to be presented on major histocompatibility complex (MHC) molecules, they display rapid pro-inflammatory reactions. We aimed to further investigate these responses by exploring the capacity of γδT cells to kill both Gram-positive *Staphylococcus aureus* (*S. aureus*) and Gram-negative *E. coli* bacteria by using an *ex vivo* co-culture model of isolated γδT cells and bacteria. We intended to elucidate the nature of this killing by comparing freshly-isolated and 14-day zoledronate-expanded γδT cells, to explore whether expansion via zoledronate impacts the bactericidal activity of γδT cells. Our results show that i) zoledronate-expanded γδT cells kill bacteria to the same degree as freshly-isolated γδT cells and that ii) freshly-isolated γδT cells can kill bacteria more significantly than other freshly-isolated immune cells and, through use of nalm-6 cells as comparators, iii) freshly-isolated γδT cells can innately kill bacteria more significantly compared to killing of cancer cells. Focussing further on freshly-isolated γδT cells, we then explored the cell markers and receptors involved in γδT cell-mediated killing of *E. coli* and *S. aureus*. Through use of blocking antibodies to the TLR-2, TLR-4 and CD14 receptors, as well as TAK-242; in combination with flow cytometric analysis of γδT cells, we have established that killing of bacteria by γδT cells utilizes innate signals and receptors. Further identification of the involvement of these receptors may enable the development of new classes of small and medium size molecules that can be used as alternative anti-microbials.

Impact Statement

The immune system is designed to recognise and target foreign invaders that might otherwise cause harm to the host. A carefully orchestrated sequence of events takes place with controlled interplay between both branches of the immune system, innate and adaptive, to eliminate potentially harmful foreign invaders i.e., through the recognition, phagocytosis, and clearing of bacterial antigens. Bacterial infections may cause damaging symptoms and without an effective immune response and/or treatment, a severe prognosis and may ultimately lead to death of the infected individual. However, there has been an increase in the number of antibiotic-resistant strains whereby adoptive T cell therapies may help in overcoming an undesirable prognosis.

Gamma delta T (γδT) cells have previously been identified as promising vessels for cell therapy of a range of cancers as they display potent cytotoxic behaviour. These unique cells demonstrate innate as well as adaptive-like qualities for overcoming the burdens which may overwhelm innate immune cells by providing rapid adaptive responses without the need for prior exposure or antigen presentation. Hence, this thesis sets out to explore the mechanisms by which γδT cells can recognise bacterial antigens and if functions of killing may further be optimised.

Our data is the first *in vitro* study of its kind to underline the varying functional capacities of γδT cells as innately bactericidal to both Gram-negative and Gram-positive bacteria using *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) as respective representatives, to showcase γδT cell function in the absence of previous priming or expansion, as previously expected as necessary for this function. We further conclude that mainly innate signals and receptors are needed for the killing of bacteria by γδT cells: CD14, TLR-2 and TLR-4 have all been implicated in bacterial killing. Thus, we show novel data displaying expression of CD14, a marker of phagocytes and has further identified a role for CD14 in γδT cell killing of bacteria. The combination of both CD14 and TLR involvement implicate the varying functional capacities of γδT cells in acting both as phagocytic killers and pAPCs and provides insight into the mechanisms employed by these cells when performing such functions.

Further identification of the involvement of these molecules may enable the development of new classes of small and medium size molecules that can be used as alternative anti-microbials. Our work utilises commensal bacteria but disturbance of microbiota, acquisition of as new virulence factors through evolution and tropism to alternative sites can result in otherwise harmless commensal bacteria causing fatal disease. Moreover, γδT cells have been shown to play a role in the initial immune response to a vast array of pathogens and as anti-microbial resistance gains prevalence among various pathogenic species, identification of how to enhance nature's oldest defence mechanism – the immune system – is of vital interest.

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Table of Contents

List of Figures

List of Tables

Abbreviations

αβ	Alpha Beta
α-	Anti-
AF (AF647/AF700)	Alexa Fluor
APC	Antigen Presenting Cell
APC *(in the context of methods section)*	Allophycocyanin
BTN	Butyrophilin
CDR	Complimentary Determining Region
CD	Cluster of Differentiation
CFU	Colony Forming Units
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
DNA	Deoxyribonucleaic Acid
EDTA	Ethylenediamine tetraacetic acid
FACS	Fluorescence Associated Cell Sorter
FBS	Foetal Bovine Serum
γδ	Gamma Delta
HLA	Human Leukocyte Antigen
HMBPP	(E)-4-hydroxy-3-methyl-but-2-enyl diphosphate
IEL	Intraepithelial Lymphocyte

IFN	Interferon
IL	Interleukin
IPP	Isopentenyl Pyrophosphate
LB	Lysogeny Broth
LPS	Lipopolysaccharide
MAMP	Molecular Associated Pattern
MHC	Major Histocompatibility Complex
MICA/B	MHC class I chain-related A/B
MOI	Multiplicity of Infection
NK	Natural Killer
NKG2D	Natural Killer Cell Group 2D Receptor
NLR	Nod Like Receptor
OD	Optical Density
OMV	Outer Membrane Vesicle
pAPC	Professional Antigen Presenting Cell
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
RLR	Rig Like Receptor
PMA	Phorbol 12-myristate 13-acetate
RNA	Ribonucleic Acid
RT	Room Temperature

RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
RPM	Rotations Per Minute
s.d	Standard deviation
TCR	T Cell Receptor
Tfh	T follicular helper
Th	T helper
Treg	T regulatory
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TB	Tuberculosis
UV	Ultraviolet
Zol	Zoledronate

1. Introduction

1.1. Introduction to the immune system

1.1.1. Defence mechanisms

In the context of infectious agents, several barriers are involved in the prevention of invasion into the body including the skin, respiratory tract, genitourinary and gastrointestinal tracts via physical/mechanical, chemical, and biological defence mechanisms. Physical and mechanical defence systems include coughing and sneezing actions of the lungs, operating to expel pathogens and irritants from the respiratory tract where they may gain access to other sites of the body and cause infection and inflammation. Other forms of physical defence mechanisms include the production of tears and urine to flush out pathogens. Chemical defence include secretion of antimicrobial peptides and enzymes or acidic pH, which assist in the destruction of pathogens (1,2).

Within specific sites such as the genitourinary and gastrointestinal tracts, commensal microbes serve as a biological defence mechanism to maintain those sites by competing with other, more infectious pathogens for nutrients. However, when these first-line defence mechanisms fail, the various components of the immune system are triggered.

1.1.2. Immune system recognition

Divided amongst two main arms: the innate and adaptive immune systems, the immune system is a complex and layered arrangement of biological processes, consisting of cells, proteins and soluble factors and involving various organ systems that together aim to protect the host organism from infection and disease. Found in all animals, the innate immune system offers a rapid, non-specific response to invading pathogens and in vertebrates, the adaptative immune system can also be later triggered to activate a more specific response. Both arms of the immune system need to be able to distinguish between self (and therefore harmless) and non-self-molecules, with the former not triggering the immune system but the latter leading to a measured response of appropriate intensity (1).

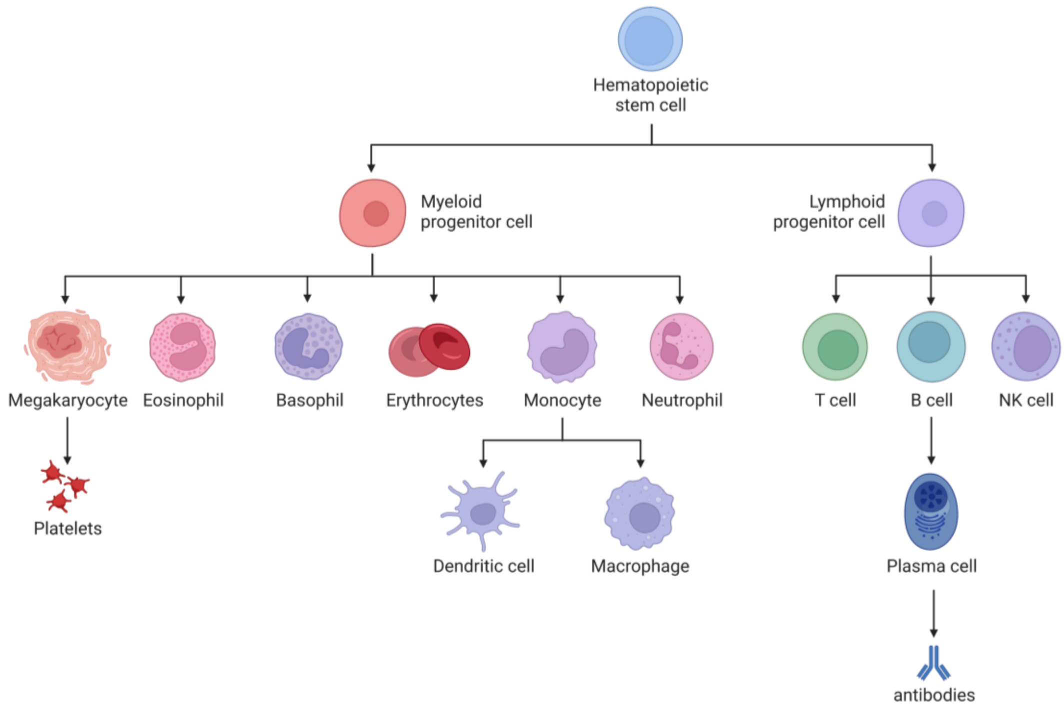

Figure 1.1 – Overview of various immune cell types and their differentiation from hematopoietic stem cells
Hematopoietic stem cells within the bone marrow give rise to myeloid and lymphoid progenitor cells which, upon activation of specific transcription factors give rise to the various immune cells. (Created on biorender.com)

Recognition of antigen (distinct molecular structures consisting of both self and non-self-classification) is a vital part of immune sensing and relies on various receptors. The most common types of immune sensing receptors, and an integral part of the innate immune response are pattern recognition receptors (PRRs) which recognise pattern-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) originating from non-self and self-molecules respectively. The main forms of PRRs are toll-like receptors (TLRs) which are expressed on both innate and adaptive immune cells as well as epithelial cells. Other forms of PRRs include nod-like receptors (NLRs) and rig-like receptors (RLRs). Upon recognition of PAMPs or

DAMPs by the appropriate PRR, chemical signals are released from cells in the form of chemokines and cytokines (interleukins *etc.*), that recruit other immune cells to the site of infection (3)

1.1.3. The innate immune system

The innate immune system consists of phagocytes such as neutrophils and macrophages, as well as an abundance of other cells such as mast cells, basophils, eosinophils, natural killer (NK) cells, innate lymphoid cells and dendritic cells (DCs). Functions and defining characteristics of these cells are described below (Table 1.1), ranging from release of cytokines and recruitment of other cells to degranulation and cytotoxic responses as well as phagocytosis.

Phagocytosis is a process by which specific cells are able to engulf microbes (bacteria and parasites) and destroy them through lytic enzymes or respiratory burst. In macrophages, phagocytosis can be paired with antigen presentation to T lymphocytes. Antigen presentation, a key component responsible for connecting the innate and adaptive immune response is the process by which antigenic peptide is presented to T cells, as a means of activating them (4,5) and is a property of various antigen presenting cells (APC).

Immune cell	Defining characteristics
Neutrophils	- First responders - Most abundant phagocytes - Release of cytokines and chemokines
Monocytes	- Phagocytic - Antigen presentation - Release of cytokines, chemokine and complement proteins - Found in blood
Macrophages	- Phagocytic - Antigen presentation - Release of cytokines, chemokine and complement proteins - Found in tissues/organs
Dendritic cells	- Antigen presentation and co-stimulation of T cells - Release of cytokines
Basophils	- Secrete chemical mediators for immune response against parasites - Associated with allergy and asthma
Eosinophils	- Secrete chemical mediators for immune response against parasites - Associated with allergy and asthma
Mast Cells	- Associated with allergy and anaphylaxis
Natural Killer cells	- Cytotoxicity against tumour or viral-infected cells via “missing-self” recognition - Perforin, degranulation, and cytokine release
Innate lymphoid cells	- Produce effector cytokines - Lymphoid organogenesis and tissue remodelling - Antimicrobial immunity and inflammation at barrier surfaces
B cells	- Antigen specificity - Memory - Antigen presentation - Production of antibodies
T cells	- Antigen specificity - Memory

Table 1.1 – Overview of immune cells
Adapted from Janeway’s Immunobiology *(4)*

1.1.4. The adaptive immune system

The adaptive immune system consists of B and T cells bearing antigen-specific receptors; the B cell receptor (BCR) and T cell receptor (TCR) respectively. T and B cells originate from hematopoietic stem cells, found in the bone marrow. Developing T cells then migrate to the thymus for further development.

B cells play a specific role via production of antigen-specific immunoglobin (Ig), which can be membrane bound, (BCR) or released in abundance via plasma B cells (6,7). Immunoglobulin exists in 5 different classes – Ig-G, -M, -A, -D and -E, which range in abundancy, structure, and function. The immunoglobulin is formed through an Fc and Fab (antigen-binding) region (8). Circulating immunoglobulin can bind to antigen on foreign microbes via Fab-binding, leaving the Fc portion of Ig exposed. Tagging of pathogens via multiple Ig is a process known as opsonisation and allows for identification by other immune cells via the exposed Fc portions of bound-Ig reacting to Fc receptor (FcR) on immune cells. Opsonisation of targets allows for antibody-mediated cytoxicity on cells like NK cells and phagocytes and is a means by which the adaptive immune system assists the innate immune system to react to more specific targets (9).

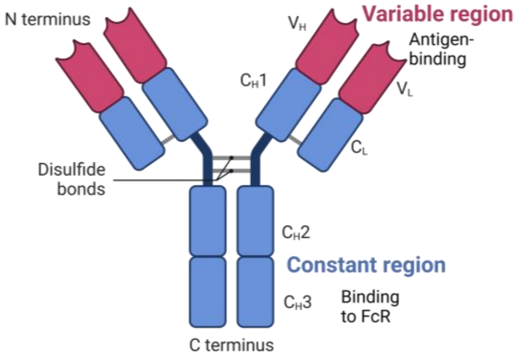

Figure 1.2 – The structure of the B cell receptor
The B cell receptor is made of variable (antigen-binding) and constant (Fc receptor-binding) regions, linked through disulfide bonds. B cell receptors can be released as immunoglobulin to act in circulation via means of antigen-binding and opsonisation. (Created on biorender.com)

T cells are a population of lymphocytes defined by the expression of a TCR and CD3, which regulates TCR development and function (10). TCRs are made up of two glycoprotein chains and for the majority of TCRs found on peripheral blood derived T cells, this consists of an alpha (α) and a beta (β) chain, thereby classifying the T cell as an alpha beta T (αβT) cell. αβT cells can then be further categorised by the presence of cell-surface markers CD4 and CD8 (11), which determine their biological properties and subsequently, their immunological function. The TCR functions by recognition of antigen on other cells; and in the case of αβT cells, these antigens are required to be presented in the context of major histocompatibility complex (MHC). The MHC is a protein expressed on almost all cells and occurs in two forms: MHC class I and MHC class II. MHC class I presents endogenous peptides to CD8-expressing T cells whereas MHC class II presents exogenous peptides to CD4-expressing T cells. This mechanism of presentation to αβT cells thereby acts as a recognition tool for the immune system as to the status of the cell. αβT cells require the surface CD4 and CD8 as co-receptors to their TCR, as well as co-stimulation through CD28 (on APCs) for full T cell activation.

Both B and T cells exist in various subtypes (Table 1.2), with various functions. However, one key theme amongst B and T cells is the capability to generate memory responses to antigen, which is a vital component for vaccines, allowing for rapid immune responses to previously detected and previously deemed-pathogenic foreign antigen.

Type of T and B cells	Defining marker(s)	Defining characteristics
T helper cell	- CD3+CD4+ - αβTCR	Release of various cytokines depending on type of helper cell; known types of T helper cells include: - Th1: IFN-γ, TNF-α - Th2: IL-4 - Th9: IL-9 - Th17: IL-17 - Th22: IL-22, TNF-α
T follicular helper cell (Tfh)	- CD3+CD4+ - αβTCR	Provide B cell help and maturation
Cytotoxic T cell	- CD3+CD8+ - αβTCR	Cytotoxicity against tumour or viral-infected cells via perforin, degranulation, and cytokine release
T regulatory cell (Treg)	- CD4+CD127^{low}CD25^{hi}FoxP3+ - αβTCR	Releases anti-inflammatory cytokines upon recognition of self-peptide - IL-10
T memory cell	- CD3+CD45RO+CD45RA−	- Rapid activation upon restimulation by specific antigen
Mucosal invariant T cell (MAIT)	- CD3+ - Semi-invariant αβTCR	- Innate immune T cell - Recognizes small-molecule biosynthetic derivatives of riboflavin synthesis
γδT cell	- CD3 - γδTCR	- Recognise cognate antigen (phosphoantigen) - Innate immune T cell - Cytotoxicity via perforin, degranulation, and cytokine release
B cell	- CD19/CD20+ - CD27-	- Release immunoglobulin - APC function -
Regulatory B cell	- CD19/CD20+ - CD1d+CD5+CD24^{hi}CD38^{hi}	- Tolerogenic - Present antigen to naïve T cells to promote Treg differentiation - IL-10
Plasma cell	- CD19/CD20+ - CD27^{hi}	Secrete high amounts of antibody
Memory B cell	- CD19/CD20+ - CD27+	Produce antibody upon restimulation by specific antigen

Table 1.2 - Overview of B and T cells
Adapted from information found in the following sources: *(1–4)*

1.2. Gamma delta (γδ) T cell background

In contrast to αβT cells, gamma delta T cells (γδT cells) are a unique subset of T cells defined by their distinctive TCR made up of a γ chain and a δ chain (12), making up 0.5–16% of all T cells in human peripheral blood, but can be found in larger abundances in peripheral tissues and organs, such as the gut and skin, rather than residing in lymphoid organs, like B and other T lymphocytes (13). This, combined with the fact that γδT cells can recognise cognate antigen thereby allows for an accelerated immune response in peripheral tissues and other sites that may otherwise not be well served by B and αβT cells, such as the mucosal sites Moreover, the conservation of γδT cells in all animals with an adaptive immune system and all jawed vertebrates makes them particularly interesting as a minor T cell population, indicating a non-redundant role for them within the immune system (14). Thus, much research is dedicated to elucidating the unique, specific roles that γδT cells play in the context of infection and immunity.

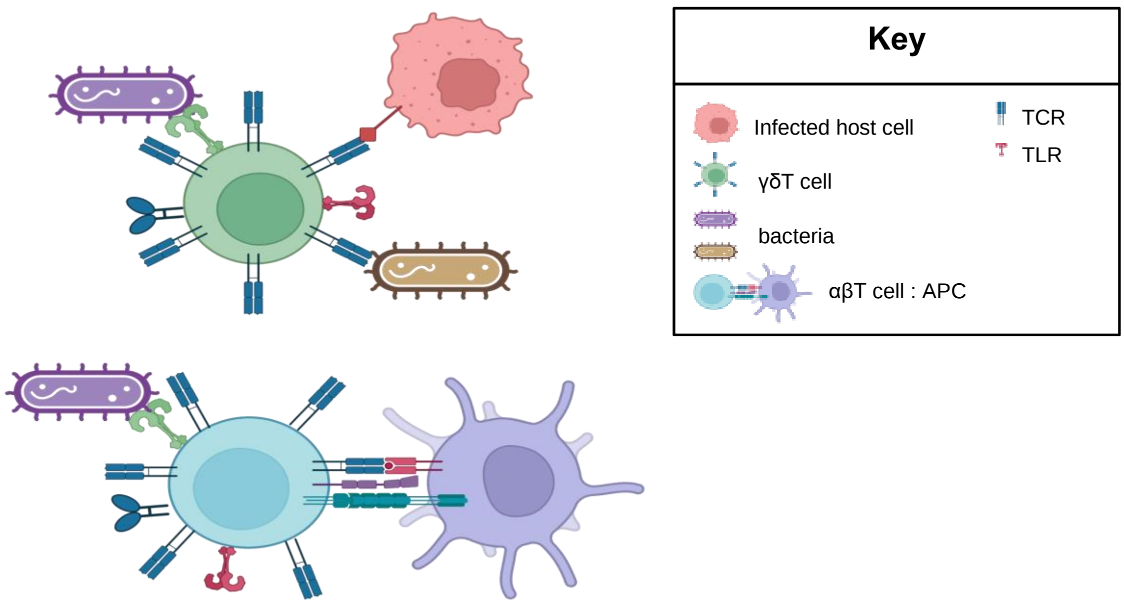

Figure 1.3 – Activation of γδT and αβT cells
γδT cells can react to direct stimulation of γδTCR via stress signals on infected or cancerous host cells or via external signals from microbial ligands to the γδTCR or TLRs whereas αβT cells react to endogenous or exogenous peptide presented on MHC on APCs. (Created on biorender.com)

In contract to αβT cells, and what defines their rapid "innate-like" response in comparison to αβT cells, γδT cells rather uniquely do not require antigen presentation via MHC, nor are they reliant on co-receptor interaction. Moreover, whilst αβT cell receptors (αβTCR) are restricted to only recognising peptide antigens, human γδT cell receptors (γδTCR) can recognize a variety of small phosphorylated non-peptide antigen. They have been specifically shown to recognise phosphate-containing compounds with non-bulky and short carbon chains (15,16). As such, the γδTCR has the capability of binding soluble or membrane proteins which include bacterial and viral proteins as well as heat shock proteins (HSP) (17–19).

Despite these differences, in many other ways, γδT cells share similarities with αβT cells. Interaction with antigen leads to similar downstream immune processes as αβT cells such as clonal expansion, cytokine release and cytotoxicity. This is of no surprise as γδT cells express most of the T cell lineage-specific genes that αβT cells express, including transcription factors, cell surface receptors and cytokines. Moreover, the phenotypic and functional states of both resting and activated γδT cells resemble that of counterpart αβT cells (12). Likewise, T cells can enter a state of dysfunction, known as T cell exhaustion, whereby they express sustained levels of inhibitory receptors leading to poor effector function, often as a result of chronic infection. This state is a characteristic of both αβT and γδT cells.

Like other immune cells, γδT cells express a range of other cell surface receptors. There is a large overlap in the expression of receptors between Natural Killer (NK) cells and γδT cells. These include the expression of NKG2D (an activating receptor) as well as FasL which engages with Fas on DCs to engage in effector functions and upregulation of costimulatory markers. As such, a common assumption of γδT cells is that their repertoire is largely directed against self, this is due to the expression of these receptors and their anti-tumour activity (20). However, there is evidence of γδT cells recognition of foreign stimuli such as the herpes simplex virus and mycobacteria (21,22). Aside from receptor expression and antigen recognition, the NK cell and γδT cell similarities extend to their biological roles also. Both cell types are known as innate cytotoxic cells, with the capabilities of acting in an adaptive immune response. Interestingly, both of these cells also play a role in the context of pregnancy, through high levels of CD56+ γδT cells and NK cells (23,24).

Other receptors expressed by γδT cells include CXCR4, which plays a role in T cell adhesion and chemotaxis (25) as well as Fc receptors and other pattern recognition receptors (PRRs) such as TLRs common to innate immune cells. Interestingly, whilst most γδT cells do not express the αβT cell characteristic markers CD4 and CD8, around 30% express CD4 and less than 1% express CD8 (26). Expression of CD4 by γδT cells may enable the activation of other γδT cells acting as helper cells, as well as presentation of peptide to CD8 T cells (27). Whereas expression of CD8 is associated with more directly cytotoxic potential of γδT cells (24). γδT cells have also displayed ability to express co-stimulatory receptors (28). Recognition of antigen by γδT cell can thereby occur via the defining γδTCR or the numerous other aforementioned cell surface receptors. The multifaceted array of receptors aid in the response to both non-self antigens and self-antigens including unconventional antigens such as the stress molecules MIC-A (29). MIC molecules exhibit class homology with human leukocyte antigen (HLA) molecules. However, they do not associate with β2 microglobulin and thus do not bind peptide. In addition to this, the γδTCR interactions with other immune cells via binding of CD1d. CD1d is a surface molecule expressed by professional antigen presenting cells (pAPCs) such as DCs. Thus, these APCs can activate γδT cells via CD1d leading to proliferation, expansion and activation of the γδT cell whilst also stimulating αβT cells to do the same via MHC-antigen interactions (30). However, interaction with the CD1c molecules is independent of any foreign lipid or glycolipid presence (18). Further data regarding the impact of γδTCR in aiding recognition of pathogenic stimuli, their interactions with other immune cells and their role in health and disease will be discussed in more detail further into this chapter.

1.3. The gamma delta T cell receptor (γδTCR)

1.3.1. Background

γδTCRs showcase the ability for both innate and adaptive ligand recognition via either germline-encoded regions of the receptor, reminiscent of PRRs or adaptive antigen binding via the CDRs. However, despite this information, exact mechanisms on how human γδTCRs recognize majority of their ligands remains unclear and many γδTCR-ligand combinations and groups are still largely unknown.

Like αβT cells, γδT cells also utilise somatically rearranged variable (V), diversity (D) and joining (J) genes to form their TCRs. However, the sets of genes used to form γδTCRs are distinct to αβTCRs. In comparison to αβT cells, the available germline repertoires of Vγ and Vδ genes are restricted as γδT cells contain less V, D, and J segments than αβT cells. Despite this, the combinatorial diversity of the γδTCR repertoire is as large as the αβTCR repertoire, due to other mechanisms of diversity such as the insertion of N regions and the imprecise joining of segments (31). Human γδT cells are categorised into subtypes according to their Vδ gene segment; *e.g*. Vδ1, Vδ2 or Vδ3, with little sequence variation among family members (31). Added to this are gamma chain subtypes Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, Vγ9 and Vγ11 that pair with specific δ-chains to give unique receptor combinations.

1.3.2. γδTCR development

Given that there are two known distinct forms of TCRs that a T cell can express, it is important to explore how these αβT and γδT cells evolved separately. Despite originating from common thymic precursors, phylogenetic studies have shown γ and δ TCR segments to predate α and β TCR sequences (32,33). This suggests these cells to be more primitive in nature and explains their more innate-like qualities, whilst further supporting the idea of αβT cells forming later due to more range of antigenic stimuli and providing memory and recall responses. The fact that γδT cells are capable of phagocytosis further supports the idea that γδT cells preceded αβT cells and are a more primitive T cell as phagocytosis as an immune defence strategy preceded the acquisition of adaptive immunity and the immune cell counterparts (34,35)

Despite forming distinct TCRs with differing properties and recognition profiles, there seems to be some fluidity in the state of a T cell from thymic development to its end-state as data have shown the capacity for the human peripheral CD4+ Vδ1+ T cell subset to develop into αβT cells (36). This data is particularly important when considering therapeutic potential of the potent and cytotoxic Vδ1 cells subset, which will be discussed further sections in 1.5.1 and 1.11. However, it has been argued that the molecular switches involved in lineage commitment are not homogenous for all γδT cells and thus this capability may be restricted to specific Vδ1 T cell subsets. It is therefore probable that γδT cells exist as a phenotypically heterogenous population, comprising of distinct γδTCRs with some γδT cells retaining the ability to phagocytose and others acquiring alternative functions (33,37).

1.3.3. Insights from γδT cell 3D structures

Unlike αβT cells, for which there is much data and 3D structures available for the αβTCR, there are only two human γδTCR structures currently known. These consist of an isolated human Vδ3 domain of unknown ligand specificity and the ligand-free ectodomain of human Vγ9Vδ2 (Vγ9δ2) TCR. Taken together, these structures help to highlight the structural homology between the αβ and γδ TCRs. The Vγ9 chain most often associated with the Vδ2 chain, defining the common Vγ9δ2 T cell population which accounts for 50–95% of γδT cells in the peripheral blood. This γδT cell subtype is unique to humans and other primates (38,39). Using the crystal structure formed of the ligand-free ectodomain of human Vγ9δ2 TCR, similarities between other immune components could be drawn. The crystal structure of a human γδTCR shows distinct structural similarities between the γδTCR and the heavy and light chains of immunoglobulins. For instance, parallels can be drawn between the combining site of the immunoglobulins and the cleft formation between the CDR3 loops in the γδTCR. Moreover, as the amino acid residues residing in this pocket are mostly germline encoded it can be inferred that γδT cells directly recognize non-peptide antigens in a manner similar to those of antibodies.

It is thought that γδTCR recognition of phosphoantigen requires presentation of the phosphoantigen to γδTCR through ATP synthase-F1/apolipoprotein A-1 complex and butyrophilin 3 (BTN) molecules (40,41). The existence of BTN molecules plays a vital role within the γδT cell field and are thus a well-studied part of γδT cell research. These proteins, found in both humans and mice, belong to a family of immunoglobulin-like molecules which function in an immunomodulatory way and are expressed throughout the body. For instance, during lactation, BTN1A1 is highly expressed in the secretory epithelium of mammary gland. In addition, BTN2A1 and 2, as well as BTN3A1, 2 and 3 are expressed in many other tissues (42).

The phosphoantigen activation of Vγ9δ2 T cells in particular, requires BTN3A1 molecule, whereby it acts as an antigen presenting molecule, like an APC to present the phosphoantigens to the γδTCR (43). However, Vγ9δ2 TCR-mediated recognition of prenyl phosphates via BTN3A1 seems to occur in a different manner to the classical APC molecule, but rather a sensor of intracellular levels of phosphorylated antigens (44).

1.3.4. Activation of the γδTCR

γδT cells function primarily in the immune surveillance of stress signals released from stressed tumour cells or infected cells. (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP), an intermediate of the non-mevalonate pathway in bacteria (22,45,46) and isopentenyl pyrophosphate (IPP), an endogenous pyrophosphate accumulated from disruption of the host mevalonate pathways from transformed cells, are two of the major characteristic phosphoantigens involved in activation of the γδTCR and commencement of γδT cell-mediated immune response (46,47). The mechanism by which HMBPP and IPP leads to the activation of γδT cells is unclear. However, it has been suggested, in particularly with respect to Vδ2 T cells, that it may be due to downstream induction of self-stress molecules, vis-a-vis conformational changes within BTN molecules such as BTN3A (44).

Similarities between the effects of these two pyrophosphates can be explained by their likeness in structure (48). However, whilst their mechanism of action remains alike, the potency of said action may vary, leading to variation in degree of proliferation and activation achieved. For instance, HMBPP, when acting alone, is a far more potent activator of Vδ2 cells than IPP (49). Aminobisphosphates or nitrogen-containing bisphosphates such as pamidronate and zoledronic acid have been shown to enhance levels of IPP (50). Zoledronate is an FDA-approved drug often used in the prevention of skeletal fractures in patients with cancers, as well as for treating osteoporosis and Paget's disease, a condition that results patients with bone deformity through cellular remodelling (51). Due to its causative effect in IPP accumulation, it is also used within the γδT cell field to enhance activation-status of γδT cells for study and use (50,52). The γδT cell activation through phosphoantigens is evident via proliferation, as there is a marked increase in cell numbers upon infection. These proliferated cells also exhibit an activated phenotype through the expression of MHC class II (HLA-DR), CD86 and CD69 (50,53).

1.4. Mouse γδT cells

In addition to the two structures mentioned in section 1.3, a third γδTCR structure has been identified for the murine γδTCR G8 bound to its non-classical MHC class I ligand, H-2T22 (54,55). More data have been published with regards to murine γδT cells. For instance, in mice, γδT cell subsets have been shown to carry semi-invariant TCRs and recognise a limited range of self-ligands (56). One unique feature of murine γδT cells compared to human γδT cells is their distinct expression of different Vγ segments in different tissues, dividing subtypes up by geographical organ/tissue location within the body (57). For instance, Vγ5$^+$ γδT cells are present within the skin, Vγ7$^+$ γδT cells, in the intestine, Vγ6$^+$ γδT cells are found in the mucosa of reproductive organs, and Vγ1$^+$ or Vγ4$^+$ γδT cells are found in secondary lymphoid organs (58,59). The Vγ chains pair with a range of Vδ chains and in adult mice, as much as of 75% γδT cells express either Vδ4, Vδ5 or Vδ6 (60).

In mice, the Vδ1 subset is the predominant subtype within circulation, akin the Vγ9δ2 subtype in humans, and is interestingly associated with production of various cytokines such as IL-4 and IL-17 (61,62). The Vγ5δ1+ subset is the most extensively studied γδ T cell subtype in mice. Found in the mouse epidermis and making up most of the dendritic epidermal T cell (DETC) population, this subset acts predominantly in an innate-like manner, responding to stress-associated ligands. Upon stimulation, Vγ5δ1+ T cells produce IFN-γ and promote wound-healing, thus playing an essential role as first-line defence of maintenance of the epithelial barrier and integrity (63,64).

The Vy6 subset of murine γδT cells predominantly produce IL-17 and IL-22, specifically in response to *S. aureus* infection (65). The Vy6 chain is also paired with the Vδ1 chain, with preferential pairing over the Vδ5 chain in the epidermis, giving rise to the Vγ6δ1+ subset. This subset tends to migrate to the genital tract and have been shown to play a role in immune-regulation during pregnancy of mice by responding to inflammation (66,67). The Vγ6δ1+ subset also migrates to the lungs and has been shown to inhibit the development of pulmonary fibrosis by exerting antibacterial activities (68).

A fundamental difference between mice and human γδT cells is the ability for human blood Vγ9δ2 subset to react to phosphoantigens, a phenomenon that has not yet been observed in mice (69). It is therefore unsurprising that many differences among mice and human γδT cells exist. Thus, whilst data gathered on mice γδT cells can provide insight on human γδT cells, it is important to consider that subsets described via the TCR segments across species may not correspond to one another. For instance, Vγ4 T cells in mice may differ significantly in functionality from Vγ4 T cells in humans (70).

1.5. Properties of the different subtypes of human γδT cells

Similarly to αβT cells, γδT cells come in the form of subtypes which are determined by the type of δ chain expressed to give the three major subclass of γδTCR: Vδ1, Vδ2 and Vδ3. In general, the Vδ1 subtype is the prominent subtype of the epithelia and the Vδ2 subtype is the predominant peripheral subtype of the blood (71,72). However, the exact balance of γδT cell subtypes varies between humans, with studies showing an influence of gender and age on the level of Vδ2 cells in blood. Women have been identified to have lower levels of Vδ2 cells compared to age-matched men (73). The balance of Vδ2 and Vδ1 populations in peripheral blood of humans is thought to be controlled by both environmental and genetic factors.

Subset	**Pairs**	**Anatomical Distribution**	**Cytokines**	**Function**
Vδ1	Vγ2/3/4/5/8	Epithelial and mucosal sites	IL-4, IL-17, TNF-α, IFN-γ	Infectious diseases and cancer
Vδ2	Vγ9	Peripheral blood	TNF-α, IFN-γ, IL-10, TGF-β	Infectious diseases Phagocytosis and antigen presentation
Vδ3	Vγ2 or Vγ3	Thymus	unknown	Respond to CD1d
Vδ4	Unknown	Peripheral blood	unknown	Response against autologous tumour cells
Vδ5	Vγ4	unknown	unknown	Bind the endothelial protein C receptor
Vδ6	Unknown	Peripheral blood	unknown	Response against the autologous tumour cells
Vδ7	Unknown	Peripheral blood	unknown	Response against the autologous tumour cells
Vδ8	Unknown	Peripheral blood	unknown	Response against the autologous tumour cells

Table 1.3 – Known human γδT cell subsets
Data sourced from the following references (74–76)

1.5.1. The Three main Vδ subtypes of γδT cells

1.5.1.1 The Vδ1 subtype

Despite comprising a minor population within the blood and lymph, γδT cells are significantly enriched within barrier tissues. In particular, γδT cells bearing the Vδ1 receptor comprise 70-90% of the γδT cells within epithelia and are highly present in mucosal surfaces, specifically the intraepithelial lymphocyte (IEL) compartment, with majority residing in the gut, liver and other epithelial tissues (77–79). They are also the most prevalent subtype of γδT cells at birth (80). The Vδ1 chain pairs mainly pairs with Vγ8 or Vγ9 chains (81) . In general, due to their locations, it is thought that Vδ1 play a vital role in immunosurveillance, via viral infections such as CMV as well as through tissue damage repair and stress and are able to recognize cancerous cells (82,83). With regards to ligand specificity, the only two ligands that have been identified for Vδ1 TCR are the CD1c molecules and the MIC self-antigens. No other unique ligands have been identified thus far. Vδ1 T cells can recognize both lipids and glycolipids presented by CD1 molecules (84–86). Recently a direct interaction between Vδ1 TCR with HLA-DR was revealed in CMV-induce γδT cell clones, thus showcasing an MHC-dependent immune response of Vδ1 cells (87).

Most gut-residing Vδ1 cells have a cytotoxic phenotype and express Nkp46. Expression of Nkp46 on Vδ1 T cells is associated with anti-tumour activity, particularly in colorectal cancer and higher frequencies of NKp46^{+} Vδ1 intraepithelial lymphocytes within tumour-free specimens from patients with colorectal cancer patients correlate with a lower risk of developing metastatic III/IV disease stages (88). This data, combined with the fact that their increased frequencies among tumour-infiltrating lymphocytes indicate their importance in tumour immunosurveillance (89). Tumour-reactive Vδ1 T cells can persist in circulation for many years without entering an exhausted or senescent phenotype and are also less susceptible to activation-induced cell death in comparison to other γδT cell subtypes and other T cells. In addition to increased cytotoxicity, specific populations of Vδ1 T cells can also exhibit immunosuppressive and regulatory properties (90). Overall, Vδ1 T cells show enhanced cytotoxic properties over Vδ2 T cells. However, effective harvesting of Vδ1

T cells remains a barrier for studying and harnessing the full therapeutic potential of these uniquely cytotoxic T cells.

1.5.1.2 The Vδ2 subtype

Vγ9δ2 are the prominent γδTCR combination within the Vδ2 subtype family. Vγ9δ2 T cells are known to identify microbe-derived HMBPP and host-derived IPP, showcasing their ability to respond to both self and non-self-stimuli. This interaction occurs through their association with BTN3A1 and BTN3A2 (41,44,91). Like Vδ1 cells, Vδ2 T cells also co-express the functional receptors of innate cells. For instance, despite being first described in Vδ1 T cells, NKG2D on Vδ2 T cells also interacts with MIC-A/ molecules and UL16 binding proteins (ULBP) (92,93). Other similarities with Vδ1 T cells include their activation by HSP (94,95). Moreover, Vδ2 T cells can be further sub-categorised into four populations based upon their expression of CD27 and CD45RA: naïve ($CD27^{+}CD45RA^{+}$), effector memory ($CD27^{-}CD45RA^{-}$), central memory ($CD27^{+}CD45RA^{-}$) and terminally differentiated ($CD27^{-}CD45RA^{+}$) cells. The differentiation towards these subtypes are driven by different stimuli, for example, IL-18 enhances effector memory formation (96,97).

Activated Vδ2 T cells are capable of maturing DCs into cytokine-producing APCs thereby activating αβT cells. Data has shown blood Vγ9δ2 to prime both CD4 and CD8 T cells in response to signals from microbes, as well as cross-present antigens to CD8 T cells (17,27). In comparison to traditional APCs, such as DCs, the endosomal acidification were shown to be significantly delayed in γδT cells (98). Existing evidence showing activated γδT cells' ability to activate CD8 T cell response as well as phagocytose tumour antigens and apoptotic cancer cells lead to the hypothesis that a similar phenomenon exists in response to bacterial stimuli (99). Data by Barisa *et al* showed that the transition from naïve cytokine-producing state to a phagocytic state was accompanied by a loss of cytokine production. However, the cells maintained cytotoxic degranulation and bactericidal activity (100) This suggests that circulating Vδ2 T cells can act as sensors with their immunological functions manipulated via stressors.

1.5.1.3 The Vδ3 subtype

γδT cells comprising of Vδ3 chains, known as the Vδ3 subtype are another population of γδT cells which make up only a minute portion of circulating T cells (0.2%), but like Vδ2 cells, are more present in other sites such as the liver as well as gut epithelium. This unique subset is the least studied of the 3 main human γδT cell subsets and thus limited data has been gathered on their specific properties. Mangan et al have described Vδ3 T cells as glycolipid-reactive T cells with functional similarities to NKT cells (101).

Whilst there is limited data on the impact of this specific subset in the context of health and disease, studies have shown they are particularly highly expressed in patients with leukaemia and chronic viral infections (83,102,103), indicating similar functionality to Vδ2 T cells. Expansion of Vδ3 T cells in response to cytomegalovirus activation has been shown (104). Moreover, expansion of Vδ3 T cells has been noted in the context of B cell lymphocytic leukaemia (102). Vδ3 T cells express the cell surface receptors CD56, CD161, HLA-DR and NKG2D and can be expanded via IL-2 stimulation to recognise and kill CD1d+ cells. Upon activation, Vδ3 T cells can release cytokines typical of Th1, Th2, and Th17 cells as well as induce maturation of DCs into APCs (105)**.**

Similar to Vδ2 T cells, Vδ3 T cells are capable of maturing DCs into cytokine-producing APCs. In addition to this, interaction between Vδ3 T cells and B cells showcased reciprocal expression of CD40, CD86 and HLA-DR as well as promotion of IgM antibody from B cells. The activation of B cells to specifically release IgM, as well as the reciprocated activated state of the Vδ3 T cell, without induction of Th1, 2 or 17 cytokines, displays an interesting and unique relationship between B cells and γδT cells that is different with interaction with the Vδ3 subset (106).

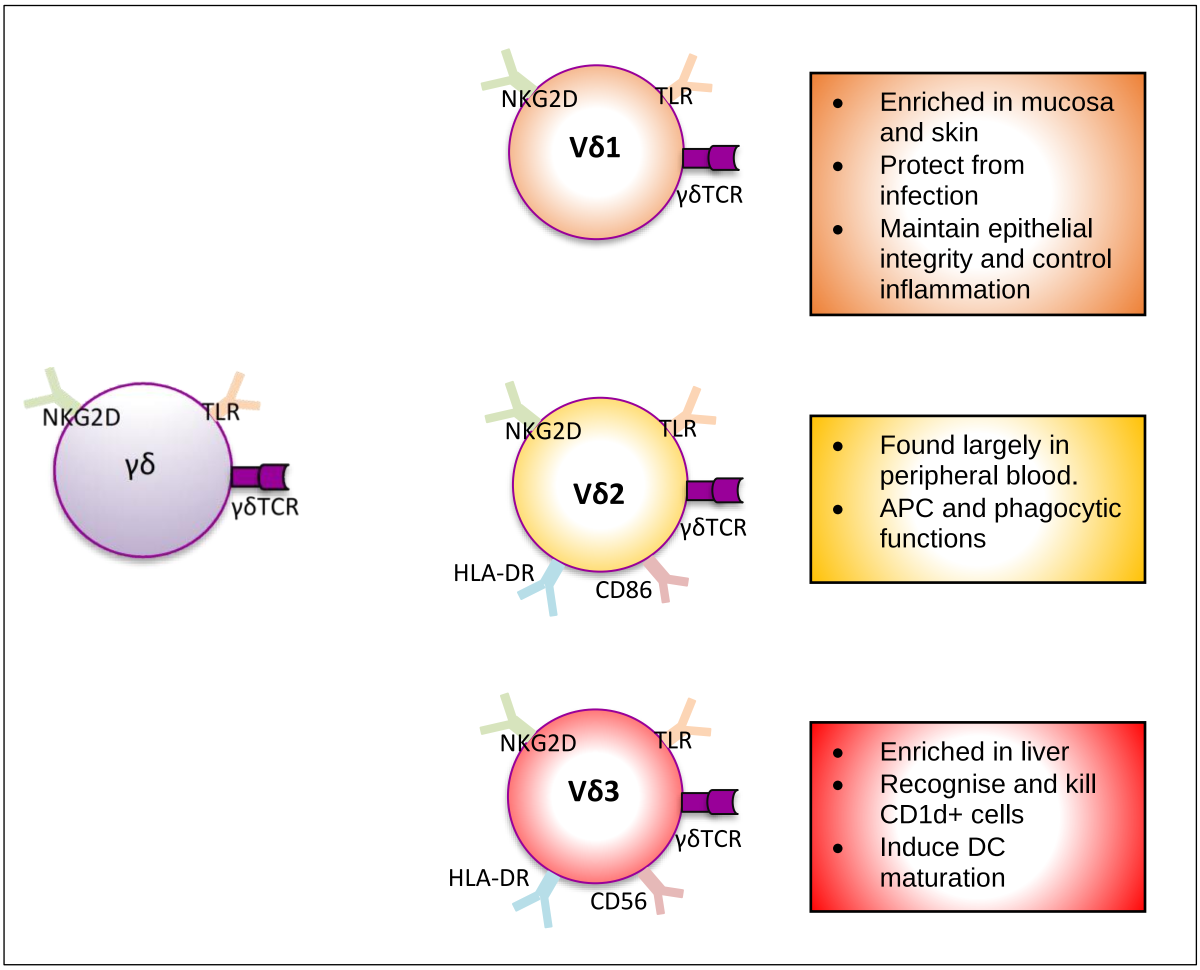

Figure 1.4 – Overview of the different subtypes of γδT cells, their localisation and general functions
Human γδT cells are characterised via the type of delta (δ) chain present in the γδTCR. Three main types of γδT cells exist, Vδ1 (orange), Vδ2 (yellow) and Vδ3 (red). Consistent amongst all γδT cell types (purple), is the presence of PRRs, NKG2D receptors and the characteristic γδTCR, whereas the subtypes possess additional receptors (such as HLA-DR and CD86) and differentiate in combination and ratios of such receptors, which translate to their differences functionally, as indicated in the corresponding text boxes.

1.5.2. Polarization of γδT cells into phenotypes similar to those of αβT cells

Aside from the δ-chain subtype classification, γδT cells also exhibit similar grouping into effector populations similar to those of αβT cells on the basis of their functional capabilities. Like αβT cells, this polarization usually occurs upon activation of the TCR and in response to cytokines, leading to Th1 (γδT1), Th2 (γδT2), Th17 (γδT17) and Treg (γδTregs) populations. (107–109). Among the Vγ9δ2 subtype of the blood which express Th1-type signatures, 50-80% of cells also produce the Th1-type cytokine IFN-γ and express CD27 whereas <5% produce IL-17 and do not express CD27 (109). Recently, a IL-15-producing γδT cell population has also been described and have been shown to induce memory T cells within experimental allergic encephalomyelitis (EAE) mice (110).

1.5.2.1 Th1-like and Th-2 like γδT cells (γδT1 and γδT2)

The early activation of resting γδT cells was characterized by the induction of IFN-γ. Priming under Th1 conditions induced a Th1 profile characterized by increased secretion of IFN-γ and TNF-α, while Th2 conditions caused increased production of IL-4 (Th2 profile) by the γδT cells. These results indicate that the major subset of human γδT cells can be polarized into either Th1 or Th2 cytokine pattern depending on the cytokine milieu in which contact with antigen occurs (107). Interaction of CD70 with CD27 promotes the expansion of Th1-biased Vγ9Vδ2 T cells (111).

1.5.2.2 IL-17-producing γδT cells (γδT17)

Like their αβ Th17 counterparts, γδT17 cells also express nuclear hormone receptor RORγt as a lineage determination transcription factor (112). Majority of the knowledge regarding lineage of γδT17 cells has come from mice studies. These works have shown γδT17 cells to occur as natural IL-17-secreting γδT cells or as induced γδT17 cells, the latter of which start to produce after antigen exposure (113). The non-lymphoid population of γδT17 cells exhibit memory phenotype and belong to CD27− CD45RA± effector or terminally differentiated (TEMRA) subtypes (114,115). Similar observations have been made in the murine γδT17 cell population (116). Data studies have shown γδT17 cells to play a role in the pathogenesis of many conditions where immune dysregulation takes place, including autoimmune disease (117) and cancer (118).

1.5.2.3 γδ T regulatory cells (γδTregs)

Suppressive functions of human γδT cells have been described, thus forming a γδT regulatory cell (γδ Treg) population. This suppressive capability was first described by Patel *et al* (119). Much of the data looking at γδTregs has specifically highlighted the capability of the Vδ2 subtype to transition into γδTregs. Like αβTregs, development of γδTregs occurs through engagement of the γδTCR, with the aid of cytokines such as IL-15 and TGF-β (108). Data has shown a role for vitamin C in supporting the conversion of human γδT cells, even in the absence of TGF-β1. This mechanism is thought to occur through the DNA hypermethylation, leading to epigenetic modification of the factor forkhead box P3 (FoxP3) gene (120). However, there are differences between γδTregs and conventional CD4+ αβTregs; γδTregs exhibit lower levels of CD25 and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and do not always express Foxp3 (121,122). Thus, their Treg-likeness is based on functional suppressive capabilities they possess over other γδT cells and not the markers they express.

1.6. γδT cell interactions with other immune cells

As previously mentioned, γδT cell effector function ranges from defence against pathogen to immunomodulatory, the latter of which is aided by interactions with other immune cells. This crosstalk has highlighted γδT cells as potential for use in adoptive cell therapy. γδT cell interactions with other immune cells remain to be thoroughly investigated and thus have not been well defined.

1.6.1. γδT cell interactions with αβT cells

Most intriguing to discuss is the interaction of γδT cells with their αβT cell counterparts. Various studies have shown the capability of Vδ2 T cells to act as professional APCs to human αβT cells, exhibiting the ability to take up and present antigens in a similar manner to DCs (17,27). This phenomenon has also been described in mouse γδT cells (123). As well as enhancement to αβT cell activity via antigen presentation, competition between γδT cells and other T cells, the CD8+ T cell population has also been reported. Baccala *et al* demonstrated competition of homeostatic cytokine, IL-15 between CD8+ αβT cells and γδT cells to play a role in inhibition of γδT cells proliferation (124), with the opposite effect also occurring. Within lymphopenic settings, γδT cells have been identified to play a role in limiting CD8 T-cell expansion. The competition between these subsets is thought to be mediated by IL-15 being trans-presented on the membrane of $CD11c^{+}$ DCs (125).

However, this competition between γδT cells and CD8 T cells is only one such aspect of the complex interplay between αβT and γδT cells. One study has shown that inactivation or depletion of the Vγ4 T cells in mice lead not only to significantly decreased levels of αβT cells, suggesting a supportive nature of these γδT cells to αβT cells, at least within this γδT cell subset (126).

1.6.2. γδT cell interactions with B cells

Studies using γδT cell-deficient mouse strains have shown the impact of γδT cells in the development and maturation of B cells via lowered levels of transitional, marginal-zone, follicular and aged B cells in the absence of γδT cells. This showcased the capability of γδT cells to regulate specific stages of B cell development and the defective cell compartments was shown to be as a result of the absent $V\gamma4^{+}$ T cells and in particular, their ability to produce IL-4. Interestingly, direct contact of marginal zone B cells with γδT cells seems to be responsible for their decrease (127).

It seems as though γδT cell aid in B cell activation and differentiation in two main ways. Firstly, in a manner identical to αβT cells, γδT cells can interact with B cells through costimulatory pathways such as CD40/CD40-L interaction as well as Inducible T-cell costimulatory ligand ICOS/ICOS-L and CD86/CD28 (33,128). These interactions and the release of cytokines such as IL-2, -4 and -10, lead to the differentiation of B cells into plasma cells capable of antibody secretion. Additionally, γδT cells have been identified to be a source of chemokine ligand CXCL13 (129), which is involved in the migration of B cells into spleen follicles. The second manner in which γδT cells promote B cell activation is through interaction with αβT cells. γδT cell presentation of Wnt ligands to T follicular helper (Tfh) cells aids in Tfh development and function (130,131).

In mice, γδT cells have been shown to regulate IgE production and reactivity (132), with different subsets exhibiting converse effects. For instance, Vγ1 T cells such as the $V\gamma1V\delta5^{+}$ subsets have been shown to enhance IgE responses, whereas $V\gamma4^{+}$ T cells have been shown to have a repressive effect on the αβT cell-dependent antigen-specific IgE responses (133).

1.6.3. γδT cell interactions with NK cells

As previously discussed, γδT cells act in an antibody- and MHC-independent manner similar to NK cells, whilst exerting cytotoxic functions similar to those of αβT cells.

Unlike αβ and γδT cell interactions, there is little data pointing in the direction of any interactivity between γδT cells and NK cells. Whilst limited, available data suggests γδT cells and NK cells regulate and enhance functions of one another. For instance, it has been shown that γδT cells play a crucial role in regulation of NK cell-mediated tumour responses (134). This is supported by data from Maniar *et al*. which showed presence of zoledronic-acid activated γδT cells lead to improved NK cell-mediated tumour cytotoxicity (135). Equally, NK cells have been shown to enhance γδT cell activity. Stimulation from antigens derived from *M. tuberculosis* lead to activated NK cells enhancing γδT cells proliferation (136).

However, the opposite observation was made within *in vivo* conditions, whereby NK cells have been shown to suppress γδT cells expansion in the absence of αβT cells. These differing observations may be due competitive advantages of the cells *in vivo*. Absence of αβT cells *in vivo* would mean an absence of homeostatic competition between αβT cells and γδT cells, leading to higher population of unchecked γδT cells, leading to NK cell adopting the role of αβT cells in regulation of γδT cells. Another explanation for these differing results may be due to observations between different γδT cell subsets which have distinctive phenotypic properties, as discussed within chapter about B cells.

Overall, these data suggest that γδT cells act in an immunomodulatory manner, to both enhance and regulate other immune cell activity, particularly with regards to lymphocytes. The balance and shift of the opposing effects seem to be driven by the presence of particular γδT cell subsets as well as αβT cells, suggesting a complex interplay between these lymphocytes.

1.7. The role of γδT cells in the gut

As discussed earlier, γδT cells are enriched within mucosal tissues and within the gut. Here, these tissue-resident lymphocytes play a role in immune surveillance and regulation of the gut microbiota via performing a protective role of either pro- or anti-inflammatory capabilities in response to barrier damage or translocation of pathogenic bacteria. In addition to this, their general capability to engage and boost crosstalk between immune cells and bridging innate and adaptive immunity provoke immune responses where required. Thus, it is unsurprising that these γδT cells are impacted by chronic inflammation in response to inflammatory conditions.

Maintenance of healthy microbiota balance and general immune regulation is a vital role for the Vδ1 T cells, particularly those that rise in colonic epithelial tissues. These cells rely on instructions from the microbiota for development and capacity for antimicrobial activity (137,138). During microbial dysbiosis, Vδ1 T cells are essential early on and are responsible for triggering protective responses as well as wound healing (63,137). However, if inflammation persists, Vδ1 T cells aid in establishment of chronic inflammatory conditions via recruitment of myeloid-derived suppressor cells and angiogenesis (139–141).

1.7.1. The impact of γδT cells in celiac disease

In celiac disease, data has shown a permanent reconfiguration of the tissue-resident TCR γδ+ intraepithelial lymphocyte (IEL) compartment. Usually, these Vγ4δ1 IEL cells display specificity for BTNL3 and BTNL8 and display innate cytolytic properties. However, in response to gluten-induced inflammation, these cells were depleted and a new IFN-γ producing Vγ4δ1 T cells formed the pool of IELs which displayed reduced specificity to BTNL3/8 (142). This is supported by other existing data which shows increased levels of γδT cells in the IEL compartment of celiac disease patients. Normal levels of these cells were not achieved after commencement of a gluten-free diet. It is suggested that in celiac disease, the IELs are cytotoxic for epithelial cells either via specific recognition of gluten presented by an epithelial cell or via IL-2 released by gluten-reactive αβT cells. This then contributes to much of the epithelial damage and loss of integrity observed in patients (143).

1.7.2. The impact of γδT cells in inflammatory bowel disease (IBD)

Interestingly, CD8αβ+ γδT cells, a novel subset which have been shown to be enriched in the gut, exhibiting cytotoxic features. These unique cells which are mostly Vδ1+ respond to IL-2, produce IFN-γ and TNF-α and express CD69, expression of which aids in T cell activation and stimulation (144). These CD8αβ+ γδT cells have been recently shown to play a protective role via gut homeostasis and mucosal healing. This was showcased via studies by Marsall *et al*, whereby the CD8αβ+ γδT cells were shown to be significantly decreased within inflammatory bowel disease (IBD) patients. However, the cell populations were increased to normal levels in patients who received anti-TNF-α therapy (144).

1.7.3. The impact of γδT cells in gut inflammation and cancer

Thus, in response to a prolonged inflammatory state, these typically protective cells aid in the formation of a pre-cancerous environment. Whilst the IPP released via stressed or cancerous cells can activate Vγ9δ2 T cells to counteract the inflammatory environment (145), the tumour microenvironment can gradually polarise these cells via release of IL-23 into a Th17-like, cancer-promoting phenotype or into γδTregs (146,147). Evidence of these cancer-promoting γδT cell populations' contribution in colorectal cancer has been identified; with frequency of γδT17 and CD39+γδTregs relating to clinical factors such as tumour size (118,148). Additionally, in rectal cancer tissues, patients exhibit higher proportions of Vδ1 T cells compared to Vδ2 T cells when compared with adjacent non-cancerous tissues and the Vδ1 T cells displayed strong inhibitory functions (149). Via similar mechanisms, a link between commensal bacteria, γδT cells and promotion of lung cancer has also been identified (150).

In summary, the delicate balance of pro- and anti-inflammatory environments within the gut is of extreme importance in shaping the γδT cells' phenotype. When overstimulated in response to chronic infection, these protective cells can display harmful cytotoxic effects, promotion of inflammatory states within conditions such as celiac disease and IBD as well as promotion of tumour environments. It is therefore interesting to decipher which microbial or non-microbial ligands may drive this process, and in the context of autoimmune conditions in the gut, whether rectification of these cell compartments can correct disease aetiology.

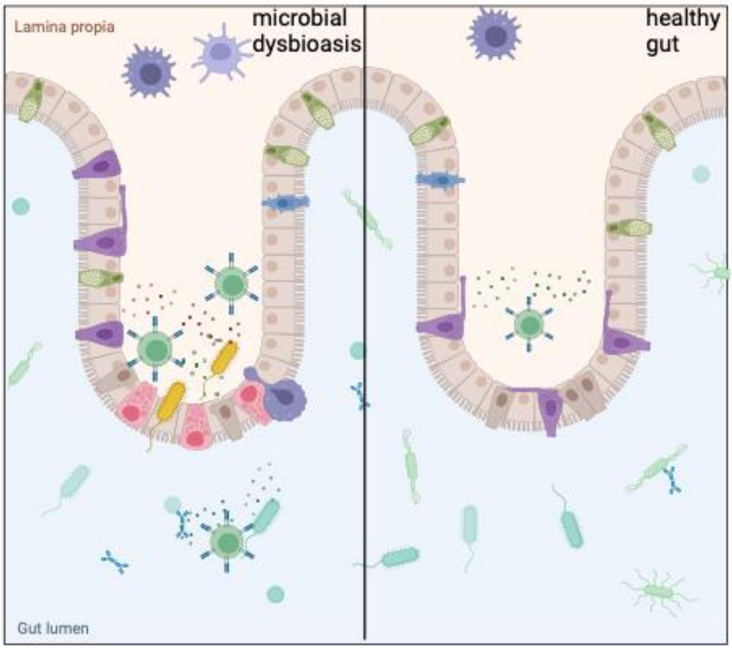

Figure 1.5 – Inflammatory γδT cell reactivity upon gut microbial dysbiosis
The cellular barrier of the gut consists of intestinal epithelial cells (brown cells) interspersed with intraepithelial lymphocytes (purple oblong cells). Microbial dysbiosis (left), occurs via disruption and damage to epithelia, or acquisition of pathogenic bacterium with enhanced tropism to mucosal sites (bright yellow), thereby leading to loss of epithelial integrity and an inflammatory environment (inflamed intraepithelial cells depicted as red) within the gut, via triggering of and release of IL-17 (red dots) from γδT cells (green cells). (Created on biorender.com)

1.8. The impact of γδT cells in the context of infections and their associated diseases

Whilst the importance of γδT cells is now clear, few studies have been able to identify an independent and unique role of γδT cells in the context of infection. This is particularly interesting considering the advantage of γδT cells over αβT cells in not being restricted by MHC with regards to antigen recognition. Moreover, the high levels and distribution of γδT cells found in the epithelia, suggests a role for these cells in the first line of defence against invading pathogens.

Observations have been made of γδT cells role in improving prognosis and playing a protective role in the context of chronic bacterial, viral, and parasitic infections in a variety of ways. For instance, studies have shown that increased IL-17 production from γδT cells to play a protective role in *Mycobacterium tuberculosis* (*M. tuberculosis)* infection (151), which has been supported by a recent study showing similar findings from IL-23-responsive dermal γδT cells in response to leprosy lesions (152). γδT cell-mediated macrophage cytocidal activity has been shown to be present within *Toxoplasma gondii* infection (153) and other studies have shown the importance of γδT cells in responding to human cytomegalovirus (HCMV) (154), and *Listeria monocytogenes* (53,155). Moreover, data has shown expansion of γδT cells in response to *E. coli* as well as *Pseudomonas aeruginosa (P. aeruginosa)* (156). Interestingly, when comparing adult peripheral blood mononuclear cell (PBMC) responses to new-born PBMC responses to *E. coli* and *P. aeruginosa* bacteria, the expanded γδT cells were mainly of Vδ2 subtype in adults and Vδ1 subtype in newborns. However, despite these differences, the presence of γδT cell response in newborn PBMC showcased γδT cells to be vital as innate responder cells, as no prior bacterial stimulation was necessary (156). Thus, taken together the above studies signify a necessary role of γδT cells in the immune system; perhaps as first-responders, as links between the innate and adaptive system, or as amplifiers of response.

In general, as γδT cells interact with other immune cells, it can be inferred that they partake in a vast array of microbe-specific immunity. For instance, rapid HMBPP dependent crosstalk has been identified between Vγ9δ2 T cells and autologous monocytes, leading to up-regulation of inflammatory ligands and production and release of inflammatory cytokines such as IFN-γ, IL-6, TNF-*α* as well as chemokines. Thus, immune recognition can aid monocytes to differentiate into a more inflammatory phenotype and uptake APC-functions, which leads onto knock-on effects of activating αβT cells (157). As well as recruitment of other cells, direct engagement of γδT cells either through the γδTCR or other cell surface receptors leads to the production of bacteriostatic and lytic molecules by the γδT cells such as granulysin and defensins.

1.8.1. The role of γδT cells in *S. aureus* infection

γδT cells can sense the dysregulation of the mevalonate pathway in response to *S. aureus* infection, leading to proliferation and activation of γδT cells to produce inflammatory cytokines and mediators (158,159). As such, γδT cell-deficient mice have been shown to have impaired bacterial clearance, recruitment of neutrophil and lower levels of IL-6 (158) This is supported by data showing a role of γδT cells in protection against *S. aureus*-induced pneumonia (160).

Whilst many murine studies have elucidated a role for γδT cells in staphylococcus-mediated immunity, few evidence has been gathered with regards to human studies. However, a study by Cooper *et al* showed capacity for human Vδ2 T cells to undergo rapid activation in response to *S. aureus*-infected monocyte-derived DCs. In this study, Vδ2 T cells were used, which are the predominant subtype of the peripheral blood and were shown to upregulate CD69 and produce high levels of IFN-γ upon exposure to the *S. aureus*-infected monocyte-derived DCs. This phenomenon occurred via direct contact with the DCs and secretion of IL-12. Interestingly, the IFN-γ produced by Vδ2 T cells further enhanced the secretion of IL-12, creating a positive feedback loop. Thus, the cytotoxic activity is enhanced indirectly by microbial ligands via DCs. This thereby acts to further promote inflammation towards the DC itself, via IL-12 secretion as well as increased expression of costimulatory molecules CD86 and chemokine

CCR7, which allow for homing to the lymph nodes and subsequent activation of αβT cells (161).

1.8.2. The role of γδT cells in *E. coli* infection

In addition to protection against *S. aureus*, data has also shown a role for γδT cells in the host immune response against *E. coli*, typically driven by accumulation of phosphoantigens from the deoxyxylulose 5-phophate pathway in bacterial metabolism (162). Moreover, data by Barisa *et al* has found that *E. coli* promotes human γδT cell transition from a naïve cytokine- producing state to a phagocytic state. Interestingly, the development of this γδ APC phenotype was accompanied by a loss of cytokine production, however, cells maintained cytotoxic degranulation and bactericidal activity (100). Moreover, it has previously described that within mice, IL-17A produced by Vγ6δ1 T cells aids neutrophil migration after *E. coli* infection (163). In contrast to Vγ4δ1 T cells, which also produce IL-17A, the proliferation of these cells occurs in an IL-23-independent but γδTCR-dependent manner. This therefore shows how subtype specificity with regards to immunity to specific pathogens, *i.e. E. coli*, within mice (164). These findings were supported by Takano et al who also described an important role for Vγ9δ1 T cells in host defence against *E. coli* infection. Studies conducted on mice showcased that depletion of γδT cells exhibited impaired protection from *E. coli* and administration of anti-IL-15 mAbs inhibited the appearance of γδT cells (165). Taken together, these findings indicate an important role for LPS-stimulated γδT cells in the host defence against *E. coli* infection, with specific reference to the Vγ9δ1 subset and suggest that IL-15, but not IL-23 could be involved in the protection via promoting γδT cell proliferation.

1.8.3. The role of γδT cells in *M. tuberculosis* infection

Expanding on the role of γδT cells within bacterial responses further, we can concur a vital role for γδT cells. For instance, it is thought that human γδT cell play an important role within early stages of infection of intracellular bacteria like mycobacterium and salmonella, with γδT cells playing an active role in the immune response against *M. tuberculosis.* This is exemplified by both mice and human studies. Mice studies have shown expansion of γδT cells upon exposure to mycobacterial ligands whilst γδT cells expressing reactivity to mycobacterial antigens have been identified in humans (166).

Studies have shown that mycobacterium-infected monocytes are potent inducers of γδT cell expansion and they are therefore primarily expressed in and around maturing granulomas and secrete high levels of IFN-γ and monocyte chemoattractant protein (MCP-1) (151,167,168). Tsukaguchi *et al* described a difference in the range of cytosolic antigens recognised by CD4+ T cells and γδT cells, with the latter reacting to a more restricted pattern and dominance for recognising antigens of 10-15 kDa. Whilst both T cell subsets were equally cytotoxic for *M. tuberculosis*-infected monocytes, comparison of ELISA and ELISPOT data suggested that whilst the amount of IFN-γ produced by the two subsets were comparable, the frequency of IFN-γ producing γδT cells was approximately 50% that of CD4+ T cells. This suggests that γδT cells were more potent producers of IFN-γ. However, CD4+ T cells produced more IL-2 and as a result, γδT cells exhibited less proliferation (169). Thus, in the context of *M. tuberculosis* infection, it is possible that they aid in the preferential skewing of Th1-type response. Especially within the initial stage of infection, by responding to a wider range of antigens, exhibiting similar effector functions to boost the CD4+ T cell population response. This is supported by the fact that IL-10 presence has been shown to strongly inhibit the activation of Vγ9 T cells within PBMCs (170).

1.8.4. The role of γδT cells in Human Immunodeficiency Virus (HIV) infection

Studies have also shown a role for γδT cells in the context of HIV infection. This is displayed by the drastic depletion of Vγ9δ2 T cells in HIV-infected individuals, coupled with the imbalance of Vδ1:Vδ2 ratio in peripheral blood (171). Since CD4 expression only exists in a small population of circulating Vδ2 T cells, it is unknown how the mechanism of depletion occurs. Viral load of infected individuals has been shown to correlate to the degree of this depletion (172). Due to the general depletion of T cells among HIV-infected individuals, gut dysbiosis occurs, leading to activation of Vδ1 T cells to exacerbate a chronic inflammatory environment. Elite controllers of HIV infection display elevated levels of Vδ1 T cells in the gut mucosa which correlates with viral load mucosa (173). On a functional level, HIV infection has been shown to impair many effects, including their APC functions (174). As γδT cells act to enhance other immune cell functions, it is thus reasonable to suggest that early depletion of Vγ9δ2 T cells acts as a vantage point for HIV dissemination by impacting activity of other immune cells and altering the cytokine milieu.

Thus, γδT cells display themselves as a subset of T cells involved in immune defence against chronic diseases and where drugs fail due to increased drug tolerance and resistance, manipulation of these unique, innately adaptive cells display a prospective tool for novel treatment options.

1.9. The impact of γδT cells in health and non-infectious diseases

In addition to playing a role in the context of infections via invading pathogens, γδT cells also play an immunoregulatory role. Much of the implications of γδT cells in the context of non-infectious diseases focuses of IL17-producing γδT cells (γδT17), as IL-17 drives inflammation, which if left unchecked, drives much of the pathology of many autoimmune conditions.

1.9.1. γδT cells in pregnancy

Pregnancy can be seen as an immunological paradox, whereby the foetus must be accepted by the maternal immune system despite consisting of foreign cells. In most cases, interactions with foreign stimuli leads to a cytotoxic response in immune cells. However, during pregnancy, the opposite effect is required. Much data has shown an important role for NK cells to maintain this effect and, CD56, also known as neural cell adhesion molecule (NCAM), for the maintenance of pregnancy.

With almost 50% of CD3+ CD56+ cells consisting of γδT cells, it is therefore not surprising that γδT cells also play an important role in pregnancy. Especially when considering the data that suggests that γδT cells play an active role in the tolerance of paternal antigens within pregnancy (23,175–177). This CD56+ γδT cell population is higher in pregnant women compared to non-pregnant women and expands in the 2nd and 3rd trimesters (24). Moreover, whilst some γδT cells have been shown to express CD4, the CD56+ γδT cells are CD4- with some being CD8- and some being CD8+. Conversely the CD4+ γδT cells present in pregnancy are CD56-. These cells showed high cytotoxic potential through expression of CD107a whilst also continuously showing a higher prevalence of PD-1 expression, in a correlated manner (24). Like engagement of inhibitory NK cell receptors, it is thought that these PD-1-expressing γδT cells become inhibited after binding of their ligands in the placenta. This is supported by the fact that levels of PD-1 expression fell after the 1st trimester, where rates of foetal rejection and pregnancy failure is at its highest.

To sum, the decidual γδTCR repertoire and, surface receptor expression and cytotoxicity changes dynamically during pregnancy. Interestingly, decidual γδT cells express less perforin than blood γδT cells but higher levels of granzyme. This was paired with reduced levels of proinflammatory cytokines IFN-γ and TNF-α in early pregnancy compared to end-of-term pregnancy (178).

1.9.2. γδT cells within the central nervous system (CNS)

As previously discussed, a population of γδT cells have been shown to co-express αβ and γδTCRs (179,180). Studies have shown a link between these unique cells and inflammation within the central nervous system (CNS), whereby they protect against infection via triggering of encephalitogenic Th17 cells. This is supported by data which showcases γδT cell implication within inflammatory diseases of the CNS such as multiple sclerosis (MS) as well as neuroinflammation associated with spinal cord injury (SCI) (181,182).

The ability of γδT cells to produce IL-17 in the absence of activated αβT cells is a vital factor for CNS inflammation. Data has shown that IL-17 to play a crucial role for CNS inflammation, particularly in the context of ischaemic brain injury, and that γδT cells are a potent producer of this cytokine (116,183,184). High levels of γδT cells have been observed in both mice and humans: in the peripheral blood and cerebrospinal fluid of MS patients as well as the brains of mice with EAE (184–187). A specific role of IFN-γ-producing γδT17 cells (188), as well as increased frequencies of CD161^{hi} and CCR6+ γδT cells in MS patients with relapse has also been observed (189). In mice, Vγ4 T cells have been identified as the major γδT17 cells (190). A majority of studies on γδT cells within the CNS thus far have focussed on mouse models and it is important to consider that γδT cell properties of the mouse are not always directly relatable to humans.

γδT cells can be activated by the HSPs; HSP60 and HSP90 which are overexpressed in MS plaques and colocalization of HSP with γδT cells has been observed as well as HSP-mediated proliferation of γδT cells (191), suggesting a role for these antigens in relation to γδT cell involvement within MS pathogenesis. Data has shown a role for γδT cells in a protective manner against CNS bacterial infections. It thus may be that the usual protective effects of γδT17 cells within the CNS (192), when exacerbated or overstimulated, can lead to pathogenesis indicative of autoimmune conditions.

1.9.3. γδT cells in spondyloarthritis and associated conditions

Findings have demonstrated a role for γδT cells in the pathogenesis of spondyloarthirtis (SpA). SpA describes inflammatory diseases of the rheumatoid compartment which specifically effect the axial skeleton and presents as inflammation and new bone formation at connecting tendons and ligaments. Genomic studies have linked SpA to the IL-17/IL-23 axis. The impact of γδT cells in SpA is an observation made in both humans and in mice. Various mouse models have shown increased levels of γδT cells and IL-17-producing γδT cells in inflamed tissues (193–195). In addition to this, a direct association of γδT cells and IL-17 secretion in human SpA was observed by Kenna *et al* and has been shown to be in-part driven by IL-9-mediated expansion of these γδT17 cells, specifically within the synovial fluid (196,197). IL-23R-expressing IL-17-producing γδT cells were enriched in the blood of ankylosing spondylitis patients (197). Interestingly, this observation was not present in rheumatoid arthritis patients, suggesting IL-17 involvement in the spondylitis pathogenesis rather than arthritic inflammation. Considering the fact that many SpA patients exhibit extra-articular manifestations such as IBD, it can be proposed that initial inflammation driving the γδT17 cells could exacerbate inflammatory conditions of the gut, leading to microbial dysbiosis and alteration of the γδT cell populations within the gut (198), as described in other conditions (149,171)

1.9.4. γδT cells in asthma

In further relation to inflammatory conditions perpetuated by γδT cells, few inflammatory conditions point to lower levels of γδT cells within the condition. Studies have investigated the link between asthma and γδT cells have shown lower levels of γδT cells in asthmatic patients. However, there has been discrepancy and contrasting results in terms of γδT cell levels observed between asthmatic patients and healthy controls; with some studies indicating no difference (199,200) and others showing lower levels of γδT cells in the blood (201–203).

Decreased levels of γδT cells in peripheral blood have been supported by studies observed in atopic patients under 30, particularly of CD8+ γδT cells as well as in older asthmatic patients (204,205) Despite decreased levels of γδT cells observed in the blood, Hamzaoui *et al* observed increased levels of activation markers, such as CD25 on γδT cells from sputum of asthmatic patients, as well as increased FasL expression and cytotoxicity (206).

1.10. The role of γδT cells in cancer biology

Despite the potential for γδT cells to promote the tumour environment in response to gut microbiota dysbiosis and inflammatory disease conditions, as covered in previous sections, there is also clear evidence for protective effects of γδT cells in response to cancerous cells. A meta-analysis of studies which covered 18,000 human tumours, consisting of 25 different types has shown a correlation between γδT cells and positive outcomes. This link was present in both pan-cancer and solid tumour analyses (207).

γδT cell reactivity in cancer is both due to general cytotoxicity of T cells, as well as specific features of the γδTCR. For instance, CD56+ γδT cells have shown enhanced IFN-γ production and display increased cytotoxicity against tumours (208,209). Moreover, data has shown that Vδ1 T cells are able to persist in circulation, providing a long-lived immune response and showcasing an important immunoregulatory role. This is supported by studies which have demonstrated the cytotoxic activity of these cells in a variety of cancers such as lymphoid and myeloid malignancies, lung, colon and pancreatic cancer as well as neuroblastoma (210–212). Moreover, recent findings have shown enhanced antitumour effects by stimulation of tissue-resident γδT cells from the liver, indicating additional benefit in stimulation of localised as well as circulating γδT cell subsets (213).

γδ TILs consisting predominantly of the Vδ1 subtype are found in high levels in patients with colorectal cancer and have been associated with early controlling of the tumour. Furthermore, transcriptomic analyses revealed that patients with abundant γδT cells displayed significantly longer 5-year disease-free survival rates (214). This is supported by additional transcriptomic analysis which shows higher levels of γδT cells to be positively associated with lower progression to severe clinical stages and improved overall survival in patients with head and neck squamous cell carcinoma (215). It is thought that this interaction was mediated by high expressions of BTN molecules. There was also a positive correlation between the abundance of γδT cells and αβ CD4+ or CD8+ T cell infiltration, accompanied by increased levels of T cell activation, proliferation, and effector functions such as chemokine and cytokine production and general cytotoxicity state of cells (215). This is supported by data by Wang *et al* which displays γδT cell infiltration as an independent prognostic factor in patients with gastric cancer. It is suggested that increased levels of γδT cell infiltration in patients with TNM II and III stage disease is a predictive prognostic factor of survival benefit from adjuvant chemotherapy treatment. Thus, this study rings to light the possibility of using γδT cell infiltration alongside disease staging systems to predict efficacy of chemotherapy treatment (216).

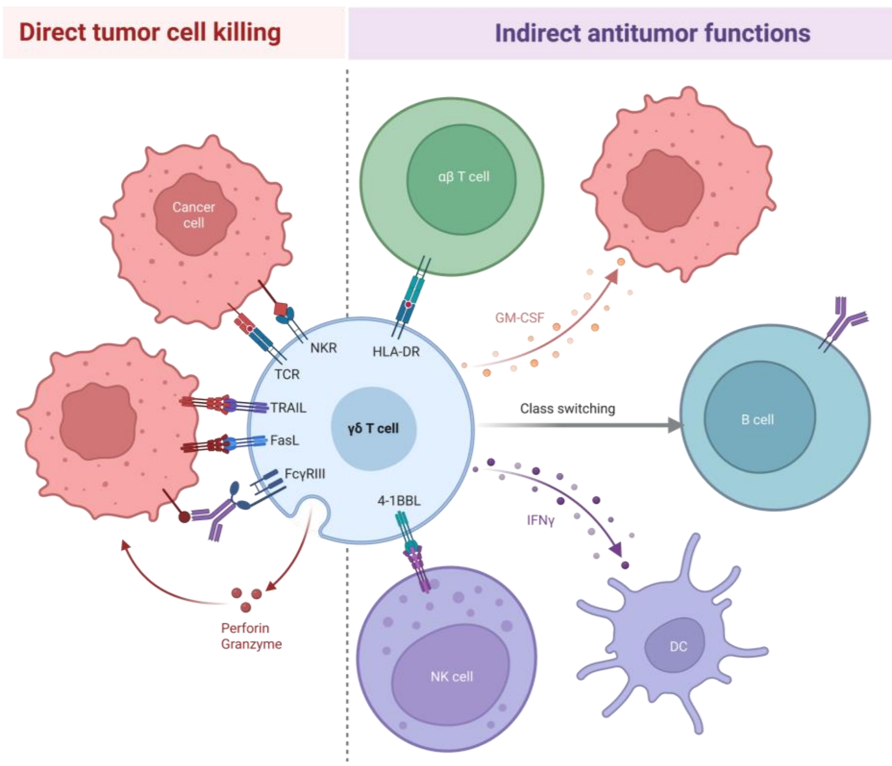

Figure 1.6 – Antitumour activity of γδT cells
γδT cells exhibit vast amounts of antitumour functions via direct (receptor recognition of cancer cell/stress ligands) and indirect mechanisms (influence of other immune cells: αβT cells, NK cells and B cells) (Created on biorender.com)

1.11. The therapeutic potential of γδT cells

1.11.1. Chimeric antigen receptor (CAR) T cells

Cancer immunotherapy aims to combine our knowledge of the human immune system with the available and capable components of the host's immune system to eradicate cancerous cells and tumours. Chimeric antigen receptor (CAR) T cell therapy is a form of cell-based gene therapy within cancer treatment that utilises T cells by manipulating them via insertion of gene constructs to express additional receptors. Most CAR T cell therapy is customised for each patient, via collection of T cells from the patient blood. The chimeric antigen receptor placed on the CAR T cell is thus specific for an antigen expressed on the target tumour cells and consists of external (binding) and internal (signalling) segments. Upon antigen-binding on the external segment of the receptor, the internal segment of the receptor then transmits signals into the cell and leads to cell activation and effector function. This labour-intensive, personalised therapeutic option is currently considered for patients not responding to multiple forms and phases of chemotherapy and other treatments or have relapsed. However, a few clinical trials have been initiated for administration of CAR T cells in children with ALL more early on.

The first CAR T cell, tisagenlecleucel, also known as Kymriah is a CAR T construct directed against CD19 and was approved by the FDA in 2017 for treatment of acute lymphoblastic leukaemia (ALL) in children. It has also been used to treat adults with relapsed or refractory non-Hodgkin's lymphoma (NHL). Other CD19 CAR T cells include Tecartus and Yescarta for use in adults with relapsed or refractory mantle cell lymphoma or acute B cell lymphoblastic leukaemia and follicular lymphoma or NHL respectively. Two other CAR T cells have been developed against the BCMA antigen, Abecma and Carvykti, for use in adults with relapsed or refractory multiple myeloma.

Over the past few decades, great advancements have been made in the undertaking of this more personalised approach to medicine. However, a vast majority of these achievements have been made using the more common αβT cell subset. Whilst these cells provide ease of access to starting material, they are restricted in performance by their dependency on MHC molecules for presentation of peptide and subsequent activation. One of the first hallmarks of a cancerous cell is to down-regulate HLA molecules and thus this is one of many obstacles with regards to usage of αβT cells for cancer immunotherapy. Recognition of mutated peptides on MHC via αβT cells is short-lived via mutation of cancer cells allowing for tumour escape and failed clearance. This has therefore primed the journey of manipulation and enhancement of existing αβT cells and/or the seeking of alternative cell treatments for cancer therapy (217). Whilst autologous αβ CAR T cell therapies have displayed significant clinical benefit in many hematologic malignancies, their efficacy within solid tumours remains limited. This largely due to tumour heterogeneity (variation in target antigens) and the impact of the immunosuppressive tumour microenvironments.

1.11.2. γδ CAR T cells

Thus, recent renewed interest into the use of γδT cells has grown, as these cells avoid the traditional challenges presented by αβT cells. γδT cell's innate and adaptive-like qualities, as well as their capabilities to crosstalk to other immune cells have made the cells promising candidates in cancer immunotherapy. In addition to this, γδT cells have often presented themselves as a more potent counterpart to the αβT cells, thereby exhibiting the idea that these cells may be able to surpass the immunosuppressive tumour microenvironment better than αβT cells. Despite interest only peaking in recent years, evidence of γδT cell antitumour effects were discovered over 20 years ago by Girardi *et al* in mice (218) . This study showed γδT cells were directly found to prevent the development of squamous cell carcinoma.

The unique capability of γδT cells to act as cytotoxic killers without being reliant on MHC, thus bypassing tumour escape based on MHC class I downregulation, as well as their dual capacity as antigen presenting cells, makes them a putative tool for CAR therapy against tumours. A study by Rozenbaum *et al* showed effectivity of γδ CAR T cells against killing CD19-expressing cell lines *in vitro* and *in vivo*. The CAR T cells engaged in effector functions consisting of cytokine production, target cell killing and clearance of bone marrow leukemic cells (219). Within the mouse model used, priming the mice with zoledronate lead to an enhanced reduction of tumours. Interestingly, in contrast to αβ CAR T cells, these γδ CAR T cells were also able to target the CD19-negative leukemic cells. This therefore shows an advance of γδ CAR T cells over αβ CAR T cells in overcoming the obstacle of tumour escape (219). Modified γδT cells are now being engineered to express chimeric antigen receptors and are currently being assessed within clinical phase 1 trials as allogeneic candidates for the treatment of various forms of malignancies including leukaemia, lymphomas, and solid tumours (220,221).

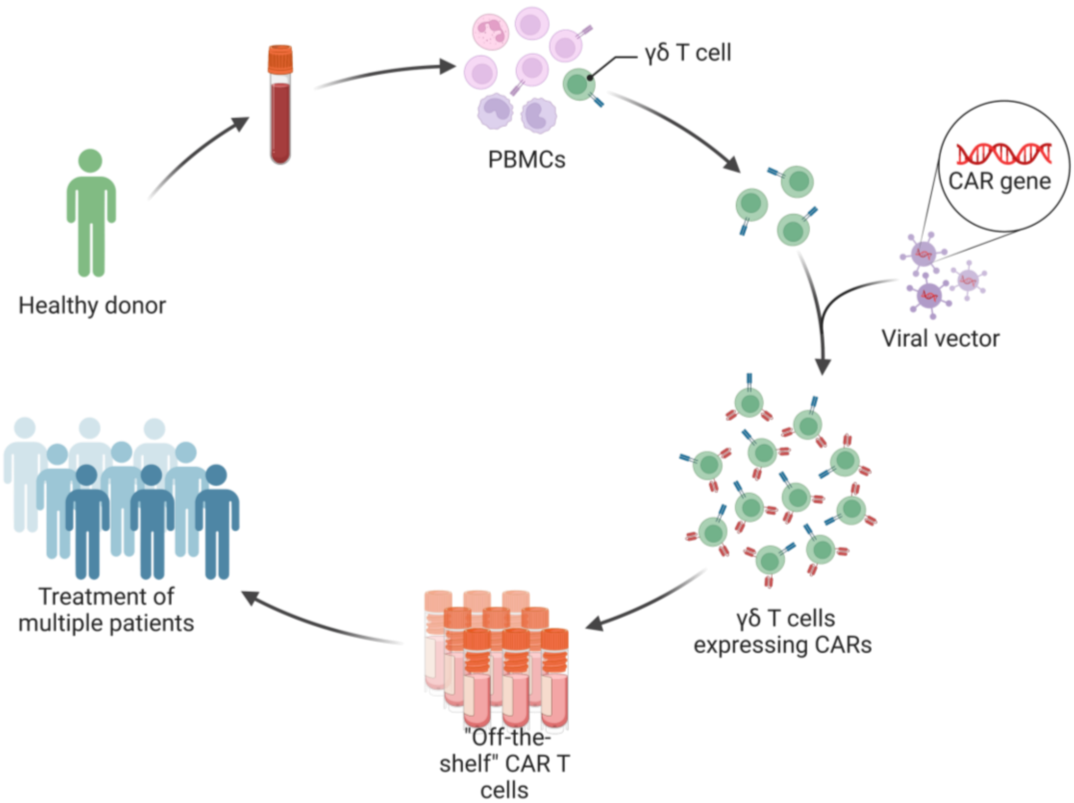

Figure 1.7 – Utilisation of γδT cells in the manufacturing and administration of CAR T cell therapy
γδT cells are harnessed from PBMCs of healthy donors and genetically modified to express specific chimeric receptors to antigen expressed on target cancer cells. The resulting CAR T cells can used for treatment of many patients. (Created on biorender.com)

1.11.3. Harnessing Vδ1 T cells for treatment

Tumour-reactive Vδ1 T cells can persist in circulation for many years without entering an exhausted or senescent phenotype and are also less susceptible to activation-induced cell death in comparison to other γδT cell subtypes and other T cells. Despite this, current cancer immunotherapies primarily focus on and utilise the Vγ9Vδ2 subtype. This subtype is most commonly present within the blood and thus proves as an easier subtype to harvest, study and manipulate for therapeutic use compared to Vδ1 subtypes, despite the latter being more cytotoxic. Utilisation of Vδ2 T cells in clinical trials for treatment of colorectal cancer have not been as promising as hoped and is an example of how more encouraging results could be achieved through utilisation of Vδ1 cells (222,223). In addition to increased cytotoxicity, specific populations of Vδ1 T cells can also exhibit immunosuppressive and regulatory properties, another function which could be exploited for therapeutic purposes.

Effective harvesting of Vδ1 T cells remains a barrier for studying and harnessing the full therapeutic potential of these uniquely cytotoxic T cells. However, a few number of studies have aimed to overcome this barrier. The use of glypican-3, an oncofoetal protein which is highly expressed in various solid tumours for use of development of Vδ1 CAR T cells has been identified as a promising avenue for therapy against hepatocellular carcinoma (224). In this study, Vδ1 T cells were expanded from PBMCs and genetically modified to express a CAR against GPC-3 and encoded a s-IL15. These γδ CAR T cells showed strong proliferation and robust in vitro antitumor activity against GPC-3-expressing tumour cell lines. Overall they showcased long-term cytotoxicity and increased levels of proliferation, indicating them as a promising candidate for therapy (224). The Vδ1 subset is preferentially localized in peripheral tissue, existing in more abundance in comparison to peripheral blood. Thus, engineering these subsets with CARs to augment intrinsic antitumor activity represents an attractive approach in comparison to the Vδ2 subtype and αβT cells.

γδT cells display potent antileukemia activity and perform their functions despite a lack of MHC-recognition (in stark contrast to αβT cells). Thus, these cells should be considered in the context of successful clinical outcome of high-risk acute leukaemia patients undergoing HLA-haploidentical haematopoietic stem cell transplant that may be depleted of TCR αβ T/CD19+ B lymphocytes (225,226). In addition to this, allogeneic CAR T cell therapy utilising γδT cells rather than αβT cells, may overcome the hurdles faced such as graft-versus-host disease, specifically in relation to the cytotoxic Vδ1 T cells located in the peripheral tissues (227).

Increasingly, immunotherapy has been utilised to treat human diseases, especially in the context of cancers. However, with the rise of antibiotic resistance, manipulation of the immune system to harness potent and optimal action against infections remains a putative potential for therapy. Data discussed in this chapter has covered the therapeutic use of γδT cells in cancer therapy but considering the established role of these cells in the context of infection, it is important to explore how effects of γδT cells can be primed or enhanced for treatment against infection, as well as in cross-reactive anti-cancer effects.

1.12. Study Aims

Having only been discovered 30 years ago, knowledge within the γδT cell field has expanded vastly in a short amount of time. However, data within the field still draws short in comparison the vast specifics available on αβT cells. One aspect of γδT cell biology that is left to be properly understood is the antigen recognition mechanisms involved at a molecular level. Much evidence exists to exhibit the TCR-dependent manner in which γδT cells interact with antigen. However, this is coupled with evidence of non-TCR-dependent antigen interactions and thus, raises many questions on the exact ligands involved in γδT cell stimulation and their varying levels of importance.

Our group have found that *E. coli* promotes human γδT cell transition from a naïve cytokine-producing state to a phagocytic state. Interestingly, the development of this γδ APC phenotype was accompanied by a loss of cytokine production, however, cells maintained cytotoxic degranulation and bactericidal activity (228). What is left to be answered is whether freshly isolated, day 0, non-expanded γδT cells can also display this potent antimicrobial activity in the context of a direct γδT cell: bacteria killing assay.

We therefore hypothesised that 14-day expanded γδT cells displayed an enhanced potency to kill bacteria in comparison to fresh γδT cells. To test this, we investigated antibacterial activity against both *E. coli* and *S. aureus*, exemplifying both a Gram-negative and a Gram-positive bacterium. γδT cells have been shown to play a role in immune response against both bacteria. In *S. aureus* infection, γδT cell-deficient mice were shown to have impaired bacterial clearance and recruitment of neutrophils, suggesting a role of γδT cells in protection against *S. aureus*-induced pneumonia (160). In *E. coli* infection, accumulation of phosphoantigens from the deoxyxylulose 5-phophate pathway in bacterial metabolism drive γδT cell immune response (162).

Through the identification of receptor interaction and enhanced cell-signalling pathways, our findings will help to elucidate mechanisms whereby γδT cells functionality against bacteria can be manipulated and enhanced.

2. General methods and materials

2.1. Bacterial Cell Culture

2.1.1. Maintenance of *E. coli* and *S. aureus* culture

All experiments were conducted using the category 2 lab-strain of *E. coli*, DH5α or *S. aureus* NCTC 6571 obtained from cryogenically preserved aliquots and performed in a class II biosafety hood. Bacteria was grown on LB agar plates which were restreaked every 2 weeks, *i.e*., a sterile loop was used to pick up a single colony and streak it onto a sterile LB agar plate, with new sterile loops being used for every streak. The newly streaked LB agar was then kept at 37°C 5% CO_2 overnight with expression of colonies the following day. The bacterial plates stored at 4ºC until further use, or until 14 days, where the plates were streaked again.

2.1.2. Growth of *E. coli* and *S. aureus* cultures

Using a sterile loop, bacterial culture was streaked from the appropriate bacterial stock onto an LB-agar plate. The plate was incubated overnight at 37°C 5% CO_2. The next day, a single colony from the agar plate was inoculated with 6ml of LB broth and left in a shaking incubator to grow overnight at 220 RPM, 37°C. This allows for growth of young exponential bacterial cells.

2.2. Cell isolations and expansions

2.2.1. Ethical considerations

All experiments using peripheral blood-derived cells in this thesis were approved by the UCL Research Ethics Committee and were performed according to relevant guidelines and regulations. Whole blood or buffy coat was retrieved from consented healthy and willing donors. Donor blood and its derived materials, in this case PBMC, were used for the experiments stated within this thesis, with cells being disposed of after use.

2.2.2. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from blood

Blood was obtained and PBMC isolations were performed, on the day of collection from the subject. Sources of healthy donor-derived blood:

- For PBMC isolation for use of expansions only, 50ml of blood was sourced from readily available healthy donors.
- For PBMC isolations for use of testing freshly isolated γδT cells and comparable expanded γδT cells, whole blood was either purchased from:
    1) Cambridge Bioscience (UK) in 500ml citrate phosphate dextrose-coated bags

       or
    2) 50ml of buffy coats was purchased from NHS Blood and Transplant services.

  The purchase of primary material from third parties enclosed participant consent and all associated ethical considerations and responsibilities.

2.2.2.1 PBMC isolation from 50ml of donor blood

Blood was taken from healthy and willing donors and PBMCs were isolated through ficoll-paque^{TM} PLUS centrifugation (GE Healthcare). Blood was diluted with RPMI-

1640 (Gibco) at a 1:1 ratio and carefully layered across falcon tubes filled with 15ml of ficoll before being centrifuged at 800g for 25 minutes, with acceleration of 5, and deceleration of 3, at RT. The PBMC layer was removed using a pasteur pipette into a clean 50ml falcon tube and topped up to 50ml with media before being centrifuged again at 600g for 10 minutes break high, acceleration high at RT. The supernatant was discarded and cell pellet was resuspended in 10ml of medium for counting.

2.2.2.2 PBMC isolation from 500ml of donor blood or buffy coats

Blood or buffy coats were diluted with RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (pen/strep, Gibco) at a 1:1 or 1:3 ratio (blood or buffy coats respectively). 30ml of diluted blood or buffy coat was then carefully layered onto each SepMate^{TM} tubes containing 15ml of Lymphoprep^{TM} density gradient medium (Stemcell Technologies), before being centrifuged at 1200g for 15 minutes with the breaks off at RT. The PBMC layer was removed using a pasteur pipette into a clean 50ml falcon (with PBMCs from a maximum of 2 lymphoprep tubes being transferred into 1 falcon tube) and topped up to 50ml with media before being centrifuged at 500g for 10 minutes, the supernatant was discarded and cell pellet was resuspended before being topped up with medium and centrifuged at 400g for 10 minutes. Supernatant was discarded and falcons pooled together for a final centrifugation at 150g for 10 minutes with breaks off. The supernatant was discarded and cell pellet was resuspended in 10ml of medium for counting.

2.2.3. Cell counting

Cells were counted using a haemocytometer via trypan blue staining (for donor blood of 50ml volume) or otherwise counted using the NC-3000 automated cell counter.

2.2.4. γδT cells expansions from PBMC

2.2.4.1 IL-2 preparation

Lyophilized stock of IL-2 was purchased from Miltenyi Biotec (UK) and reconstituted using 100µL distilled water (Gibco) and stored at -20ºC. Further dilutions were made using PBS and 0.1% BSA.

2.2.4.2 γδT cells expansion

PBMCs were plated in Nunclon™ 24-well plates (Thermo Fisher) at a concentration of 1.5M/ml with RPMI 1640 + GlutaMAX™ media (Gibco) supplemented with 10% FCS and 1% pen/strep (Gibco). Cells were stimulated with 100IU of IL-2 (Miltenyi Biotec) and either 5µM/ml of Zoledronic acid monohydrate (Sigma-Aldrich) for zoledronate expansions or no stimuli. Cells were restimulated with IL-2 and media was changed every two or three days.

2.3. Flow Cytometry Cell Staining

2.3.1. Cell Preparation

Cells were harvested from culture and counted using a haemocytometer before being washed with staining buffer (PBS (Gibco) with 1% FBS and 2mM EDTA (Invitrogen)) in a 15mL falcon tube at 400g brake 9/9 for 5 minutes at 4°C. Cells were then transferred to a 96-well plate, $1x10^7$ cells/ml were resuspended in staining buffer and 100µL added to each well. Wells were topped up with another 100µL staining buffer for washing again at 400g brake 9/9 for 5 minutes at 4°C. Supernatant was removed from the wells by flicking of the plate. After cells were washed and supernatant was removed, 5µL of FC block (BD Biosciences) was added per well, for 10 minutes at room temperature (RT).

2.3.2. Flow Cytometry staining

Cells were then stained with the following antibodies in the anchor marker panel as well as additional antibodies (mentioned where appropriate, in chapter-specific methods sections):

Panel	Antibody marker	Fluorochrome	Clone	Manufacturer
Anchor marker panel	CD3	BV711	UCHT1	Biolegend
	αβTCR	PE	IP26	Biolegend
	Vδ1	FITC	B6	Biolegend
	Vδ2	PerCP	TS8.2	Thermo Fisher
	Blue live/dead	-	-	Thermo Fisher
	CD86	AF700	L243	Biolegend

Table 2.1 - Antibody markers used for γδT cell classification

Antibodies were added to the cells and incubated for 30 minutes at 4°C in the dark before being washed twice at 400g for 5 minutes. Cells were resuspended in 200µl of staining buffer before being transferred to appropriately labelled FACS tubes.

Gating of cell populations was determined using a combination of use of FMOs and isotype controls. Table of isotype controls used throughout flow cytometry staining are detailed in the appropriate chapter-specific method sections. A representative of isotype control staining is shown below.

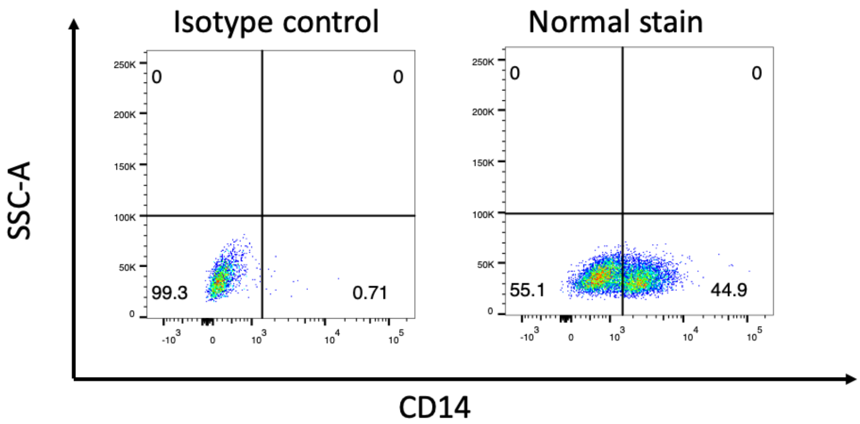

Figure 2.1- Representative plot of isotype control staining
CD3+ PBMCs were stained with CD14 antibody (right) or its isotype control (left) to determine non-specific binding of antibody and true CD14 expression.

For flow cytometry acquisition, the entire sample was acquired at medium speed or until 100,000 events were recorded and took place with a flow cytometers equipped with FACsDiva software. Experiments within this thesis used BD LSR II and LSRFortessa^{TM} X-20 (Beckon Dickinson, UK), at the Zayed Centre of Research and University College London Rayne Flow Cytometry facility respectively.

2.4. Data analysis

All flow cytometry data was analysed with FlowJo V10.6.1. Moreover, all graphs and figures were made using GraphPad Prism V9.0.0.

All statistical analyses within this thesis were also performed using GraphPad Prism software and consisted of paired t-tests between the selected data sets. Statistical significance was determined as $P < 0.05$ and denoted as (*). The degree of statistical significance was thereby depicted as * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$) and **** ($P < 0.0005$).

3. Optimisation

3.1. Aims and Objectives

This chapter aims to detail and discuss the optimisation of assays which laid the foundations of all experimental work within this thesis, providing context for any parameters used. These include growth of *E. coli* and *S. aureus* bacteria, UV irradiation of bacteria for use in γδT cell expansions, Titrations of MOI of bacteria for use in killing assays. In addition, data discussed within this chapter includes phenotypic information on γδT cells upon expansion with zoledronate, as well as expansion rates. This data provides informative background material which will be important to take into consideration when discussing the results of experiments detailed in subsequent chapters within the thesis, and as such will be referred to where necessary.

Due to the numerous assays and cell types used for the varying experiments, optimisation of each experimental procedure was undertaken. These include:

1. Optimisation of bacterial assays: Establishment of growth curves to ensure bacteria used in experiments were in log-phase; utilising varying UV irradiation parameters to ensure killed, but physically intact, bacteria were used in expansion protocols
2. Optimisation of expansion assays: Testing and validating the use of bacteria to expand γδT cells in comparison to zoledronate.
3. Optimisation of killing assays: These involve all experimental optimisations required **before** killing assays can take place such as isolation of γδT cells from PBMC: comparing purity of different γδT cell isolation kits.
4. Optimisations of cell-based assays: These largely refer to flow cytometry-based optimisations, such as gating strategies and use of

controls but also include the investigation of different detachment methods to remove THP-1 cells from flasks, without effecting levels of cell surface receptor expression *e.g.* CD14 levels.

3.2. Introduction to bacterial assays

This thesis sets out to identify the mechanisms and receptors responsible for immune activity of γδT cells. Following data shown by our group, which showcased the impact of cells to *E. coli* bacteria (100); we further investigated the use of bacteria to expand γδT cells.

There are numerous forms of classification for bacteria. One such method of classification is based on the properties of the cell membrane of the bacteria; Gram-positivity or Gram-negativity. This classification is based on a test to determine whether the bacterial cell membrane consisted of thick (Gram-positive) or thin (Gram-negative), peptidoglycan layer. We therefore utilised this classification criteria when designing our experiments. In order to achieve a wider understanding of γδT cell-mediated bacterial killing, we utilised both Gram-positive and Gram-negative bacteria. Thus, we used *E. coli* bacteria, as a previously confirmed stimulant of γδT cells and as a representative of Gram-negative bacteria. In addition to this, we also tested the use of *S. aureus*, as a representative of Gram-negative bacteria.

3.2.1. Growth of bacteria

As with all prokaryotic organisms, growth of bacteria is affected by various factors which include but are not limited to; nutrient availability *(e.g.* LB agar supplemented or unsupplemented), temperature and CO_2 levels. For instance, data has shown that *E. coli* can grow faster at warmer temperatures but its death rate is also higher in warmer temperatures. Whereas cooler temperatures seem to slow down both *E. coli* growth and death. As such, unsurprisingly, genotype of *E. coli* can influence growth and survival of *E. coli* depending on factors. *E. coli* O157:H7 experienced lower rate of growth in manure under fluctuating temperature conditions compared to stable temperatures, with lower growth rate observed as the intensity of temperature fluctuation increased (229).

Growth rate curves of bacteria in culture therefore show five phases of growth (230):

i) **Lag phase**: slow growth phase; delay before exponential bacterial growth commences

ii) **Exponential (Log) phase:** Growth phase where cell division occurs at a constant rate

iii) **Stationary phase:** Growth conditions become unfavourable and therefore bacterial replication stops

iv) **Death phase:** Loss of cell viability (bacterial cell death)

v) **Long-term stationary phase**

Bacterial growth starts within lag phase; this is the most poorly understood period of bacterial growth. In early studies focussing on *Salmonella enterica* bacteria, this phase was briefly referred to as the 'latent period' and described as an equilibrium phase controlled by an unknown regulatory mechanism (230).

There are no known physical or biochemical changes between bacteria in lag phase and those in log phase, it is even shown that lag-phase bacteria are metabolically active (231). Moreover, the underlying physiological and molecular process which drive bacteria to commence growth in this slowed down, lag phase as well as the driving force for transitioning into exponential growth have not yet been determined. However it is thought that within the lag phase, bacterial cells are adapted to the environmental conditions – which could include synthesis of cellular components and/or mechanisms to allow for growth or even the initiation of repair processes for macromolecular damage acquired during stationary phase (232).

Once in exponential phase (also referred to as log phase), bacteria begin to grow at an exponential rate. Thus, use of bacteria within log phase occurs within experimental procedures and studies as most bacterial cells will be at optimum health. This will also mean that the bacteria of interest will express characteristic levels and distribution of relevant protein and cell constituents (233). For use within immunology studies, this is important as cells under stress will alter metabolic profiles as well as expression of characterising markers, which may skew experimental data. For instance, genomic and transcriptomic analysis of *Mycobacterium avium* 104 has shown altered expression profiles of proteins within log and exponential phases of growth which play a role in metabolism and stress response to environmental conditions. Proteins such as aminopeptidase, choline dehydrogenase, oxidoreductase and ATP binding proteins were all up-regulated in stationary phase, whilst acetyl-CoA acetyltransferase, universal stress protein and catalase peroxidase were down-regulated (234).

After bacterial cells have gone through several rounds of replication within log phase, growth conditions can become unfavourable, usually due to a lack of nutrients or a change in environmental conditions (temperature, oxygen availability). This then pushes bacterial growth into stationary phase, where growth then reaches a plateau due to the number of dividing cells and growing cells being equal. In stationary phase, bacterial cells often become spherical and smaller; bacterial cell walls also become highly cross-linked and gene expression is adapted to respond to stresses. In some bacteria species, biofilm formation occurs during the stationary phase (235). Where the number of dying cells slowly outweigh the number of living and dividing cells, bacterial cells then enter the death phase.

3.2.2. UV irradiation of bacteria

When using bacteria as stimulants for γδT cell expansion, we hypothesized that intact bacteria is required, so that the component of bacteria responsible for stimulating γδT cell expansion is still present, in the correct conformation and undamaged. However, most antibacterial agents cause alteration to the bacterial cells and associated components via their varying methods of action which include: interference of cell wall synthesis, protein synthesis inhibition of various cellular processes such as protein synthesis, nucleic acid synthesis, membrane function, of ATP synthase and of metabolic pathways (236). Therefore, other methods of rendering the bacteria dead or inactive without loss of bacterial integrity should be executed. One such method for this is the use of ultraviolet (UV) irradiation. UV irradiation renders bacteria inactive and prevents bacterial growth (237). This occurs due to the UV irradiation damaging the nucleotides within nucleic acids causing mutagenic DNA lesions, leading to a cease in DNA replication. Interestingly, some microorganisms can bypass this via repairing of the UV damage in processes known as photoreactivation or dark repair.

Data has shown that depending on bacteria type, susceptibility to UV varies. For instance, *E. coli* has been shown to require lower dose of irradiation when compared to *Mycobacterium avium*. Another factor for efficiency of UV irradiation is also the amount of suspended particles in the medium, *i.e.* the blocking or perturbing of UV light to the 'target' bacteria (238).

Of importance to note, is the presence of UV resistance among some bacterial strains. It has been noted that environmental bacteria have an increased resistance to UV compared to laboratory strains. Moreover, data has shown that UV exposure as a form of environmental stress, can lead to bacterial entering non-culturable states in which they may halt growth but are still viable. For instance, a study by Blatchley *et al* has shown that resistance to UV-imposed loss of viability in *E. coli* cultures can be partially attributed to agglomeration during the irradiation process (239). This therefore needs to be taken into consideration when choosing genotype and bacterial strains for use adequate UV irradiation, with the intended result of rendering bacterial cells as non-viable, yet intact cells needs to be considered for use in γδT cell expansion assays.

3.2.3. Background to *Escherichia coli (E. coli)*

E. coli is a heavily-studied microorganism, uniquely displaying itself as both a common commensal and otherwise 'harmless' inhabitant of anatomical sites mainly consisting of the mucosa and the gastrointestinal tract, whilst also exhibiting virulent and pathogenic characteristics under specific conditions (240). Usually, *E. coli* co-exists with the human host, with virtually no detection and a mutual benefit for both parties; via production of vitamin K2, and providing healthy competitive environment amongst other bacterial species in the mucosa (241). Severe *E. coli*-mediated diseases therefore are a rare occurrence, following mostly in immunocompromised patients, where GI barriers are damaged. Regarding commensal strains of *E. coli*, its niche occurs at the mucous layer within the colon. Here, *E. coli* express tropism; proving itself as a successful competitor of resources and therefore most abundant anaerobe within the crowded colon (242).

However, as with most commensal bacteria; several adapted *E. coli* variants exist which have gained virulence factors. These factors occur via mutations in the *E. coli* genome leading to variation in the genetic elements coded which can lead to advantageous characteristics in the form of metabolic or physical differences (243). These characteristics confer the *E. coli* strains with the ability to access new sites and adapt to new niches, causing a broader spectrum of disease, across different anatomical sites. *E. coli* strains that have acquired virulence factors are thus known as pathotypes; the most common clinical syndromes arising from infections from these *E. coli* pathotypes are i) Urinary tract infections (UTIs), ii) Sepsis/Meningitis, iii) Enteric/diarrhoeal disease (244).

Category of *E. coli* pathotype	Virulence factor(s)	Associated diseases
Enteropathogenic *E. coli* (EPEC)	• Attaching and Effacing ability (A/E) • EPEC Adherence Plasmid (EAF) • Bundle-forming pilus (BFP) • LifA/Efa	Gastroenteritis
Enterohaemorrhagic *E. coli* (EHEC)	• Shiga toxin (stx) • Attaching and Effacing ability (A/E) • Enterohemolysin (Ehly) • *E. coli* secreted protein (EspP) • Katalase Peroxidase (KatP) • ToxB • Efa-1/LifA	Hemolytic-uremic *syndrome*
Enterotoxigenic *E. coli* (ETEC)	• Enteroaggregative heat-stable toxin (EAST-1) • Cytolysin A (clyA) • eastA • leoA • tia • tibC	Travellers' diarrhoea
Enteroaggregative *E. coli* (EAEC)	• Enteroaggregative heat-stable toxin (EAST-1) • Plasmid-encoded toxin (Pet) • Protein involved in colonization (pic) • Shigella enterotoxin 1 (ShET-1) • Hemolysin E (HlyE) • Dispersin (Aap) • Aggregative adherence factor II (AAF/II)	Acute or persistent diarrhoea
Enteroinvasive *E. coli* (EIEC)	• Vir/lesA cluster • IpaA, IpaB, IpaC, IpaD, IpaH • Shigella enterotoxin-1 (ShET1)	Shigellosis-like conditions and diarrhoea
Diffusely adherent *E. coli* (DAEC)	• Fimrial adhesin F1845 • Adhesin in diffuse adherence (AIDA-I)	Diarrheal *disease* in children >12 months of age
Uropathogenic *E. coli* (UPEC)	• Type 1 fimbriae • Dr adhesion • S fimbriae • Type 2, P fimbriae • F1C fimbriae	Urinary tract infections
Meningitis-associated *E. coli* (MNEC)	• Adhesins • Toxins • iron acquisition factors • lipopolysaccharides • polysaccharide capsules • invasins	Sepsis/Meningitis

Table 3.1 – Virulence factors exhibited by the different *E. coli* pathotypes
Data adapted from the following references *(242,245–255)*.

3.2.4. Background to *Staphylococcus aureus (S. aureus*)

S. aureus is a Gram-positive bacterium species. Making up part of the regular human microbiota, it is usually found in the upper respiratory tract as well as other mucosal sites such as the skin and gastrointestinal tract. It's main site of inoculation are the nostrils, with bacterial load positively correlating with likelihood of colonisation to other sites (256).

Despite its existence as a commensal, upon disruption to the microbiota balance or loss of integrity at its homing sites, *S. aureus* can develop tropism for and colonise other areas. These occurrences lead to *S. aureus* transitioning from commensal to pathogenic bacterium, causing infections and disease in humans that range from curable and manageable to severe and life threating. Minor infections of *S. aureus* are usually localised to skin and soft tissue, which take the form of abscesses, furuncles, and cellulitis (257). However, more serious infections occur when the bacteria gain access across these mucosal sites and cause more systemic infections. Examples of more severe *S. aureus* infections include pneumonia, bloodstream or bone and joint infections, toxic shock syndrome and food poisoning (258).

S. aureus hosts a range of toxins and virulence factors which allow it to evade the immune response (259). These include protein A located in the bacterium cell wall, which binds IgG at the Fc region, causing the bacterium to be covered by host IgG and thus prevents immune cell recognition; as well as the polysaccharide capsule, which inhibits phagocytosis by DCs and macrophages (260,261). Other toxins produced by *S. aureus* are classified as cytotoxins, pyrogenic (fever-producing), enterotoxins and exfoliative toxins described in table 3.2. and contribute to the symptoms caused by *S. aureus* infections.

Category of Toxin	Examples	Virulence factor(s)
Cytotoxin	• Leukocidins: Panton-Valentine leukocidin (PVL), leukotoxin ED (LukED), and leukotoxin AB/GH (LukAB/GH) • Gamma (γ)-hemolysins: (HlgAB and HlgCB)	Forms pores in the plasma membrane of cells leading to osmotic lysis of target cells, causing necrotic cell death.
Pyrogenic toxins (PTSAgs)	• Toxic shock syndrome toxins (TSST) • *Staphylococcal enterotoxins* (SE)	Cause widespread inflammation via: • T cell activation • release of proinflammatory cytokines by APCs • increased sensitivity to LPS
Exfoliative toxins	• ETA • ETB • ETD	Loss of cell-cell adhesion of keratinocytes in the superficial epidermis, allowing for bacterium invasion to deeper sites.

Table 3.2 – Overview of the different classes of toxins produced from *S. aureus*
Data sourced from the following references *(262–267)*

3.3. Culturing γδT cells for experimental use

3.3.1. Expanding γδT cells from PBMC

As γδT cells exist in low numbers within blood, making up 0.5-5% of total T cells (268), experiments using γδT cells rely upon expansion protocols to achieve yields of adequate cell numbers for assays of interest. When expanding γδT cells from human PBMC; common procedure is to use zoledronate in combination with IL-2 (222). This method often achieves yields of at least 60% (of T lymphocytes). However, a number of different culture methods to expand γδT cells have been reported globally by various laboratories since the 21st century. Despite the varying procedures, the common characteristic within these varying methods is the requirement to expand γδT cells from PBMCs rather than from purified T cells, or expansion through isolation, before in vitro culture or experimental usage. γδT cell culture via PBMCs is required due to the necessity of cell-to-cell contact. Addition of stimulating agents *e.g*. zoledronate to PBMC lead to accumulation of IPP in cells within the PBMC culture such as monocytes, which subsequently activates γδT cells through cell-to-cell interactions (269). As sensors of their local environment, both cytokine and antigen stimulation is required for effective γδT cell expansion (270).

3.3.1.1 Antigen stimulation

Addition of antigens within the PBMC cultures is required to stimulate expansion of γδT cells; these antigens include: IPP, HMBPP and bromohydrin pyrophosphate (BrHPP) pamidronate and zoledronate (271–273). An overview of antigen stimulants of γδT cells: zoledronate and IPP and their role in expansion is briefly discussed in chapter 1.3.4.

γδT cells are triggered by an accumulation of IPP, which occurs due to disruption of the host mevalonate pathway (46,47). The mevalonate pathway is a metabolic pathway in cells that is responsible for numerous cellular processes and generates vital products necessary for cell functions such as the intermediate product used in the cholesterol pathway, farnesyl pyrophosphate (FPP) leading to downstream products such as vitamin D, bile products and steroid hormones (274). With IPP being a key intermediate in this pathway, disruption therefore leads to an accumulation of IPP (275).

Despite having the same downstream effect of expanding γδT cells, zoledronate acts in a different manner to cause γδT cell expansion. Zoledronate and pamidronate are nitrogen-containing bisphosphates that inhibit the FPPS enzyme, which is responsible for conversion of IPP to FPP, via competitive inhibition (276). This therefore leads to enhanced levels of IPP, causing γδT cell expansion (50). Both zoledronate and BrHPP share structural homology with IPP and thus, as a synthetic analog to zoledronate, BrHPP is seen to act in a similar manner (277).

3.3.1.2 Cytokine Stimulation

Using IL-2 for expansion of γδT cells

In addition to the antigenic stimulation, presence of cytokine is also needed in the expansion of γδT cells. Interleukin 2 (IL-2) is regarded as an essential cytokine for general T cell expansion and is therefore commonly used in the expansion and culture of any T cell subtype, including γδT cells (278). Polyclonal expansion of T cells is caused via the binding of IL-2 to IL2R on T cells. Once bound, the complex is internalised, the a-subunit of the IL-2R, known as CD25 then resurfaces to bind to IL-2. This sequence of events will lead to diminishment of IL-2 signal over time and it is therefore important to introduce IL-2 into the PBMC culture every 2-3 days during T cell expansion (279).

Studies have shown varying effects on expansion of T cell populations depending on concentration of IL-2 cytokine used introduced into the culture. Low-dose IL-2, regarded as 100 U/ml has been largely used throughout expansion protocols as the optimum dosage (280). A study by Wang *et al* further investigated usage of different concentrations of IL-2 alongside IPP for γδT cell expansion. Investigators first used 100 u/ml IL-2 until γδT cells reached a logarithmic phase (indicated by visible clusters under a microscope), where they were the predominant T cell subset within culture. Hereafter, the dose of IL-2 was increased to 1000 U/ml to expand the now predominant γδT cells more extensively. This was compared to utilising high dose IL-

2 (1000 U/ml) for γδT cell expansion from day 0. Results from these experiments showed that despite initial higher levels of γδT cells and Vγ9δ2 T cells in the high dose group, by the end of culture (16 days) the first group (low dose, then high dose) yielded the higher total cell count and the highest number of Vγ9δ2 T cells (280). This shows a benefit of low dose IL-2 at the beginning of culture with regards to long-term culture of Vγ9δ2 T cells, which is necessary for therapy utilising the genetic modification of T cells through lentiviral transduction.

Interesting to note, is the phenomenon of low-dose IL-2 to yield generation of early memory T cells over naïve and/or effector T cells during expansion of CAR-T cells (281). Such findings have not yet been identified in γδT cell expansion but as memory-like γδT cells have recently been discovered within murine models of listeria infection (282), it is important to consider how concentration of stimulating antigens and/or cytokine dosage may impact γδT cell proliferation in terms of effector functionality.

<u>Alternatives to IL-2</u>

Although IL-2 is widely regarded as the common cytokine of choice for T cell expansions, limitations related to its toxicity to cells warrant investigations into the use of other cytokines for expansion (283). As such, recent studies have looked into cytokines with similar properties to IL-2. IL-15 positions itself as a promising candidate as an alternative to IL-2 for a number of reasons. Firstly, it is closely related to IL-2; similarities between IL-2 and iL-15 include the nature and structure of the cytokine molecule and how reacts with its corresponding receptor; with both cytokines consisting of alpha-chains, beta-chains, and gamma chains (284). Secondly, it is regarded as highly ranked drug in terms of immunotherapeutic potential even for broad usage within cancer therapy (285). Moreover, when comparing effects on T cells and NK cells, functional similarities between the two cytokines can be seen, which include the impact on lymphocyte proliferation and promotion of cytotoxicity (286). Addition of IL-15 results in a more activated phenotype of γδT cells; with cells exhibiting higher levels of proliferation, cytotoxicity and Th1 polarisation (287). Moreover, in T cell therapy studies, IL-15 has shown efficacy where IL-2 has failed (288). With regard specifically to γδT cells, data has shown that γδT cell expansion from PBMC from both healthy donors as well as acute myeloid leukaemia patients to be boosted in the presence of IL-15 and to also boost the antitumour properties of the cells (227,289).

Thus, the rationale for utilising IL-15 +/- IL-2 is apparent and clear; especially since it has been shown that IL-2 stimulation alone can result in activation-induced cell death and seems to favour preferential maintenance of Tregs (290,291).

However, whilst the proliferative and pro-inflammatory capabilities of IL-15 are promising for therapy, using IL-15 in our assays to explore the interactions between γδT cells and bacteria would skew the results in favour of high-performing γδT cells. Our investigations are more explorative in nature; therefore, IL-2 was chosen as the stimulating cytokine during our experiments as the resulting γδT cells from expansion would be more representative of *in vivo* γδT cells.

3.3.2. Harnessing fresh γδT cells from PBMC

Whilst many studies investigating the role of γδT cells within bacterial infections exist, few studies have investigated the impact of γδT cells in direct exposure to bacteria within *in vitro* experiments, particularly when considering the use of fresh, non-expanded γδT cells. This is largely due to the fact that γδT cells exist in low numbers within the PBMC fraction, therefore acquiring a sufficient amount of γδT cells from blood, without expanding, is challenging and demands large volume of blood as well as being labour intensive (when considering the PBMC isolation required of such large blood volumes). The use of fresh γδT cells therefore presents itself as a challenge as when undertaking our assays, larger volumes of blood are required; for PBMC isolation to yield a sufficient number of γδT cells after isolation. These large-volume PBMC isolations also need to be achieved as timely as possible so that γδT cell isolation can occur whilst PBMCs were as fresh as possible, allowing for day 0 unstimulated γδT cells to be the subject of experiments.

Interestingly, γδT cells are distributed within mucosal sites and are present at these sites in far larger numbers. Moreover, PBMC γδT cells are Vδ2 whereas those at mucosal sites are Vδ1 cells. However, accessing and harnessing the γδT cells within these sites is not easily achieved. Upon expansion with zoledronate, γδT cells from PBMC, which consist largely of the Vδ2 subtype, leads to a further expansion of this subtype in particular, meaning that experimental data gathered from zoledronate-expanded γδT cells is specific to the Vδ2 subtype (272). Imploring the use of unstimulated, freshly isolated γδT cells allows for data to be revealed on the impact of mixed subtype γδT cells interactions, rather than specifically Vδ2 cells.

3.4. Methods and materials

3.4.1. Establishing a growth curve of *E. coli* and *S. aureus* cultures

To accurately determine the correct optical density (OD) to use for co-culture and killing experiments, growth curves were generated for both the *E. coli* and *S. aureus* strains. A growth curve measuring OD and CFU over time was generated to use as a reference for all future experiments.

Growth and maintenance of bacterial cultures have been described within the general material methods chapter 2. Using the overnight culture produced in methods 2.1.2 which give rise to young exponential phase bacterial cells, 5ml of the overnight grown culture was inoculated in a flask of 250ml autoclaved LB broth.
OD measurements were taken at the zero-hour time-point and the new LB-bacterial culture was sampled every hour for 10 hours for:

1) Measurement of OD at 600nm
2) Measurement of CFU: 1ml of bacterial culture was diluted over several dilutions (ten-fold until 10^{-8}). 100µL of each dilution was then plated onto an LB agar plate and left at 37ºC 5% CO_2 overnight for counting of CFU the next day.

3.4.2. Assessing the effect of UV irradiation on bacterial growth

Log-phase (viable) bacteria cells were subjected to UV irradiation. First, the bacterial cells were centrifuged at 1500g for 10 minutes and resuspended at densities of 300 or 100 $x10^6$ CFU/ml. Bacteria were then placed in a trans-illuminator chamber (UVITEC), equipped with 8 UV-C (250-280nm) lamps for 5, 10, 15 and 30 minutes. Cultures were then sampled for CFU growth and compared to non-UV irradiated bacteria. Irradiation times were determined by investigating the minimal time needed to prevent growth of bacteria in overnight liquid culture.

3.4.3. γδT cell expansions from PBMC using various stimuli

PBMCs were plated in Nunclon™ 24-well plates (Thermo Fisher) at a concentration of 1.5 million cells per ml with RPMI 1640 + GlutaMAX™ media (Gibco) supplemented with 10% FCS and 1% antibiotic – Pen/Strep (10,000 U/mL, Gibco). Cells were stimulated with 100IU of IL-2 (Miltenyi Biotec) and either:

1) 5µM/ml of Zoledronic acid monohydrate (Sigma-Aldrich) for zoledronate expansions
2) MOI of 5 of relevant bacteria for E. *coli* and S. *aureus* expansions
3) No stimuli (control)

Cells were restimulated with IL-2 and media was changed every two or three days. All *E. coli*- and *S. aureus* -stimulations took place with UV-irradiated bacteria.

3.4.4. γδT cell isolation via MACS human TCRγ/δ+ T Cell Isolation Kit (Miltenyi Biotec)

γδT cells were isolated after PBMC isolation and isolation kit was used according to the manufacturer's recommendations and volumes as described below (Table 3.3).

Firstly, cell number was measured, and single-cell suspension was obtained via passing the cells through 30µm nylon mesh pre-separation filters (# 130-041-407). Cells were then centrifuged at 300g for 10 minutes. Supernatant was discarded and PBMCs were resuspended in buffer (PBS supplemented with 0.5% BSA and 2mM EDTA). The appropriate volume of biotin-antibody cocktail was added before incubating the cells at 4°C for ten minutes. Cells were then washed via addition of buffer and centrifuged at 300g for 10 minutes. Supernatant was discarded and cells were resuspended in the appropriate volume of buffer before anti-biotin microbeads were added. Cells were then further incubated for 15 minutes at 4°C and washed via addition of buffer and centrifugation at 300g for 10 minutes. Supernatant was discarded and cells were resuspended in buffer at concentration of 500µl/10^8 cells.

Number of cells	Volume of buffer for resuspension	Biotin antibody cocktail	Volume of buffer (cell wash step)	Anti-biotin microbeads
$< 10^7$	80µL	20µL	2mLs	20µL
$> 10^7$ $n \times 10^7$	n x 80µL	n x 20µL	n x 2mLs	n x 20µL

Table 3.3 – Volumes of reagent required for γδT cell isolation via MACS isolation kit

An MS column (Miltenyi Biotec) was placed in the magnetic field of the MACS separator and prepared by rinsing 500µL of buffer through the column. The cell suspension was then placed under the column, effluent was collected as unlabelled γδT cells. The MS column was then washed three times with 500µL of buffer to collect as many γδT cells as possible, only adding new buffer when the column was empty.

3.4.5. γδT cell isolation via EasySep™ human TCRγ/δ+ T Cell Isolation Kit

γδT cells were isolated after PBMC isolation and isolation kit was used according to the manufacturer's recommendations and volumes as described below:

Firstly, cell number was measured, and suspended in buffer (PBS containing 2% FBS and 1 mM EDTA) at 5×10^7 cells/mL at minimum volume of 0.5mL and maximum of 2mL in a sterile polystyrene round-bottom tube. 50µL of isolation cocktail was added to cells before incubation at room temperature for 15 minutes. Magnetic particles were vortexed and added at 50µL/mL of sample before further incubation for 10 minutes at room temperature. Samples were then topped up to 2.5mL, mixed and placed (without a lid) into the EasySep magnetic separator for 5 minutes. After incubation, the magnet with the tube is inverted and contents (consisting of enriched cell suspension) poured into a new tube. Magnetic particles were then vortexed again, 37µL of which was added to the new tube now containing the enriched γδT cells and incubated at room temperature for 5 minutes. After this, the tube was placed in the magnet for 5 minutes before inversion of the magnet and tube, with contents being poured into a third and final tube.

3.4.6. Growth, maintenance, and PMA-stimulation of THP-1 cells

THP-1 cells were cultured in RPMI supplemented with 1% FBS and 1% penicillin/streptomycin at 37°C within a 5% CO_2 incubator. Cells were maintained at a cell density between 2.0×10^5 to 1.0×10^6 viable cells/mL.

THP-1 cells were stimulated with phorbol 12-myristiate-12 acetate (PMA, Sigma-Aldrich) at concentration at 10ng/ml for 24 hours across four different flasks. PMA was then removed, and cells were then rested for 24 hours in fresh media before THP-1 cells were detached from flasks.

3.4.7. Detachment of THP-1 cells from flasks

PMA-stimulated THP-1 cells were detached from flasks via 1 of the following four methods:

PBS – Flask bottom was flooded with 15ml PBS, flask was then tapped to gently detach adherent cells. Cell/PBS suspension was then decanted into a 50ml falcon tube and another 15ml of PBS used to flood the flask again. The flask was tapped again and decanted to expel any last adherent cells.

EDTA – Flask bottom was flooded with 15ml PBS with 5mM EDTA for 10 minutes. The flask was tapped and 10ml of cell suspension was removed into 1 50ml falcon tube and the flask was then incubated at 37°C for 5-10 minutes. The flask was then tapped again, and the rest of the cell suspension was decanted into the 50ml falcon tube.

Trypsin/EDTA – Flask bottom was tapped, and culture medium was removed and decanted into a 50ml falcon. Any resulting serum was removed by rinsing with 5ml of PBS and decanted into the same 50ml falcon. 2.5ml of 0.25% trypsin-EDTA solution was slowly added to the flask to cover the cell monolayer and incubated at room temperature for 45 seconds. The trypsin/EDTA solution was removed, and the flask was incubated at room temperature for a further 1 minute. The remaining cell suspension was decanted into the 50ml falcon tube.

Sodium citrate – A 10X sodium citrate solution was made using 1.35M potassium chloride and 0.15M sodium citrate in 500ml of Millipore water, this was then diluted 1 in 10 with sterile water before use. All media was removed from the flask and decanted into a 50ml falcon tube; the flask was then flooded with 15ml of 1xsodium citrate solution pre-warmed to 37°C. The flask was then incubated at 37°C for 5 minutes. After incubation, the flask was gently tapped to detach cells, and solution decanted into the 50ml falcon.

3.4.8. CD14 staining of THP-1 cells

PMA-stimulated THP-1 cells were washed at 1250RPM with PBS and counted. 1.0×10^6 cells in 100µL of MACS buffer (D-PBS + 2 mM EDTA + 1% FBS) were placed in a 96-well plate. Cells were then washed again, and supernatant removed. 10µL of Fc block (BD Pharmingen™) was added to the cell pellet and incubated at room temperature for 10 minutes. After this, 20µL of FITC anti-human CD14 antibody or isotype control (Biolegend) was added to the cell pellet and incubated for a further 30 minutes at 4C in the dark. Cells were then washed with MACS buffer 3 times, resuspended in 200µL MACS buffer and transferred to FACS tubes for flow cytometric analysis.

3.5. Results

3.5.1. Log-phase of *E. coli* (DH5α) and *S. aureus* (NCTC 6571) was achieved after 4 hours of culture

In order to ensure both optimal stimulation of PBMC by bacteria, and thus, appropriate levels of γδT cell expansion, bacteria needed to be in log-phase of growth. Therefore, growth curves of OD and CFU/ml measurements over the course of 10 hours from both the *E. coli* and *S. aureus* cryogenically preserved stocks which were used in our experiments were calculated. We calculated the log-phase for *E. coli* (DH5α) to be between 4-7 hours after placing in a shaking incubator, resulting in 250-1800x10^6CFU/ml and the log-phase for *S. aureus* (NCTC 6571) to be between 3-6 hours after placing in a shaking incubator, resulting in 300-1900x10^6CFU/ml (Figure 3.1). We therefore aimed to use mid-log phase bacteria at approximately 4 hours post-culture, to ensure bacteria used were healthy and in their optimal growth cycle range.

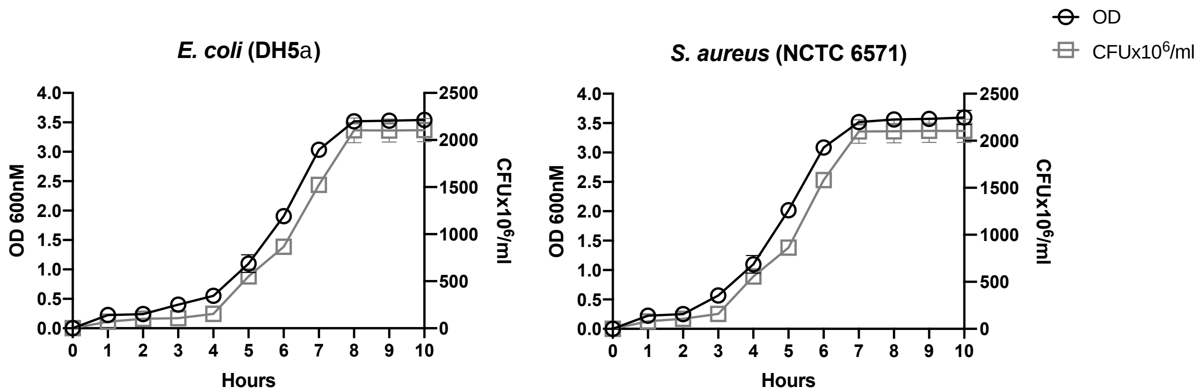

Figure 3.1 – Growth curves of *E. coli* and *S. aureus* bacteria
E. coli (DH5α) and *S. aureus* (NCTC 6571) bacteria were grown for 10 hours in a shaking incubator (220RPM) at 37ºC 5% CO_2 in liquid LB. Cultures were sampled every hour for OD measurement and plated on LB agar plates for CFU measurement. Data shown is n=3

3.5.2. Successful UV irradiation of *E. coli* and *S. aureus* occurred after 30 minutes of UV light exposure with bacteria at lower densities

Our lab has previously utilised a method for expansion of γδT cells. We aimed to build upon this method by testing additional stimuli for capability of expanding γδT cells within the same PBMC environment. Thus, we also examined the role of *S. aureus* in expanding γδT cells, alongside *E. coli*, as previously tested by Barisa *et al*, and zoledronate, a known stimulus for γδT cell expansion (52). All stimuli were investigated under the same conditions of fresh media and IL-2 supplementation every 2-3 days. Mock stimulation of PBMC involved no IL-2 supplementation or addition of stimuli but did follow the same pattern of media supplementation as the rest of the conditions tested.

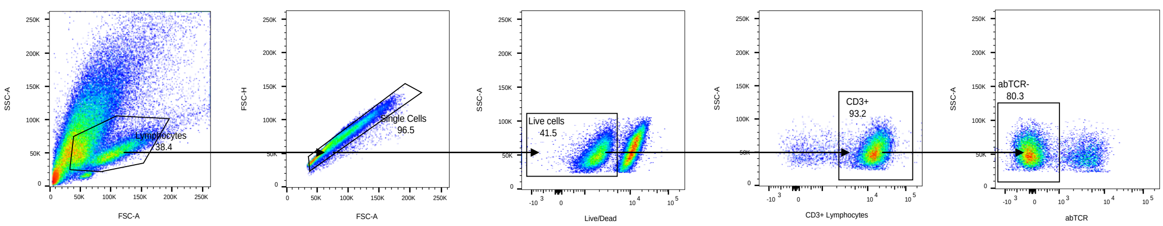

Figure 3.2 – Gating strategy
PBMC were gated on live lymphocytes determined via FSC and SSC distribution, doublets were excluded, and single cells were identified before gating on live cells. Live cells were then gated for CD3 expression and αβTCR expression measured. γδT cells were gated as the αβTCR$^-$ fraction of CD3+ cells.

For measurement of γδT cell expansion, as well as general phenotyping, γδT cells were gated as the αβTCR-CD3+ lymphocytes. This method of gating allows for subsequent measurement of Vδ1/Vδ2 subtype distribution of the γδT cells, as using a pan-γδTCR antibody would mean all γδTCRs would be stained and further subclassification of the γδTCR would not be feasible due to antibody already being bound to the receptor.

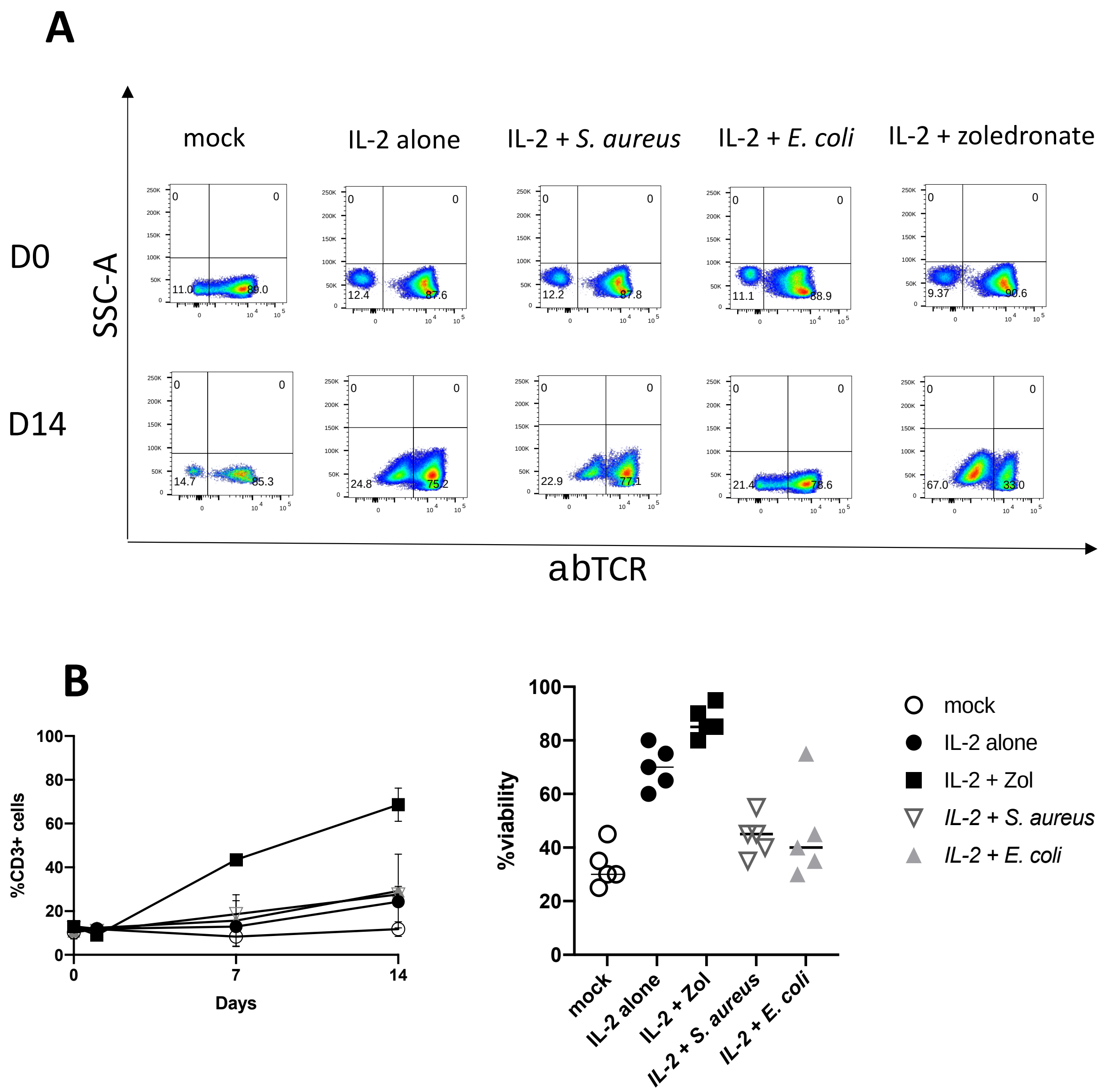

Figure 3.3 – Zoledronate stimulation of PBMCs results in repeated successful expansion of γδT cells compared to other stimuli

Freshly-isolated PBMC were stimulated with UV-irradiated *S. aureus* or *E. coli* at MOI 5 or zoledronate (5μm/ml) and left to expand in IL-2-supplemented media for 14 days with additional media and IL-2 supplementation every 2-3 days (**A**) The proportion of γδT cells (bottom left quadrant) vs αβT cells (bottom right quadrant). Data shown is gated on live CD3+ lymphocytes and is a representative of n=5. (**B**) Percentage of γδT cells within CD3+ lymphocyte population over 14 days of expansion (top) and viability of γδT cells after 14 days of expansion (bottom). Data shown is n=5.

As expected, and previously shown, IL-2 + zoledronate was a potent stimulus for the expansion of γδT cells within PBMC. Unsurprisingly, mock conditions showed no change in percentage of γδT cells within the CD3+ lymphocytes from day 0 to day 14 of culture and also exhibited low viability of cells of 30% live γδT cells after 14 days of culture (figure 3.3). IL-2 alone as well as IL-2 + *S. aureus* both showed little expansion of γδT cells of less than 20%, therefore indicating that *S. aureus*, as a representative of Gram-positive bacteria is not able to expand γδT cells. Of importance to note and in contrast to previous findings within our group, is that consistent repetition of *E. coli* stimulation of PBMC also often lead to poor cell expansion profiles and cell numbers. Similar to *S. aureus*-stimulated PBMCs, *E. coli*-stimulated PBMCs exhibited an average of less than 20% γδT cells (figure 3.3B). This was unexpected, as the same protocol as stated in the previous study by our lab was employed. Moreover, viability after 14 days of culture was lower than the IL-2 alone stimulations, meaning that PBMCs were dying after stimulation with *E. coli*. This led us to believe that the *E. coli* was perhaps not optimally UV-irradiated.

Unsurprisingly, there was visible donor-to-donor variation observed upon stimulation of PBMC with zoledronate, with variable levels of γδT cells among donors that could not be accounted for by age or gender of donor. It is therefore of note that some individuals may possess inherent factors within PBMC, or more reactive immune cells that leads to a more potent reaction to zoledronate than others.

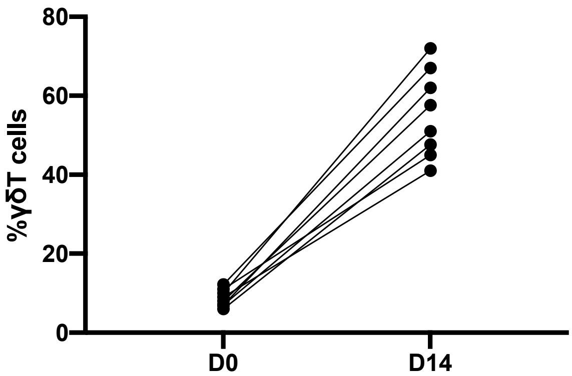

Figure 3.4 - Donor-to-donor variation in γδT expansions via zoledronate stimulation of PBMCs
Freshly-isolated PBMC were stimulated with zoledronate (5µm/ml) and left to expand in IL-2-supplemented media for 14 days with additional media and IL-2 supplementation every 2-3 days. Percentage of γδT cells from CD3+ lymphocytes measured at day 0 and day 14 of expansion from 8 donors.

3.5.3. *E. coli* stimulation was not able to consistently expand γδT cells despite successful UV-irradiation of bacteria

Therefore, upon finding these inconsistencies within the *E. coli*-stimulated expansions, we decided to reassess the previous method of UV irradiation being used. We tested UV irradiation of both bacteria to ensure the lack of γδT cell expansion from *S. aureus* stimulation wasn't due to the same issue as the *E. coli* expansion inconsistencies. Log-phase bacteria (at similar volumes and CFUs/ml for the desired stimulation) were exposed to UV light at varying densities, and contained within different vessels, for varying times of exposure. After optimisation, our findings indicated that lower densities of $100x10^6$ CFU/ml for a longer irradiation time of minimum 30 minutes, were able to inhibit the growth of bacteria successfully and consistently.

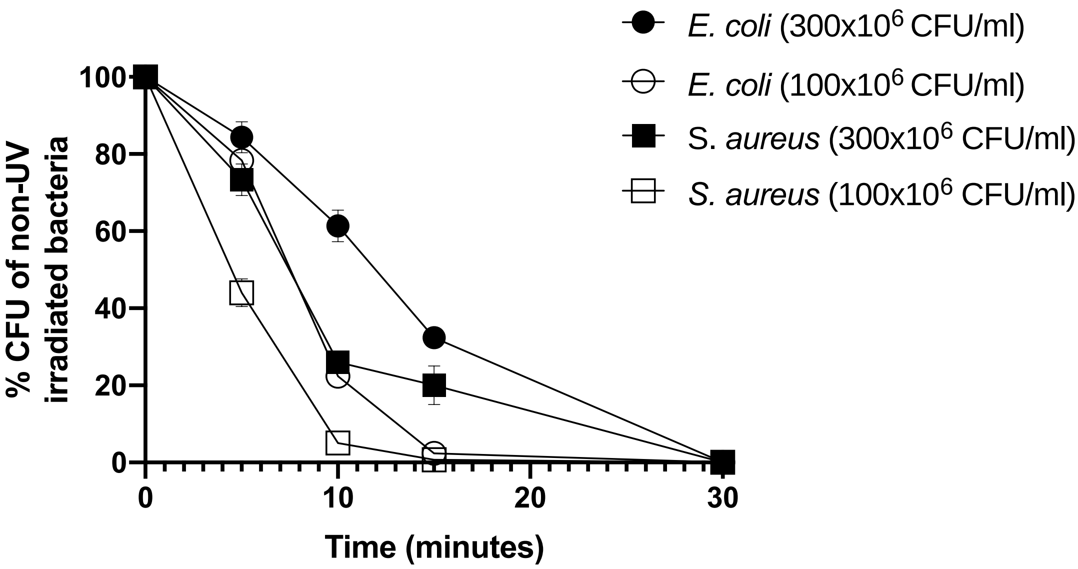

Figure 3.5 – Impact of UV irradiation times on bacterial growth
Log-phase *E. coli* (circle) and *S. aureus* (square) bacteria were exposed to UV light for 5, 10, 15 and 30 minutes in either $300x10^6$M CFU/ml (black) or $100x10^6$M CFU/ml (white) liquid cultures. Cultures were then plated onto LB agar plates and CFU/ml calculated as percentage of CFU/ml from non-UV irradiated bacteria. Data shown is n=3

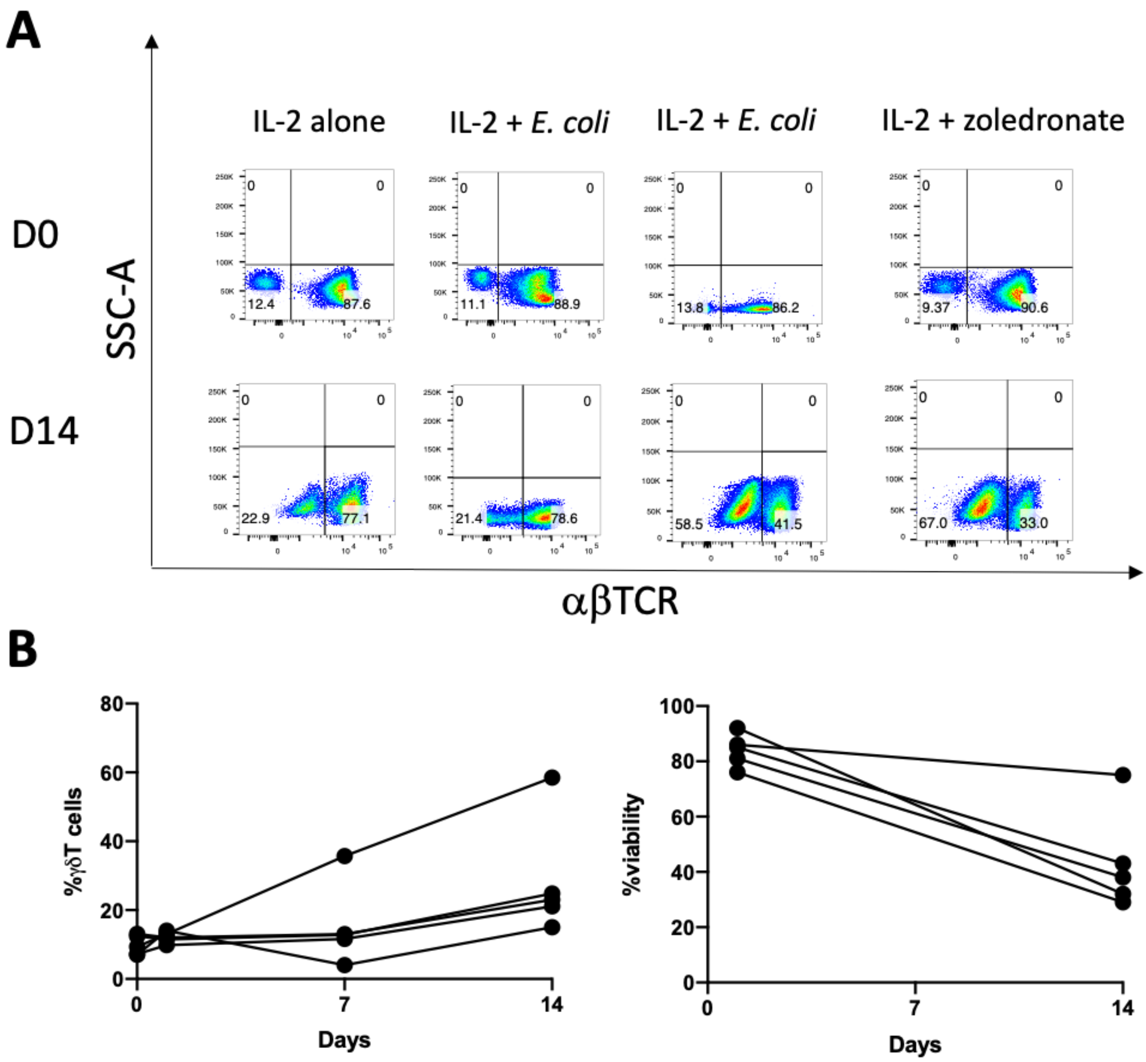

Figure 3.6 – *E. coli* stimulation of PBMCs for expansion of γδT cells
Freshly-isolated PBMC were stimulated with UV-irradiated *E. coli* at MOI 5 or zoledronate (5µm/ml) or not stimulated and left to expand in IL-2-supplemented media for 14 days with additional media and IL-2 supplementation every 2-3 days (**A**) The proportion of γδT cells (bottom left quadrant) vs αβT cells (bottom right quadrant). Data shown is gated on live CD3+ lymphocytes and is a representative of n=5. (**B**) Percentage of γδT cells from *E. coli* expansions within CD3+ lymphocyte population over 14 days of expansion (top) and viability of γδT cells after 14 days of expansion (bottom). Data shown is n=5.

However, despite this, the UV-irradiated *E. coli* and *S. aureus*-stimulated PBMC still were not able to yield γδT cell expansions. As shown in previous literature, our findings showed IL-2 + zoledronate to be able to successfully and consistently expand γδT cells to levels of approximately 65% of total CD3+ lymphocytes. Therefore henceforth, we used IL-2 + zoledronate as the stimuli to expand γδT cells required for our assays.

3.5.4. Age or gender of donor does not affect the expansion rate of Vδ1/Vδ2 distribution of γδT cells expanded via IL-2 + zoledronate stimulation of PBMCs.

As our experiments required PBMCs from various donors, we then assessed that expansion rates and Vδ1/Vδ2 distribution of zoledronate-expanded γδT cells were not impacted by donor age or gender, allowing us easier access to donor PBMCs. As expected, γδT cells consisted of over >90% Vδ2 T cells after 14 days of expansion. Our findings showed no significant difference across age-group or gender on both the expansion rates and Vδ1/Vδ2 distribution of γδT cells as well as the viability of cells after 14 days of expansion.

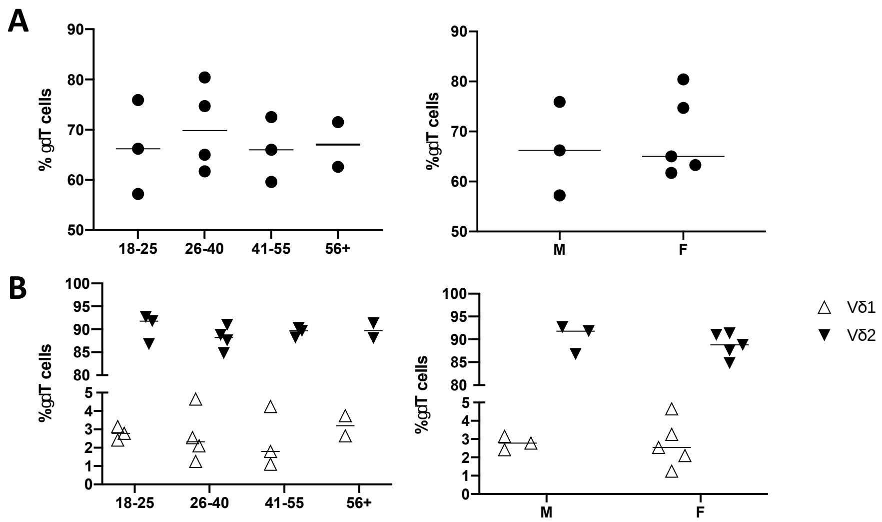

Figure 3.7 – Expansion of γδT cells using Zoledronate stimulation is not influenced by age or gender of donor

Freshly-isolated PBMC were stimulated with zoledronate (5µm/ml) and left to expand in IL-2-supplemented media for 14 days with additional media and IL-2 supplementation every 2-3 days (**A**) The proportion of γδT cells after 14-days of culture with zoledronate were shown in different age groups (left) and across male/female donors (right) and are gated on CD3+ lymphocytes (**B**) The proportion of Vδ1 and Vδ2 T cells among different age groups (left) and gender (right). Vδ1/Vδ2 subtype percentages are determined as percentage of total γδT cells.

3.5.5. Miltenyi and EasySep γδT cell isolation kits yield 95% and 98% purity of γδT cells respectively

As we aimed to assess and compare γδT cells against their IL-2 + zoledronate expanded counterparts, we needed to also harness enough fresh γδT cells for experimental use. This relied on the use of commercially-available γδT cell isolation kits. We tested two kits for use, which both work by labelling all cells within the PBMC fraction except for γδT cells, thereby not disrupting or activating the γδTCR. These kits were the Miltenyi Biotec human γδTCR negative isolation kit and the EasySep γδT cell negative isolation kit. Equal amount of PBMCs from 3 donors were subjected to isolation by both kits and %purity from resulting γδT cell fractions was measured as well as percentage from the starting PBMC and viability of the γδT cells. We did not observe any statistically significant differences between the two isolation kits within the parameters measured. However, it is important to note that our sample size was small and although not statistically significant, we observed slightly higher purity from the EasySep isolation kit (98%) compared to Miltenyi (95%).

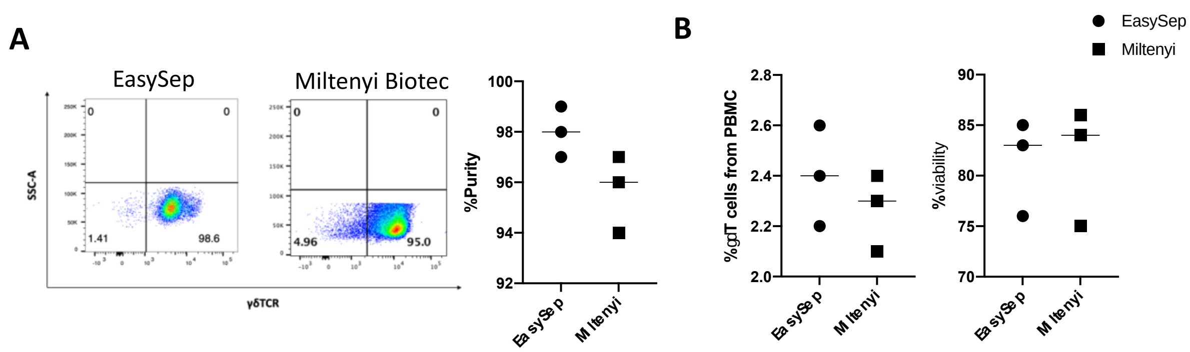

Figure 3.8 – Comparison of purity of two γδT cell negative isolation kits.
γδT cells were isolated from freshly isolated from 25M PBMCs from the same three donors using two different isolation kits (Miltenyi Biotec or EasySep) and then stained for γδTCR to compare purity. (**A**) The proportion of γδT cells (bottom right quadrant) within the isolated fraction of cells. Data shown is a representative of n=3. (**B**) percentage of γδT cells within isolated fraction of cells (left) and percentage of γδT cells of starting PBMC (right). Data shown is n=3

3.5.6. Detachment of THP-1 cells

THP-1 cells originate from a human leukaemia monocytic cell line, which have been used extensively within the monocyte/macrophage field to study characteristics such as functions, mechanisms, phenotypic expression profiles and changes and signalling pathways.

During our assays we aimed to measure the expression of CD14 on γδT cells. Thus, we used PMA-stimulated THP-1 cells as a positive control for these assays. Upon

PMA-stimulation, THP-1 cells differentiate into macrophage-like cells and when doing up-regulate various cell markers. They also become more adherent. Typically cultured within flasks, most methods of detachment of THP-1 cells from flasks suggest the use of trypsin and EDTA for removal. However, there are conflicting statements on the effect of trypsin on removal of receptors from the cell surface (292,293), thereby skewing the subsequent measurement of markers and tarnishing the use of PMA-stimulated THP-1 cells as a positive control. Thus, we tested 4 methods of detachment to remove PMA-stimulated THP-1 cells from flasks – flushing the flasks with either PBS, EDTA, trypsin/EDTA or citric saline solutions. The PMA-stimulated THP-1 cells we were then measured for CD14 expression.

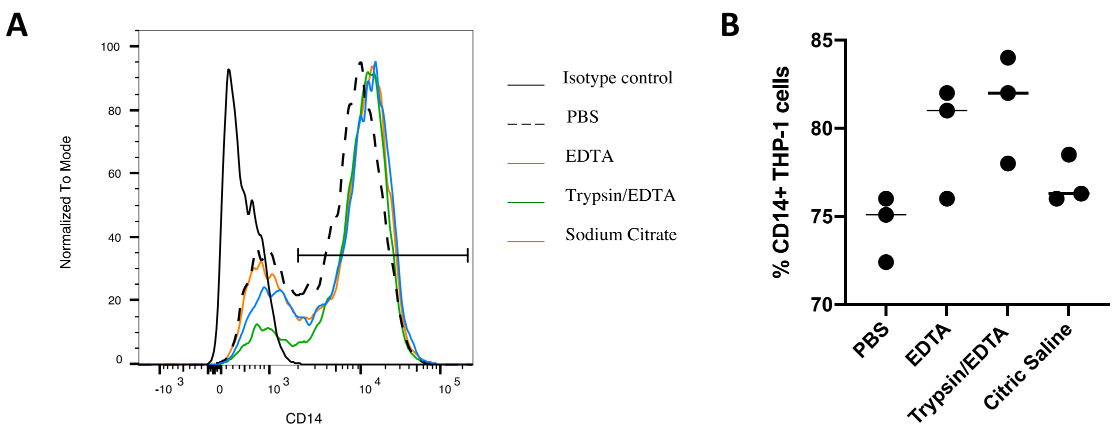

Figure 3.9 – Testing the effect of different detachment methods of THP-1 cells on CD14 expression.
THP-1 cells were stimulated with PMA at 10ng/ml and removed from flasks using different methods of detachment. The impact on CD14 expression measured by flow cytometry. (**A**) Representative histogram displaying CD14 expression after different methods of detachment. (**B**) Graph of cumulative data showing effect of detachment methods on CD14 expression. Data shown is n=3.

We found no significant difference in levels of CD14 with the four methods of detachment. However, the yield of THP-1 cells removed were greatest with trypsin/EDTA, thus moving forward, trypsin/EDTA was used to remove THP-1 cells from flasks.

3.6. Discussion

As the experiments conducted in this chapter vary in nature, covering the diverse procedures involved in the optimisation of bacterial assays, γδT cell assays and combination there within. As such these results will therefore be discussed here in sections.

3.6.1. *E. coli* expansion of γδT cells is variable and subject to successful inactivation of bacteria by UV

The first observation to discuss was the inability for *E. coli* and *S. aureus* bacteria to expand γδT cells. This finding was particular perplexing as Barisa *et al* was previously able to expand γδT cells using the same *E. coli* bacterial strain and methods (100). However, we proposed that the UV apparatus (transilluminator chamber) used for our experiments may be producing irregular UV wavelengths, leading to inefficient UV irradiation.

Moreover, the repeated freeze-thaw cycles of *E. coli* stocks over time can lead to evolutional or mutational changes. For instance, it is suggested that the ability to survive multiple freeze-thaw cycles can come at a trade-off within certain bacterial species, with regards to performance in minimal glucose medium at 37°C (294). This suggests that the freeze-thaw cycle can affect bacterial performance and by theory also effect bacterial morphology and other such characteristics which may in tern effect its survival after UV irradiation and/or its ability to cause γδT cell expansion in PBMC culture. It is also possible that the *E. coli* bacteria within our stocks consist of a heterogenous bacterial population consisting of *E. coli* that have obtained ability to tolerate UV stress. Data has shown that the growth-rate of the cultivating bacteria to play a role in the success of UV irradiation, as well as presence of the stress-response gene RpoS (295,296). This is supported by findings by Blatchley 3rd *et al* investigating dose-responses to UV, where it was suggested that bacterial cultures were comprised of two sub-populations – low resistance and high resistance population in response to

UV (239). They showed that the high-resistance population conferred variable behaviour, which is a similar observation made in our *E. coli* expansions (figure 3.6).

Moreover, it was shown that the *E. coli*-mediated expansion of γδT cells was not due to the γδTCR (100), meaning that it was less likely a cause of phosphoantigenic stimulation from *E. coli* metabolism (HMBPP), but rather a response from other cells within the PBMC fraction, perhaps upon recognition of *E. coli* markers. Thus, it is possible that the *E. coli* DH5α may have acquired altered expressions profiles of these triggers which were responsible for the γδT cell expansion exhibited by Barisa *et al.* Alternatively, it is possible that the UV irradiation performed previously conferred morphological and physical changes within the bacteria, to therefore trigger expansion of γδT cells.

To conclude, UV-irradiation is a feasible method for means of inactivation of bacteria but it is unclear and unlikely that it results in 'killing' of bacteria (239). It is highly probable that the UV irradiation does not result in bacteria reminiscent of the death phase but rather leads to bacteria that are more like those in the long-term stationary phase. It is therefore important to consider bacterial repair processes in the design of UV irradiation systems and when considering this technique for experimental use (239).

3.6.2. Using zoledronate to expand γδT cells for experimental use

We were unable to consistently expand γδT cells using bacteria. Thus, moving forward in subsequent chapters, zoledronate was used to exclusively expand γδT cells as this provided us with the ideal, consistent results. Moreover, the use of one stimulus to expand γδT cells, one that is employed throughout γδT cell literature (IL-2 + zoledronate), allowed us to draw direct parallels between killing activity in comparison to other studies. Barisa *et al*. showed *E. coli* and zoledronate-expanded γδT cells to display similar phenotypic characteristics after 14 days of expansion, thereby justifying the use of zoledronate-expanded γδT cells for use in our assays (100).

Moreover, use of zoledronate provides a better stimulus for comparing the killing activity bacteria. Using *E. coli* for γδT cell expansions would add the additional variable of *E. coli* priming of γδT cells that may lead to enhanced killing of *E. coli* compared to *S. aureus*. This is particularly important to consider as few studies have shown the capacity of γδT cells to acquire a memory phenotype (297,298). For instance, studies on γδT cells and mycobacteria have shown expression of surface markers distinctive of central memory T cells ($CD45R^{-}CD27^{+}CD62L^{hi}$) on *M. bovis*-specific γδT cells as well as expression of tissue-homing receptors CXCR3 and CCR5 (299).

3.6.3. Acquiring freshly isolated γδT cells for experimental use

Whilst adequate cell numbers were achievable in experiments utilising 14-day expanded γδT cells, testing fresh γδT cells in the same experimental assays presented a problem due to their low numbers within PBMC (13). Thus, for experiments using fresh γδT cells, large volumes of blood (500mL) were used for PBMC isolations to harness best possible yields of γδT cells, before cell isolation for obtaining pure γδT cells was performed. As the subsequent yield of PBMC from these larger volume isolations was extremely high (often >200 million PBMC), FACS sorting for γδT cells was not feasible. In addition to this, FACS sorting for γδT cells would require the use of CD3 and αβTCR staining and gating/sorting for the αβTCR- CD3+ cells to ensure capture of the γδT cells. This is due to the fact that using a pan-γδTCR antibody or even Vδ2-specific antibody to sort for γδT cells would mean possible activation of receptors (via binding of the staining antibody) and activated TCR states of γδT cells would thereby nullify and invalidate any pro-inflammatory results from the subsequent experiments. Therefore, when isolating γδT cells from PBMC via flow cytometry, staining of the CD3 receptor (for gating on T cells) and then αβTCR (for distinguishing between γδT cells and αβT cells), would be the suggested method to avoid disrupting or engaging with the γδTCR and prevent activation of the receptor before use in the experimental assays of interest. However, the biggest issue with using FACS for γδT cell isolation and reasons for which it was not the optimum method used for the killing assays are as follows:

1) The yield of PBMC from the 500ml blood isolations was extremely high (often >200 million PBMC), subsequent staining and sorting would require extremely large volumes of antibodies and extensive use of FACS cytometers; to a degree that was not feasible.
2) By gating on αβTCR-CD3+ for the presumed γδT cell population, there is no guarantee of >99% purity; possible contaminants include NKT cells which express CD3 but do not express the αβTCR.
3) Sorting an appropriate number of γδT cells via FACS is time-consuming, thus not allowing for experiments to be performed the same day.
4) In relation to point 3) The resulting γδT cells will require 24 hours of rest due to disturbance and/or activation occurring from sorting, as the sample passing through the flow cytometer could lead to potential TCR internalization in response to purification. Thus, the resulting fresh γδT cells will in fact not be D0 γδT cells when used for assays.

Therefore, as an alternative to this, we implemented the use of commercially available negative isolation kits by Miltenyi and EasySep. Both these kits utilise the use of magnetic beads and a cocktail of antibodies to bind to all non-γδT cells, leaving behind a pure population of intact, unbound and otherwise untouched, γδT cells. Each of these kits work in a similar manner and solve both problems that arose from using FACS for γδT cell isolation; they are less labour intensive, which provides the added benefit of the isolated γδT cells being as close to their 'fresh' state as possible and they allow for a pure population of γδT cells to be isolated, without activation of the γδTCR.

We therefore assessed both kits for purity of γδT cell within the isolated fragments to determine which kit was to be used for further experiments. As well as being quicker than the MACS protocol, the EasySep kit and protocol yielded higher purity of γδT cells consistently, thus was used henceforth for γδT cell isolation.

3.7. Summary

Data in this chapter show that use of bacterial stimuli, namely *E. coli* DH5α and *S. aureus* NCTC 6571 strains lead to inconsistent and repeatedly unsuccessful expansions of γδT cells (*E. coli* and *S. aureus* bacteria respectively) compared to use of zoledronate. These observations and findings are evident despite UV irradiation successfully inhibiting bacterial growth. This lead us to primarily utilise zoledronate as our stimuli for expansions going forth, for which we exhibited repeated successful, but donor-variated expansion rates.

We also found that both commercially negative isolation kits for γδT cell isolation harness γδT cells with over 95% purity, with high viability for use when needing high volumes of fresh γδT cells from large volumes of blood that would otherwise not be feasible from FACS sorting.

4. Exploring the cytotoxic activity of freshly isolated and zoledronate-expanded γδT cells against bacteria

4.1. Aims and Objectives

This chapter aims to utilise findings from the previous chapter with regards to:

i) bacterial assays (utilisation of growth curve findings for CFU values against OD measurement)

ii) γδT cells cell isolation methods

iii) γδT cells cell expansion methods

Using co-infection assays of γδT cells with bacteria, we aimed to assess the bactericidal activity of γδT cells, utilising both zoledronate + IL-2 stimulated, 14-day expanded as well as freshly isolated (day 0) γδT cells (with no prior exposure to zoledronate or IL-2 stimulation). These findings were paired with flow cytometry analysis of γδT cell phenotype. Together, this data aims to answer the following questions:

1. *What is the impact of zoledronate + IL-2 stimulation on the phenotype of γδT cells?*

Through the use of flow cytometry, we have aimed to look at characteristic markers of inflammation and cytotoxicity, to assess how zoledronate stimulation shapes γδT cells into a more activated phenotype.

2. *Can γδT cells kill bacteria?*

In conjunction with data on activation of γδT cells after zoledronate stimulation, we are subsequently interested in whether these γδT cells can exhibit inflammatory properties that are sufficient to kill bacteria. In addition to this, we also explore whether this killing capacity is specific to zoledronate-expanded γδT cells or whether freshly isolated γδT cells can also kill bacteria, and whether they can achieve killing to the same degree as zoledronate + IL-2 stimulated, 14-day expanded γδT cells.

3. *Is the killing capacity a unique feature of γδT cells and are E. coli and S. aureus bacteria unique targets?*

To understand how unique this killing capacity was to γδT cells, we explored whether other freshly isolated lymphocytes were able to kill *E. coli* and *S. aureus* bacteria. To further elucidate the cytotoxicity involved, we also explored whether freshly isolated γδT cells were able to kill cancer cells, to a similar degree as their killing of bacteria.

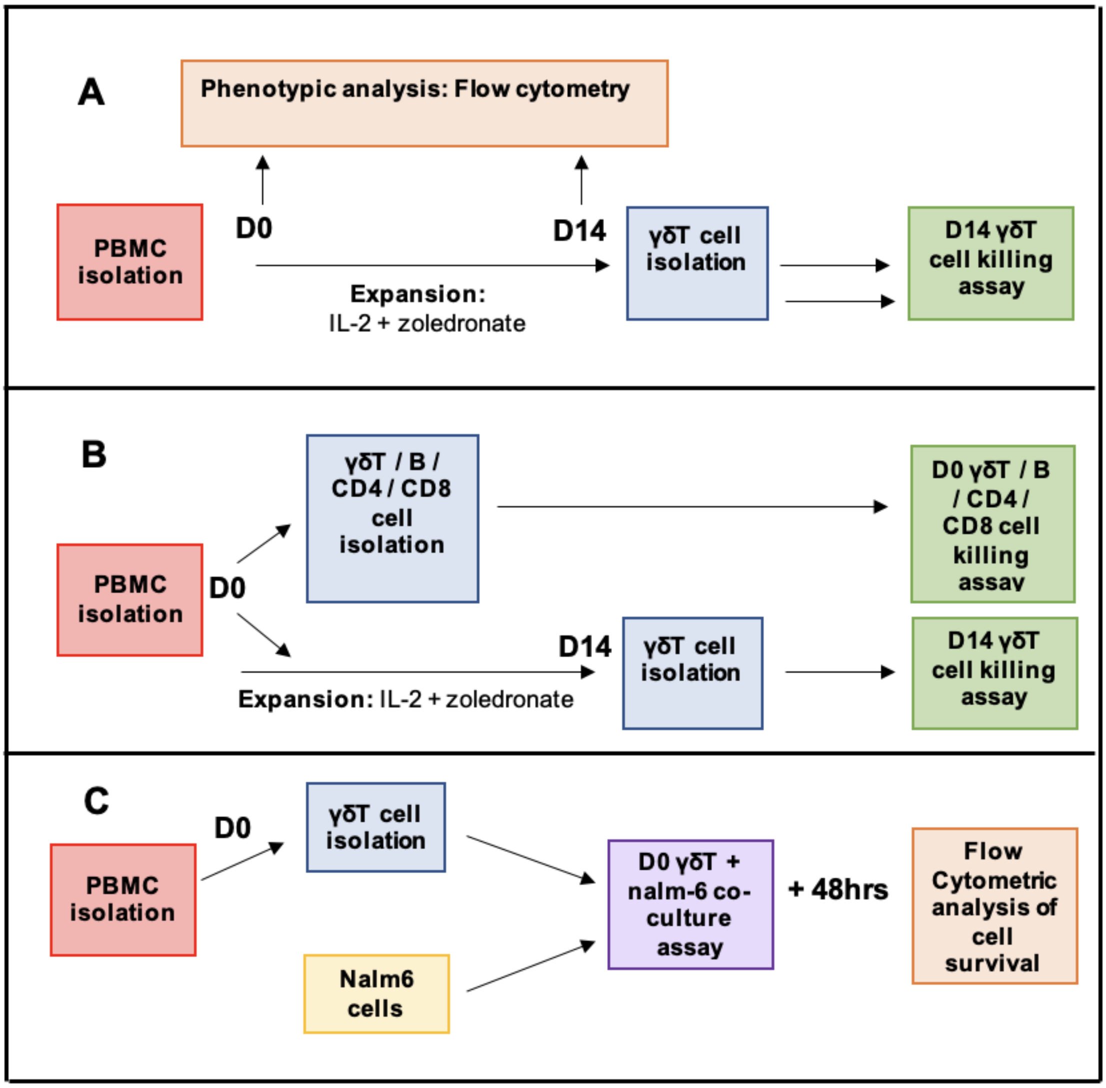

Figure 4.1 – Visual schematic of workflow

(A) PBMCs were isolated from fresh whole blood donated by healthy donors and divided across two assays for use towards characterisation and expansion. The staining assay was performed according to methods outlined in section 4.3, with relevant cell surface markers to gather data on initial phenotype distribution of γδT cells before and after expansion with zoledronate. PBMCs were stained for flow cytometric analysis at day 14 whereby the remainder was collected for FACS sorting to acquire pure γδT cells for use in killing assays. **(B)** PBMCs were isolated from fresh whole blood donated blood by healthy donors and divided for γδT cell expansion and D0 killing. The PBMCs in the D0 killing arm of the experiment were then further isolated for cell population of choice. The purified γδT, CD4, CD8 or B cells were then tested for killing of bacteria. After 14 days, the expanded γδT cells were also tested for killing of bacteria. **(C)** PBMCs were isolated from fresh whole blood donated by healthy donors and then further isolated for purified γδT cells. The γδT cells were co-cultured with Nalm-6 cells and assessed for cell survival after 48 hours.

4.2. Introduction

4.2.1. γδT cells as cytotoxic killer cells

γδT cells are known as potent effector cells, playing a vital role in controlling infection from pathogenic microbes as well as potent antitumour activity. However, key features of their cytoxicity specifically with regards to antimicrobial activity, including the mechanisms, receptors and stimulating antigens involved have not yet been identified.

More understood and of substantial focus in the therapeutics field is the promising capacity of γδT cells to kill tumorous cancer cells. Typically, their activation is mediated via the γδTCR, or through other activating receptors akin to NK cells such as NKG2D (74). Upon activation, γδT cells are able to produce IFN-γ and TNF-α cytokines reminiscent of a Th1 response. γδT cell activation also results in maturation of monocyte-derived DCs, which subsequently promotes activation of αβT cells (27). By this means, γδT cells can not only exhibit cytoxicity themselves but also promote cytoxicity of their more common counterparts. A meta-analysis of 18,000 human tumours of 25 different types showed correlation between γδT cells and positive outcomes in both pan-cancer and solid tumour analysis (300). Thus, the inflammatory nature and cytotoxic capability of these cells is of crucial host benefit.

Other cytotoxic abilities of γδT cells include their ability to exert immunomodulatory effects via signalling through their PRRs, as well as expression of CD16 (FcγRIII), which plays a vital role in their innate immune responses. Like NK cells, CD16 expression on γδT cells enables antibody-dependent cellular cytoxicity (ADCC) via interaction with antibodies bound to antigen targets. Cross-linking of multiple bound CD16 receptors leads to release of cytotoxic granules within the γδT cell or up-regulation of other cell death-inducing molecules (301). Importantly, γδT cells have been shown to display ADCC against tumour cell targets in presence of anti-CD20 monoclonal antibodies (mAbs). This cytotoxicity was increased in the presence of phosphoantigen bromohydrin pyrophosphate (302), indicating specific and enhanced ADCC capabilities of γδT cells.

Granzymes are another essential aspect of γδT cell cytotoxicity; they are a form of membrane-bound proteases present in lymphocytes such as NK cells and T cells. There are 5 known types of granzymes present in human cells; granzymes A, B, H, K and M, which share structural similarities but vary in their substrate specificity (303). Granzyme release is a prominent feature of γδT cell cytotoxicity against tumour cells (304). This is supported by studies showing a reduction in frequency and function of γδT cells in breast cancer patients, whereby association with the known granzyme B gene polymorphism was found to be significantly associated with these patients (305). Studies have shown granzyme A produced by the Vγ9δ2 subset of γδT cells to impact monocytes infected with intracellular mycobacteria. Granzyme release by these γδT cells leads to TNF-α released by the infected monocytes which subsequently lead to suppression of growth of the intracellular mycobacteria. The inhibitory effect displayed by γδT cell mediated-granzyme release on the infected macrophages was shown to be independent of other cell death-related mechanisms such as autophagy, perforin, nitric oxide production, Fas/FasL interaction and apoptosis (306).

Another vital aspect of γδT cell cytoxicity and pathogen inhibition and/or killing is the ability of γδT cells to engage in phagocytosis. Phagocytic capabilities have been described in few studies and involve a number of different pathogens (100,307–309). One such example of this resides in investigations of γδT cells and the parasite *Plasmodium falciparum (P. falciparum*), which causes malaria. The Vγ9δ2 subtype of γδT cells has been shown to kill infected red blood cells (iRBCs) via two mechanisms. The first; TCR-dependent reaction to phosphoantigens produced by iRBCs via immune synapse formation and degranulation and the second; CD16-dependent phagocytosis and degradation of opsonized iRBCs (308). Additionally, studies using the *Listeria monocytogenes* bacteria showed morphological characteristics of phagocytosis within γδT cells via transmission electron microscopy and upregulation of antigen presentation markers (307). The γδT phagocytosis performed by γδT cells in response to *L. monocytogenes* was coupled with increased CCR7 expression, suggesting that phagocytosis triggers antigen presentation in γδT cells.

Lastly and as previously described by our group, is the ability for γδT cells to behave as pAPCs, which contributes to their cytoxicity (100,307). Himoudi *et al*. showed licencing of Vγ9δ2 T cells, whereby the cells acquired APC functions after recognition of antibody-opsonisation of tumour cells. Interestingly, γδT cell targeted killing of these tumour cells also attributed to the release of tumour-associated antigens into the microenvironment, leading to potentiated licencing and killing of tumour cell targets (310). Other APC functions of functions have been highlighted by Howard *et al,* whereby infections with *P. falciparum* were investigated. Findings showed increased expression of APC-associated markers HLA-DR and CD86, similar to Barisa *et al* (2017). Moreover, the Vγ9δ2 T cells were shown to induce naive αβT cell as well as exhibit cross-presentation of protein to antigen-specific CD8+ T cells (311).

In summary, although not fully understood γδT cells clearly exhibit a vast cytotoxic capacity, through many mechanisms. The cytotoxic killing capacity has been harnessed for therapeutic uses through the licencing of zoledronate and γδ CAR T cells for cancer patients (52,219). However, further investigation and understanding is needed to utilise other features of γδT cells, such as their antigen presenting and phagocytic capabilities which not only play a vital role in rapid and innate recognition but are able to successfully recruit adaptive components of the immune system. In addition to this, it is important to consider γδT subtype, both in terms of the δ-chain subtype classification and of effector population classification, such as the γδT, γδT2, γδT17 and γδTregs populations, where preferential stimulation and expansion of particular subsets may be required to enhance specific effector functions (107–109).

4.2.2. γδT cells and *E. coli*

A few studies have shown a direct interaction between *E. coli* and γδT cells and it is clear that γδT cells provide an innate immune reaction to *E. coli* bacteria. Data has been shown that a large percentage of PBMCs that respond to *E. coli* and *Pseudomonas aeruginosa (P. aeruginosa*) infections were γδT cells. Comparisons of early T cell responses from PBMC in both the adult and newborn populations showed intense γδT cell proliferation of both Vδ1 and Vδ2 subtypes. The proliferation of γδT cells from PBMCs in the newborn population showcases the importance of γδT cells in natural immunity to these bacteria, as prior exposure was not needed for an adequate γδT cell response. Protease treatment of the bacterial preparations still yielded these expansions, meaning the stimulating antigens were not proteins. Interestingly the expanded γδT cells in adults mostly comprised of Vδ2γ9 T cells, whereas the expanded γδT cells in newborn's PBMC comprised of mainly the Vδ1 subset (156).

Our lab has published data to show *E. coli* stimulation to promote human γδT cell transition from a cytokine- producing state, representing γδT cells in a naïve state, to a phagocytic state, representing effector γδT cells. Similar experimental procedures were used in our experiments with zoledronate being the stimuli of choice for γδT cell expansion. Data from these experiments also displayed phagocytic capabilities of 14-day *E. coli* expanded, γδT cells (100). This shows that γδT cells have a direct response to *E. coli* bacteria. γδT cell response to bacteria is driven by the accumulation of phosphoantigens from the deoxyxylulose 5-phophate pathway in bacterial metabolism (162). Based on experiments utilising mutant *E. coli* genes products: *gcpE* and lytB, HMBPP has been identified as responsible for the accumulation of IPP biosynthesis (312). Interestingly, the development of the γδ APC phenotype described by Barisa *et al*., was accompanied by a loss of cytokine production. However, cells maintained cytotoxic degranulation and bactericidal activity (100). The ability of γδT cells to behave as APCs is supported by various studies (313)

Thus, as a known antigenic stimuli of γδT cells can be identified in *E. coli*, it is therefore unsurprising to find scientific data to support an immune-related role of γδT cell upon *E. coli* infection. Most of the responses identified in γδT cells involve the signalling and recruitment of other immune cells and pathways, proving γδT cells to be amplifiers of immunity. A large amount of the data showing this in the context of *E. coli* infection has occurred in mice. For instance, murine studies have displayed an increased number of γδT cells within the peritoneal cavity after inoculation with *E. coli* ATCC 26 (314). Interestingly, this increase in number was shown to be most noticeable 5 days post-inoculation, despite pathogens already being successfully eliminated from the host. This points to the evidence of lasting immunity from γδT cells, which can also contribute to lasting inflammation present in conditions where γδT cells play a key role, such as IBD (198). Many autoimmune inflammatory conditions such as these showcase an unclear and poorly understood mechanism by which initial inflammation occurs. As *E. coli* is a commensal present in the mucosa, it can be inferred that a disruption of microbiota can lead to triggering of specific γδT cell subsets and disruption of subtype proportions, leading to excessive inflammation, like that of coeliac disease (77).

Additional studies in mice have shown that γδT cells of the Vγ6δ1 subtype to release IL-17A after *E. coli* infection, which aids in neutrophil migration (163). Data has pointed to both IL-23 and IL-15 playing an important role in γδT cell response to *E. coli* depending on subtype; with Vγ4δ1 T cells influenced by IL-23 and Vγ9δ1 influenced by IL-15 (10). This therefore shows how subtype specificity with regards to immunity to specific pathogens, i.e. *E. coli*, within mice (164). However, any similar pattern of data in humans is yet to have been identified.

4.2.3. γδT cells and *S. aureus*

Although it is clear that γδT cells play a role in the immune response to *S. aureus* infection, unfortunately much of the data that elucidates more detail into this relationship has been gathered through murine studies as described in chapter 1. Such studies have highlighted an impaired bacterial clearance in γδT cell deficient mice, through an inability to promote the correct inflammatory environment (158,160).

In a study investigating impact on human γδT cells by various types of bacteria, it was found that stimulation with *S. aureus* amplified γδTCR expansion (315). However, it is important to note the sample size for this was limited to 6 donors and the expansion was only visible in 33% of donors, which can be attributed to donor-variability as shown by our data in chapter 3, in response to zoledronate stimulation. Interestingly, this study highlighted evidence of specific antigen recognition with regards to γδT cell recognition by bacteria, as different donors responded to different bacteria species. This is supported by findings in a more recent study by Cooper *et al*, whom have showed human Vδ2 T cells to undergo rapid activation in response to *S. aureus*-infected monocyte-derived DCs (161). Co-culture of the γδT cells and infected DCs showed increased surface expression of CD86, in a contact-dependent manner. Thereby suggesting *S. aureus* to express a γδT cell-activating molecule. The results suggest that *S. aureus* infection of monocytes, as well as activation of γδT cells leads to Th1 recruitment in response to *S. aureus*.

Other data on human γδT cells responses to *S. aureus* have been gathered though mice models utilising human γδT cells. Wang *et al*. explored the use of Vγ2δ2 cells which are only found in primates, within a chimeric severe combined immunodeficiency *(*SCID) mouse model (hu-SCID) (316). Their findings showed impact of Vγ2δ2 cells on *S. aureus* and *E. coli* and *Morganella morganii* bacteria within the mice, with bacterial clearance being evident 1 day post-infection, which occurred before the expansion of the γδT cells. This phenomenon leads to the idea of possible memory response of γδT cells, due to the rapid immune response. Interestingly the converse was observed in the Vδ2 T cell–depleted hu-SCID mice, coupled with decreased IFN-γ levels. Moreover, the treatment of the Vδ2 T cell–depleted hu-SCID mice with pamidronate, lead to an increased antibacterial effect of the Vγ2δ2 T cells (316).

Another such study utilising both mice and human work was that of Dillen *et al* (317). As discussed in chapter 3, *S. aureus* can cause skin infection in humans. However, the mechanisms by which protection against these infections and subsequent reinfections occurs remains unclear and does not seem to involve memory T cell or antibody presence. Utilising a mouse model, they investigated *S. aureus* skin reinfection and found that γδT cells in draining lymph nodes responded to *S. aureus* bacteria. Evidence of TLR-2 signalling of the γδT cells was found to produce inflammatory cytokines TNF-α and IFN-γ leading to neutrophil recruitment and bacterial clearance. This is supported by various studies indicating the importance of inflammatory mediators produced by γδT cells, including IL-22, in neutrophil recruitment and bacterial clearance (158,160,318). Further RNA sequencing of the lymph nodes identified a clonotypic expansion of the γδT cells and additional studies using PBMCs from IRAK4-deficient humans no longer predisposed to *S. aureus* skin infections showed expansion of γδT cells (317).

To sum, the γδT cell involvement in response to *S. aureus* bacteria is multifaceted; with roles in recruitment of other immune cells, which seems to be the ‘innate’ aspect of their response; to direct recognition and rapid inflammatory triggers, indicating a memory and ‘adaptive’ aspect of γδT cell response.

4.3. Methods and materials

4.3.1. PBMC isolation

PBMCs were isolated and prepared for experimental use using the same methods described in section 2.2.2, with the exception of the experiments described in section 4.4.2, whereby X-VIVO™ 15 (Lonza) media was used as a serum-free alternative to RPMI.

4.3.2. Isolation of γδT, CD4+, CD8+ and B Cells from PBMC using EasySep negative isolation kits

All cell isolations took place directly from freshly isolated human PBMC using the EasySep negative isolation kits. Isolation of γδT cells took place as previously described in chapter 2, with isolations of CD4+, CD8+ and B cells taking place as follows:

Preparation of cells:

PBMCs were counted using automated cell counter as previously described, before being centrifuged at 300g for 10 minutes. After this the supernatant was discarded and the cell pellet was resuspended in buffer (prepared as stated in chapter 2). The appropriate volume of buffer was used to obtain a concentration of 5×10^7/ml total cells, with a minimum volume of 0.25mL and a maximum volume of 2mL.

4.3.2.1 Isolation of CD4+ or CD8+ T cells:

The PBMCs at the appropriate volume of buffer were transferred to a 5mL polystyrene round-bottom tube and 50µL of isolation cocktail added per mL of cell suspension, mixed and incubated at room temperature for 5 minutes. The rapidspheres™ were then vortexed for 30 seconds and added in equal volume. The sample was then topped up to a volume of 2.5mL with buffer. The tube containing the magnetically stained PBMCs were then placed, without a lid, into the crevice of the EasySep magnet and left for incubation at room temperature for 3 minutes. The magnet, containing the tube was then inverted in one continuous motion, with contents being poured into a new sterile 5mL polystyrene round-bottom tube. The new tube houses the isolated cells of interest, depending on the exact isolation kit used.

4.3.2.2 Isolation of B cells:

The PBMCs at the appropriate volume of buffer were transferred to a 5mL polystyrene round-bottom tube and 50µL of cocktail enhancer and 50µL of isolation cocktail added per mL of cell suspension, mixed and incubated at room temperature for 5 minutes. The rapidspheres™ were then vortexed for 30 seconds and added in equal volume. The sample was then topped up to a volume of 2.5mL with buffer. The tube containing the magnetically stained PBMCs were then placed without a lid, into the crevice of the EasySep magnet and left for incubation at room temperature for 3 minutes. The magnet containing the tube was then inverted in one continuous motion, with contents being poured into a new sterile 5mL polystyrene round-bottom tube. The new tube containing the enriched B cell suspension was then placed without a lid into the crevice of the EasySep magnet again and left for incubation at room temperature for a further 1 minute. The magnet containing the tube was then inverted in one continuous motion again, into a final tube.

4.3.3. Bacterial killing assays

4.3.3.1 Assessment of γδT, CD4+T, CD8+T and B cell killing of E. coli and S. aureus

For the assessment of cell involvement in killing of bacteria, experiments required preparation of both cells and bacterial cultures for 24 hours before the intended day of experiment.

<u>Preparation of Bacteria:</u>

As such, a single colony of bacteria was transferred to liquid culture of LB (Sigma Aldrich) and left in a shaking incubator to grow overnight at 220 RPM, 37°C.

Preparation of cells:

Freshly isolated cells were left to rest overnight before being tested for killing capacity of bacteria at 37°C 5%CO_2.

The next day, optical density (OD) of the bacteria was measured using a spectrophotometer (U-1800, Hitachi). Overnight culture in a shaking incubator typically resulted in an OD of 1.2, which was diluted 100-fold the following morning and re-grown in the shaking incubator to an OD of 0.5-0.8 for cultivation of bacteria within log-phase (figure 3.1). Once at optimal OD, bacterium culture was centrifuged at 1500g for 10 minutes. The supernatant was discarded, and the resulting bacterial pellet was resuspended in the appropriate volume of LB broth to achieve an MOI of 5 for the killing assays. Dulbecco's phosphate buffered saline (PBS) without $CaCl_2$ and $MgCl_2$ ions (Gibco) was used to resuspend the bacterial pellet and used throughout the killing assays.

Bacterial killing Assay:

20μL of bacteria at the appropriate MOI was added and mixed to the appropriate wells. *E. coli*- and *S. aureus* with PBS alone (no addition of cells) were also incubated for 30 minutes and used as a control for background growth levels of bacteria after 30 and/or 90 minutes. After the appropriate time, cell-bacteria cultures were serially diluted with PBS and 100μL of the appropriate dilutions was dispensed and spread onto LB-agar plates. LB-agar plates were left to grow overnight at 37ºC 5%CO_2 and colonies counted the next day. All *E. coli*- and *S. aureus* -killing experiments took place with non-UV-irradiated bacteria.

4.3.3.2 Assessment of γδT cell killing of Nalm-6 cells

Maintenance of Nalm-6 cells:

Nalm-6 cells (CRL-3273; ATCC), a human -cell lymphoblastic leukaemia cell line, was gifted for use from the UCL department of Biochemical engineering. These cells were originally derived from the peripheral blood of 19-year-old male. The nalm-6 were cultured using RPMI-1640 (Gibco) and 10% FBS.

<u>Killing Assay:</u>

For assessment of γδT cell killing of nalm-6 cells, γδT cells were co-cultured with nalm-6 cells at a ratio of 1:1 and 1:10 respectively at 37°C 5% CO_2 for 48 hours. After this time, the cells were stained with relevant markers CD19 – APC (Biolegend) for Nalm-6 cells and γδTCR – PE (Biolegend) for γδT cells, as well as live/dead stain (Thermo Fisher).

4.3.4. Flow Cytometry Cell Staining and Sorting

4.3.4.1 Cell Preparation

Cells were prepared as stated in general methods and materials chapter.

4.3.4.2 Flow Cytometry staining

Cells were then stained with the following antibodies in the anchor marker panel as well as antibodies in 1 of the 3 additional panels listed:

Panel	Antibody marker	Fluorochrome	Clone	Manufacturer
Anchor marker panel	CD3	BV711	UCHT1	Biolegend
	αβTCR	PE	IP26	Biolegend
	Vδ1	FITC	B6	Biolegend
	Vδ2	PerCP	TS8.2	Thermo Fisher
	Blue live/dead	-	-	Thermo Fisher
1*	IFN-γ	AF674	4S.B3	Biolegend
	TNF-α	AF700	MAb11	Biolegend
2	CD107α	APC	H4A3	Biolegend
	CD27	AF700	O323	Biolegend
	CD69	BV421	FN50	Biolegend
3	HLA-DR	BV421	L243	Biolegend
	CD86	AF700	L243	Biolegend

Table 4.1 - Antibodies used to assess γδT cell phenotype before and after expansion

Testing antibody	Corresponding isotype control
IFN-γ – AF647, 4S.B3	AF647 Mouse IgG1, κ Isotype, MOPC-21
TNF-α – AF700, MAb11	AF700 Mouse IgG1, κ isotype control, MOPC-21
CD27 – AF700, O323	
CD86 – AF700, L243	AF700 Mouse IgG2a, κ isotype control, MOPC-173
CD107α – APC, H4A3	APC Mouse IgG1, κ isotype control, MOPC-21
HLA-DR – BV421, L243	BV421 Mouse IgG2a, κ isotype control, MOPC-173
CD69 – BV421, FN50	BV421 Mouse IgG1, κ isotype control, MOPC-21

Table 4.2 - Isotype controls used to assess true cytokine, cytotoxicity and myeloid marker expression

For staining of panels 2 and 3, antibodies were added to the cells and incubated for 30 minutes at 4°C in the dark before being washed twice at 1250RPM for 5 minutes. Cells were resuspended in 200µl of staining buffer before being transferred to appropriately labelled FACS tubes.

*For panel 1, which included intracellular cytokine staining, antibodies within the anchor marker panel (except the live/dead antibody) were added to the cells and incubated for 30 minutes at 4°C in the dark before being washed twice at 400g for 5 minutes. Cells were then treated with monensin in media at a final concentration of 2µM/ml and incubated for 4-6 hours at 37°C 5% CO_2. Cells were then washed twice with 200µL stain buffer at 400g for 5 minutes and supernatant discarded, resuspended in 100µL of stain buffer and stained with live/dead antibody for 20 minutes at 4°C in the dark. Cells were then washed twice with 200µL PBS at 400g for 5 minutes and supernatant discarded. resuspended in 100µL fix/perm mix (Invitrogen) and incubated at 4°C for 1 hour or overnight, which was prepared in a ratio of 3 parts diluent and 1 part concentrate.

After this, cells were washed twice with perm buffer (Invitrogen) at 400g for 5 minutes and supernatant discarded. Cells were then stained with the appropriate antibodies in panel 1 and incubated for 20 minutes at 4°C in the dark before being washed twice with perm buffer at 400g for 5 minutes. Cells were resuspended in 200µl of staining buffer before being transferred to appropriately labelled FACS tubes.

All processing of FACS staining samples took place with a BD LSR II machine equipped with FACSDiva software and data analysed with FlowJo.

4.3.4.3 Fluorescence-Associated Cell Sorting (FACS) Staining

γδT Cells were prepared for negative sorting using anti-αβTCR; PE and anti-CD3; BV711 antibodies on the BD FacsAria III. γδT Cells were collected from the $CD3^+$$\alpha\beta TCR^-$ fraction.

4.4. Results

4.4.1. 14-day zoledronate expanded γδT cells display an activated myeloid phenotype

In order to assess any phenotypic changes as an effect of zoledronate stimulation and expansion of γδT cells over the course of 14 days, flow cytometry staining for various cell surface markers was performed on donor PBMCs prior to stimulation (day 0) and after the 14 days. We aimed to assess the expression of markers which indicate physiological changes of the γδT cell state as cytotoxic or APC-like. Thus, we assessed the expression of degranulation marker CD107a, the T cell activation marker CD69 as well as CD27, which is expressed on activated T cells. In addition to this, we performed intracellular cytokine staining to assess the expression of proinflammatory cytokines IFN-γ and TNF-α. In addition to this we also assessed the expression of co-stimulation markers CD86 and the MHC class II molecule HLA-DR.

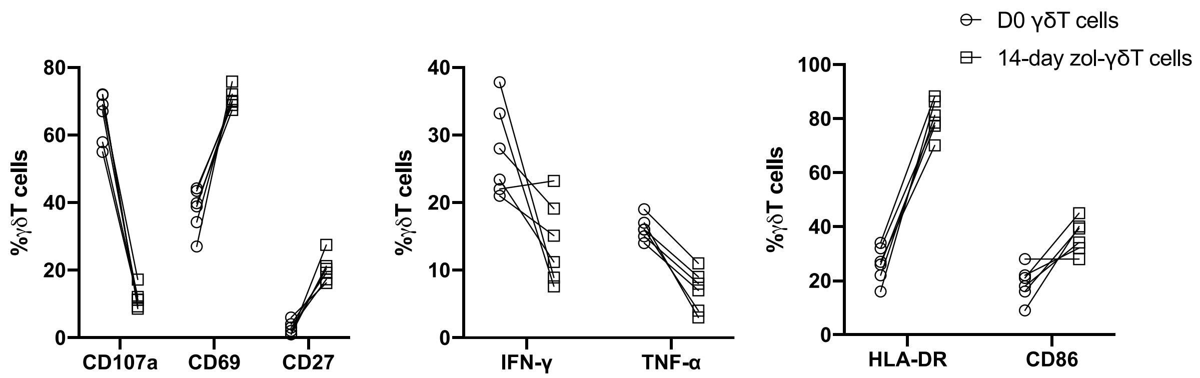

Figure 4.2 – Phenotypic analysis of γδT cells before and after zoledronate stimulation and expansion
γδT cells within PBMCs were assessed for expression of T cell activation markers of cytotoxicity CD107a, CD69 and CD27 (left), proinflammatory cytokines IFN-γ and TNF-α (middle) and T cell helper molecules HLA-DR and CD86 (right), before and after zoledronate-stimulation and 14 days of expansion. Data shown is n=6. Error bars represent s.d.

Our results indicate that zoledronate stimulation of PBMCs induced a γδT cell population that is largely Vδ2+ (figure 3.7), with increased expression of the costimulatory molecule CD86, as well as increased HLA-DR, but decreased expression of proinflammatory cytokines IFN-γ and TNF-α, as well as the degranulation marker CD107a. These findings were consistent with that of Barisa *et al*. Interestingly, whilst the expression of proinflammatory cytokines decreased, the expression of CD27 and CD69 increased. As CD27 is expressed on both early activated and effector memory T cells, the increased CD27 can be both due to expansion of activation of γδT cells or transition of the γδT cells into an effector memory phenotype capable of rapid responses after restimulation.

4.4.2. Both freshly isolated D0 and 14-day zoledronate expanded γδT cells can kill *E. coli* and *S. aureus* bacteria

Once expansion protocols and γδT cell isolation methods had been confirmed, we then embarked on the investigation of whether γδT cells exhibit the capacity to kill whole bacteria, in the absence of other immune cells and inflammatory mediators. To begin, as cell numbers of expanded γδT cells were of adequate number for use in experimental assays, we assessed the capacity for 14-day zoledronate-expanded γδT cells to kill *E. coli* and *S. aureus* via co-culture for 30 and 90 minutes at an MOI of 5. Bacteria in PBS alone (no γδT cell exposure) were used as a mock infection control to assess if there was any residual bactericidal effect of PBS. The CFU resulting from both bacterial alone and bacteria with PBS only were similar, suggesting no noticeable negative or positive effect of PBS on bacterial growth. Our data shows a remarkable bactericidal effect of γδT cells on both *E. coli* and *S. aureus*, with approximately 50% of bacteria killed after exposure to γδT cells for 30 minutes. This then increased to over 80% after 90 minutes of exposure (figure 4.1A).

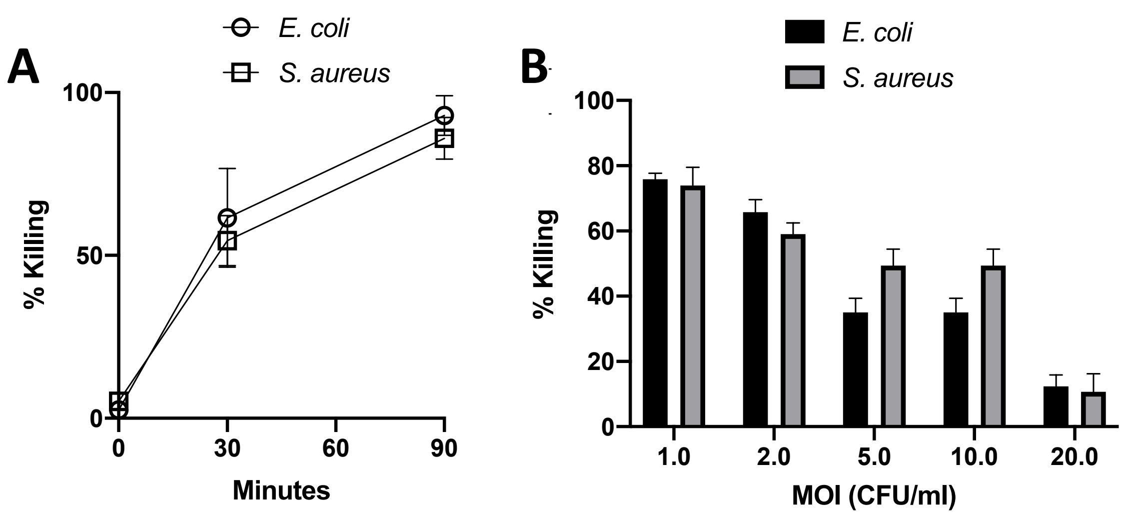

Figure 4.3 – 14-day Zoledronate-expanded γδT cells can kill bacteria
14-day zoledronate and IL-2 expanded γδT cells were tested for killing of *E. coli* and *S. aureus* bacteria. Error bars represent s.d. (**A**) γδT cell-mediated killing of *E. coli* and *S. aureus* over the course of 30 and 90 minutes at an MOI of 5. Data shown is n=5. (**B**) γδT cell-mediated killing of *E. coli* and *S. aureus* over the course of 30 minutes at various MOIs. Data shown is n=3.

We also performed a titration of various MOIs of bacteria co-cultured with γδT cells. Data from these experiments shows 14-day expanded γδT cells capable of killing both fresh *E. coli* and *S. aureus,* with enhanced killing as the MOI decreased, as expected.

This is interesting to note when considering the results to indicate inability of γδT cells to expand when exposed to *S. aureus* or *E. coli* stimulation. However, as previously stated, can be explained by ineffective UV-irradiation of bacteria prior to stimulation. *S. aureus* was unable to expand γδT cells (figure 3.3) but was still killed by γδT cells (figure 4.3A).

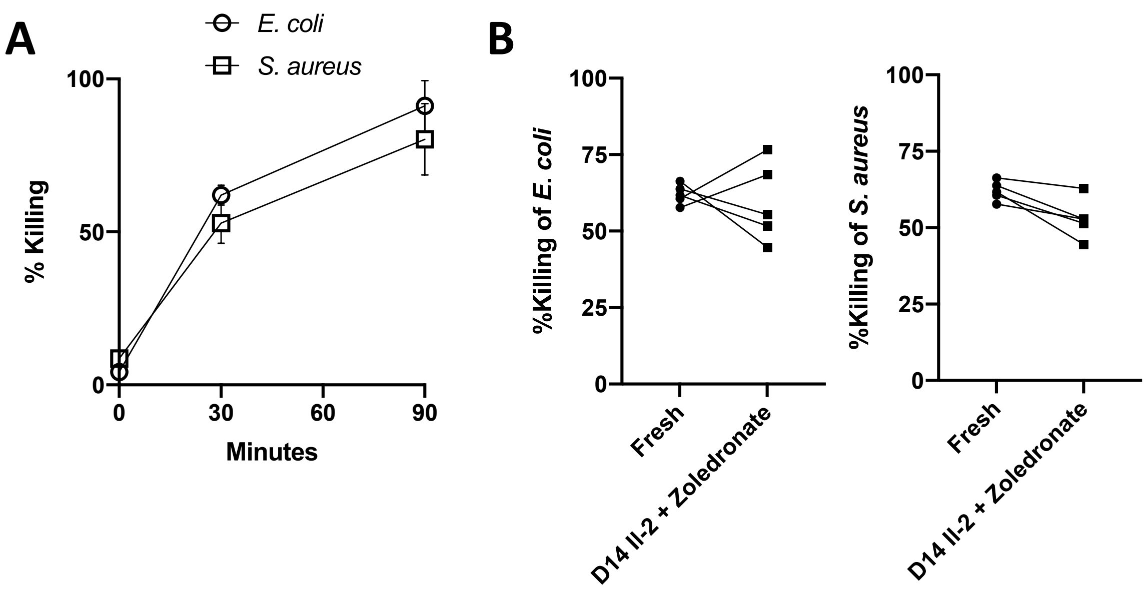

Figure 4.4 – Freshly isolated γδT cells can kill bacteria
PBMCs from the same donors were subjected to immediate isolation of γδT cells or 14-day zoledronate and IL-2 expansion. Resulting γδT cells were tested for killing of *E. coli* and *S. aureus* bacteria. Data shown is n=5. Error bars represent s.d. (**A**) Freshly isolated γδT cell-mediated killing of *E. coli* and *S. aureus* over the course of 30 and 90 minutes at an MOI of 5. (**B**) Comparisons of γδT cell-mediated killing of *E. coli* and *S. aureus* over the course of 30 minutes from freshly isolated (day 0) γδT cells and 14-day IL-2 and zoledronate-expanded γδT cells from the same donors.

Our findings are supported by various studies which show γδT cells expanded via use of zoledronate to be enhanced in cytotoxic capabilities (52,100,319). Therefore, to assess whether γδT cells express intrinsic antibacterial capabilities, without prior stimulation and away from influence of other immune cells, we then explored whether the antibacterial effects could be extended to freshly isolated γδT cells. These experiments showed novel findings of freshly isolated γδT cells to kill *E. coli* and *S. aureus* bacteria, to a similar capacity of 14-day zoledronate expanded γδT cells. Interestingly, there seemed to be less variability between killing capacity from freshly isolated γδT cells compared to the donor-matched 14-day zoledronate expanded γδT cells. This suggests a phenotypic change upon expansion with zoledronate that may affect killing capacity of γδT cells, leading to the variation observed.

Lastly, to address any concerns of the effect of media used on γδT cell acquisition of markers, receptors or any other soluble factors that may influence the killing capability exhibited against the bacteria, we performed the same bacterial killing experiments in RPMI as well as X-VIVO™ 15, as a representative of serum-free media, using the same donors. This aimed to dually assess γδT cell cytotoxicity performance in absence of serum for greater clinical translation, as well as overcome any serum-based influences of the media used. Our results were consistent with previous findings, with use of X-VIVO™ 15 media exhibiting similar levels of bacterial killing as use of RPMI media.

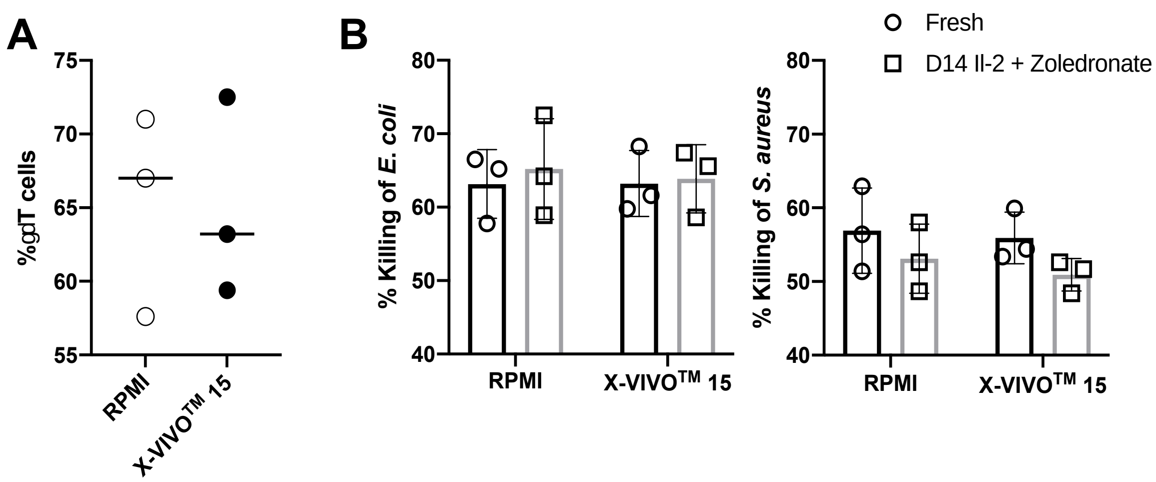

Figure 4.5 – The presence of serum in media has no effect on γδT cell-mediated killing of bacteria

PBMCs from the same donors were subjected to immediate isolation of γδT cells or 14-day zoledronate and IL-2 expansion, in either X-VIVO™ 15 media or RPMI media. Resulting γδT cells were tested for killing of *E. coli* and *S. aureus* bacteria. Data shown is n=3. Error bars represent s.d. (**A**) Percentage of γδT cells amongst CD3+ lymphocytes after IL-2 and zoledronate expansion for 14 days in either RPMI or X-VIVO™ 15 culture media. (**B**) Comparisons of RPMI or X-VIVO™ 15 culture medias on the γδT cell-mediated killing of *E. coli* (left) and *S. aureus* (right) over the course of 30 minutes at an MOI of 5. Open circles denote the use of freshly-isolated γδT cells whilst open squares denote the use of 14-day IL-2 and zoledronate-expanded γδT cells.

4.4.3. Killing of bacteria by freshly isolated lymphocytes is unique to γδT cells

To provide insight as to whether the innately cytotoxic capabilities of γδT cells to kill bacteria was indeed specific to γδT cells, we investigated the use of other lymphocytes. For consistency, ease, and efficiency, we employed the use of EasySep negative isolation kits for B, CD4+ and CD8+ T cell isolation from PBMC, alongside the previously used γδT cell isolation kit. Cell isolations were performed from freshly isolated PBMCs from the same three donors and killing of *E. coli* and *S. aureus* bacteria were tested as before. Our results showed statistical significance of enhanced capability of γδT cells to kill both *E. coli* and *S. aureus* bacteria, compared to CD4+ as well as CD8+ T cells and B cells. γδT cells exhibited 60% and 57% killing of *E. coli* and *S. aureus* respectively, whereas CD8+ T cells, which displayed the next most killing of bacterial cells, exhibited 46% and 38% killing of *E. coli* and *S. aureus* respectively.

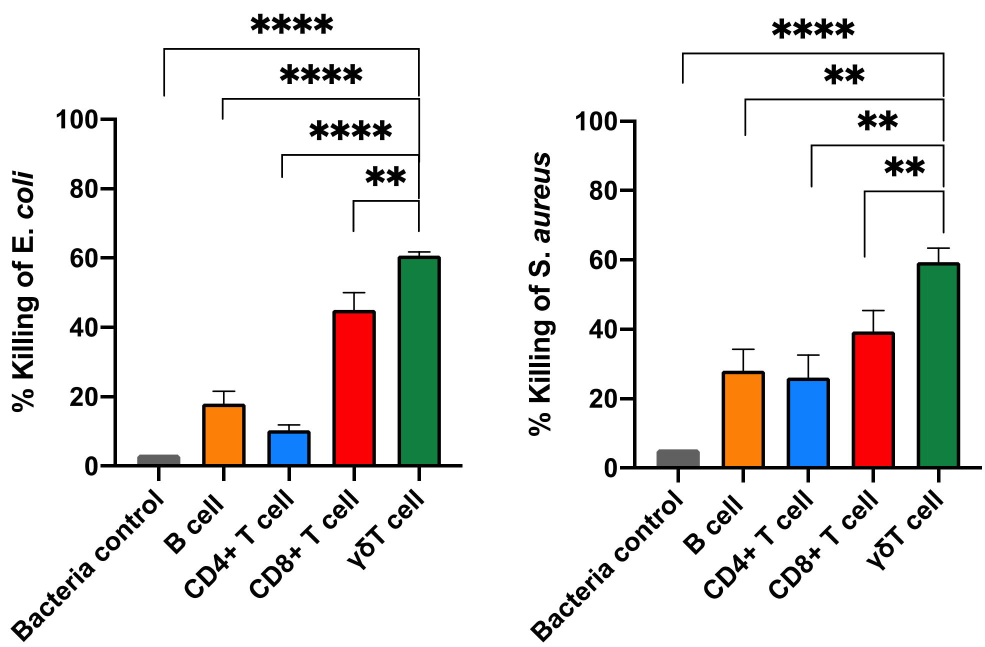

Figure 4.6 – Freshly isolated, unstimulated γδT cells can kill bacteria more efficiently than other freshly isolated lymphocytes

γδT, CD4+, CD8+ and B cells were isolated from freshly isolated PBMCs from the same three donors and tested for bactericidal killing capacity against *E. coli* (left) and *S. aureus* (right). Cells were exposed to bacteria at an MOI of 5 for 30 minutes and CFU of remaining bacteria calculated. Data shown is n=3. ** (P < 0.005), *** (P <0.0005) and **** (P < 0.0005).

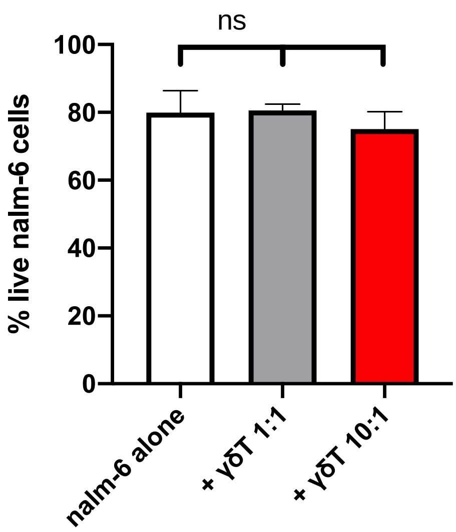

Figure 4.7 – Freshly isolated, unstimulated γδT cells cannot kill nalm-6 cells
Freshly isolated γδT cells were co-cultured with nalm-6 cells at an effector: target ratio of 1:1 and 10:1 for 48 hours and viable cells measured by flow cytometry. Data shown is n=3.

We also aimed to assess whether the enhanced cytotoxicity of freshly isolated, unstimulated γδT cells could be extended to non-bacterial targets. Thus, we employed co-culture of freshly isolated γδT cells to nalm-6 cells, a B-cell lymphoblastic leukemic cell line, representing tumour cell targets and a form of stressed-self target cells. Nalm-6 cells were cultured with γδT cells in a 1:1 or 10:1 effector/target cell ratio. However, our results did not suggest capability of freshly isolated, unstimulated γδT cells to kill nalm-6 cells. This is in direct contrast to zoledronate-expanded γδT cells, which have been shown by numerous studies to display antitumour capabilities (52,320)

4.5. Discussion

Like αβT cells, γδT cells display an ability to respond to particular antigens through interaction with the γδTCR as well as other receptors to engage in downstream effector functions, involving cytotoxicity and other inflammatory effects. It is therefore unsurprising that engagement of different receptors by varying stimuli can result in different physiological states of γδT cells. For instance, in response to zoledronate γδT cells undergo a cytotoxic phenotype change that has been advantageous for treatment of cancer. Upon initial stimulation the γδT cells display a spike in cytokines an cytotoxicity markers. However, we have shown that after 14 days of this expansion, the γδT cells then acquire a unique phenotype of CD86+HLA-DR+CD27+ that is distinct from their naïve, unstimulated state. The increased levels of CD27 from baseline (day 0), indicates that zoledronate stimulation can licence γδT cells into a phagocytic, helper-like cells capable of a rapid immune response upon re-stimulation, the latter of which was confirmed by Barisa *et al* (100).

4.5.1. Exploring the bactericidal activity of freshly isolated and 14-day zoledronate-expanded γδT cells

We therefore explored whether these γδT cells would showcase these capabilities by means of antibacterial activity. For this, we employed the use of bacterial killing assays whereby γδT cells were exposed to bacterial cells and resulting bacterial cell numbers measured via CFU counts. As zoledronate-stimulation engages the γδTCR and leads to γδT cell activation, we hypothesised that these γδT cells would kill the bacteria. Indeed, we did observe such findings, in accordance with data shown by Barisa *et al* (100). It is important to note that in experiments conducted in the preceding study, γδT cells were isolated from PBMC by means of FACS sorting, which involved staining and sorting of CD3+αβTCR- cells. This method does not account for NKT cells within the isolated fraction, which like γδT cells express CD3 but not the αβTCR, which may have impacted the results observed in this study. However, as our findings confirmed the killing and both FACS sorting (figure 4.3) and cell isolation (figure 4.4) were used, it can be deduced that any contribution observed by the NKT cells is minimal. Our results show zoledronate-stimulated γδT cells to display decreased levels of IFN-γ and TNF-α after 14 days of expansion. Therefore, it is likely that the bacterial killing observed by these cells utilises an alternative cytotoxic mechanism, possibly through the phagocytic capabilities as described by others (308,309).

As the presence of proinflammatory cytokines was not an indicator for γδT cell killing of bacteria, we tested whether the freshly isolated and unstimulated γδT cells exhibit the same killing capacity. Indeed, our findings showed capacity of freshly isolated γδT cells to also kill both *E. coli* and *S. aureus* bacteria, and although not statistically significant, a slightly higher potency to kill bacteria was observed in the freshly isolated γδT cells. A larger sample size would address the statistical significance of this observation. However, as it stands, the idea that unstimulated γδT cells may exhibit a higher pro-inflammatory capability is supported. Freshly isolated γδT cells possess a higher proportion of Vδ1 subtype compared the zoledronate-expanded and Vδ1 cells are known for their rapid proinflammatory capabilities, supported by data showing early IFN-γ release from Vδ1 cells to play a role in spinal cord-related inflammation (321). This novel finding shows direct bacterial killing without prior stimulation, thereby showcasing an innate cytotoxic capability of γδT cells towards bacteria. These findings are supported by various studies in mice which show release of pro-inflammatory cytokines by Vγ4δ1 and Vγ6δ1 T cells in response to *E. coli* infection (10, 25).

Interestingly, the donor-to-donor variation in bacterial killing by γδT cells was less prominent in the freshly isolated γδT cells compared to the zoledronate-expanded γδT cells. Thus, it is likely that the expanded γδT cells utilise a cytotoxic mechanism for killing that is separate to freshly isolated γδT cells. Moreover, in a study investigating both newborn and adult PBMC response to *E. coli*, it was found that expanded γδT cells in response to bacteria in adults mostly comprised of Vδ2γ9 T cells, yet comprised of the Vδ1 subset in newborns (156). This suggests that both Vδ1 and Vδ2 subsets can respond to bacteria, expanding and exhibiting cytotoxic effects. As zoledronate-stimulation occurs though triggering of the γδT cells, yielding a subset of γδT cells that vary in cytotoxic capability that does not involve pro-inflammatory cytokine release. Moreover, zoledronate stimulation leads to expansion of Vδ2 subset, producing a population of γδT cells which are largely Vδ2+. Differences between donors to expand Vδ2 cells may therefore exhibit itself in the downstream donor to donor differences in bacterial cell killing. This is supported by findings which have shown IPP accumulation to polarise γδT cells into Tγδ17 cells, with aid of additional cytokines in the surrounding milieu (115), meaning that cytotoxicity of resulting expanded γδT cells can be effected by various host factors.

4.5.2. Comparison of γδT cell cytotoxic activity to other lymphocytes and against non-bacterial targets

Finally, we explored whether the ability of freshly isolated, unstimulated γδT cells to kill bacteria was indeed unique to the γδT cells or whether such killing was observed with other T cells. We found that the CD4+ and CD8+ also exhibited killing of bacteria, however this was less significant and to a lesser degree than γδT cells. Thus, due to the other T cells exhibiting a baseline level of cytotoxic activity towards the bacteria, it can be inferred that the enhanced killing may be attributed to common PRRs expressed by all of the cell populations investigated but was enhanced in γδT cells due to higher cytotoxic capabilities of this cell population. Thus, when triggered, γδT cells yield a higher, more potent proinflammatory response than CD4+ and CD8+ T cells, resulting in more bacterial cells killed.

Interestingly, the capacity for γδT cells to kill bacteria, whilst extended to both freshly-isolated and 14-day zoledronate expanded γδT cells, is not translated in the same capacity to cancer cells. Much data exists to show the positive impact of zoledronate and similar molecules on γδT cell responses to tumour cells (322,323). For instance, comparisons of prostate cancer patients before and after zoledronate infusion showed enhanced activity of γδT cells to induce an antitumour immune response. However, we found that freshly isolated γδT cells failed to kill nalm-6 cells, suggesting the innate cytotoxicity exhibited by γδT cells to kill bacteria is not sufficient to kill cancer cells. This indicates that the triggering of γδTCR is vital for the recognition and killing of tumour cells and γδT cell antitumour activity. Moreover, the γδT cell-mediated killing bacteria is likely triggered by bacterial cell components engaging with receptors on the γδT cell. However, it is important to note that our experimental data was limited and could have benefitted from a larger sample size. Moreover, a direct comparison to killing of nalm-6 cells by donor-matched zoledronate-expanded γδT cells would have shed more light on exact killing potential of the freshly isolated γδT cells or the zoledronate-expanded γδT cells against bacteria compared to against nalm-6 cells. In addition to this, ideally alternative cancer cell-lines would provide more information.

4.6. Summary

Data from this chapter showcases the impact of zoledronate on γδT cells, expressing a phenotype vastly different after 14 days of expansion to their fresh counterparts, with decreased expression of TNF-α and IFN-γ and up-regulation of HLA-DR and CD86 molecules. However, despite the diminished levels of proinflammatory markers, zoledronate stimulation and 14 days of expansion yields γδT cells capable of killing bacteria.

Innately, it appears as though γδT cells express cytotoxic potential through the expression of proinflammatory cytokines. This cytotoxicity is also paired with the capability to kill both *E. coli* and *S. aureus* bacteria even without stimulation or expansion, a novel finding for fresh γδT cells and a phenomenon that is enhanced amongst the γδT cell population of lymphocytes compared to other T cells. However, the cytotoxic capacities of freshly isolated γδT cells to kill bacteria is unique to the killing of bacteria and is not extended to the killing of cancer cell targets, unlike zoledronate-stimulated γδT cells.

5. γδT Cells efficiently kill bacteria by utilizing innate signals and receptors

5.1. Aims and objectives

The previous chapter introduced novel data showcasing the ability of fresh, unstimulated γδT cells to kill bacteria. This chapter aims to utilise these with regards to the receptors involved in this killing capacity.

Using data acquired through staining and flow cytometric analysis as a rationale, we explored the use of blocking antibodies to CD14, TLR-2, TLR-4, the TLR-4 inhibitor TAK-242, as well peptide mimics of the LPS-CD14 binding site to elucidate which receptors may be involved in γδT cell recognition and killing of *E. coli* and *S. aureus* bacteria.

Our overall aim was to harness whether the blocking of innate receptors would impact the killing of bacteria, thereby providing evidence for engagement of that receptor in γδT cell-bacterial cell recognition and killing. We also aimed to assess if any impact found was consistent despite the state of the γδT cells (*i.e.* zol-expanded or freshly isolated), thereby answering the question of whether different γδT cell state could mediate different cytotoxic mechanisms of γδT cells.

5.2. Introduction

5.2.1. Innate receptor recognition on γδT cells

One hallmark of a bacterial infection includes the rapid infiltration of neutrophils, among other innate immune cells into tissues. However, this initial inflammatory response is associated with innate receptors and thereby is not associated with cells of the adaptive immune system, such as αβT and B cells. This rapid response is dependent on equally rapid recognition, which typically takes place through the identification of PAMPS via PRRS on innate immune cells such as NLRs and TLRs. Interaction of PRRs on γδT cells, is often coupled with IL-17 production and provides the first line of defence against both pathogen-derived and environmental triggers, via cytotoxic responses and signalling to other immune cells (112).

Thus, γδT cells possess unique abilities in comparison to αβT cells in their ability to behave as innate as well as adaptive (161,324). Nevertheless, γδT cells have previously demonstrated innate-like qualities via the direct killing of both bacteria-derived and parasite-derived pathogens in the absence of prior priming (228,308).

5.2.2. γδT cells and TLRs

TLRs have previously been found on γδT cells for facilitating important functions whereby several studies have set out to explore their potential (325–327). One observation found, was the up-regulation of TLR-2 and TLR-4 expression among γδT cells after early burn injury. This increased TLR expression was not present later on, suggesting the early γδT cell response to burn injuries to engage in protective mechanisms of inflammation regulation and healing, in a TLR-involving manner (328). These findings were supported by Schwacha *et al*, who showed increased expression of TLR-2 and TLR-4 on γδT cells in response to mitochondrial danger-associated molecular patterns, leading to downstream effects of tissue and cellular repair via regulatory cytokines such as IL-1β and IL-10 (329).

Quantitative PCR analysis of TLR expression among freshly isolated human γδT cells showed expression of TLR-1-10 to vary from donor-to-donor, with highest expression of TLRs-1-3 (326). Previous studies have shown TLR-mediated γδT cell activation to occur via type 1 interferon release from TLR-triggering of myeloid and plasmacytoid DCs (330,331). Interestingly, higher levels of TLR-3 were found among γδT cells in comparison to αβT cells, with substantial intracellular TLR-3 protein levels, indicating direct TLR-PAMP recognition by γδT cells (326). As TLR-3 recognises a range of RNA types; varying from viral dsRNA and self-RNA from damaged cells (332,333), this showcases uniquely heightened ability of γδT cells to detect stressed cells, as well as virally-infected cells.

Murine studies investigating mice DETCs, which consist of γδT cells do not respond to LPS but showed up-regulation of TLR-4-MD2 complex expression from emigrating cells in response to inflammation (327). Other TLR involvement on γδT cells includes TLR-2, which has been found on γδT cells as playing an important role towards innate responses aimed at commensal bacteria at the ocular surface (334) and seems to demonstrate tissue tropism to specific anatomical sites, such as the ocular surface and spleen (334,335). Increased expression of TLR-2 was observed in γδT cells in mice after *Corynebacterium mastiditis* (*C. mast*) inoculation, resulting in increased IL-17A production by the γδT cells. Conversely, TLR-2 deficient mice failed to recruit γδT cells and neutrophils (334). However, it is important to note that these studies were observed in mice and TLR-2 and TLR-4 engagement of γδT cells in humans may exhibit different conclusions.

The defining characteristic of γδT cells are their ability to respond to antigens even in the absence of APCs. However, the presence of APCs has been shown to augment the γδT cell response (336), signifying a role for additional receptors to be involved in a form of co-receptor stimulation (337). Furthermore, it has been suggested that the triggering of TLRs can provide a form of co-stimulation, alongside triggering of other innate immune receptors like NKG2D and/or the γδTCR via phosphoantigen recognition. Such activation leads to increased pro-inflammatory cytokine release and up-regulation of T cell activation marker CD69 (326). These findings have been supported by Wang *et al* who showed the co-stimulation of the γδTCR and TLR7/8 was found to promote γδT cell anti-tumour activity via mTOR pathways and APC functions (338).

5.2.3. TLR-mediated recognition of bacteria

The innate receptor recognition is important for the identification of invading pathogens where common PAMPS can often trigger PRRs on resident immune cells. *E. coli* and *S. aureus* are both examples of commensal bacteria that can be identified by PRRs of innate immune cells, a process that is vital for triggering appropriate inflammation mechanisms when commensal bacteria species invade other anatomical sites. This is particularly important as circulating adaptive immune cells, such as αβT cells and B cells, may not be present in abundance within these sites. Whereas TLRs are present on a vast range of immune and non-immune cells, do not need a co-stimulatory receptor for activation and can therefore engage in a rapid inflammatory response, recruiting the appropriate immune cells.

For instance, TLR-2 and TLR-4 signalling is known to occur following the exposure of mammary epithelial cells to *S. aureus* and *E. coli*, yet differences are found with *S. aureus* failing to activate NF-κB (339). Various studies have proven TLR-2 vital in the recognition of *S. aureus* , and has been confirmed in TLR-2 KO mice (344), with recognition of *S. aureus* via TLR-2 or NOD1/2 being thought to be dependent on bacterial load (and subsequently, concentration of peptidoglycan) (345). Interestingly, co-expression of TLR-2 with CD14 has shown enhancement of signal transmission via TLR-2-mediated recognition of peptidoglycan (342). Moreover, the lipoteichoic acid (LTA) of *S. aureus* has previously shown to activate immune cells via TLR-2, LBP, and CD14 in the absence of TLR-4 involvement (346). γδT cell response to *S. aureus* via TLR-2 is supported by experimental data using a TLR-2 agonist has implicated the role of Vδ2 cells to release IFN-γ (347). Such findings are further supported by RNA sequencing of lymph nodes which showed TLR-2-mediated signalling of γδT cells to promote bacterial clearance (318).

E. coli has been found to be a catalyst for modulating the expansion of T cells achieved via TLR-2. Use of the probiotic strain *E. coli* Nissle 1917 and TLR-2 knockout mice demonstrated the delicate balance of TLR-2-mediated T cell activation of resident / circulating T cells to maintain immunological homeostasis within the intestine (348). These findings were supported by a study by which showed purified γδT cells to respond to lipid A from *E. coli* in a TCR-independent fashion. Mukono *et al* also presented data showing LPS/lipid A-reactive γδT cells expressing high levels of TLR-2 mRNA. Moreover, TLR-2 deficient mice showed an impaired γδT cell response to lipid A (349).

LPS of Gram-negative bacteria is not just a trigger for TLR-2, but much data has shown that involvement of TLR-4 in response to *E. coli* has been through LPS-recognition, leading to NFκb activation and release of TNF-α, IL-6, IL-1β, and IL-10 cytokines. The TLR-4-LPS engagement has been implicated in phagocytosis via up-regulation of macrophage scavenger receptor and CD11b (350). Of note the lipid A of LPS activates TLR-4 though interaction with MD-2, with TLR-4 responsiveness being dependent on the degree of acylation of lipid A, as well as host genetics (351). Other roles of TLR-4 in *E. coli* infection have been identified through the use of TLR-4 antagonist TAK-242, which identified a role for TLR-4 recognition of *E. coli* fimbrae and secretion of IL-8 and IL-4 (352). As CXCL2 is the murine homologue for IL-8, this study is supported by findings by Svanborg *et al*, who showed TLR4-mutant mice failing to induce expression of CXCL2 in response to uropathogenic *E. coli* (353). The detection of enteroinvasive forms of *E. coli* has also been connected to TLR-5 mediated recognition of flagella (354).

5.2.4. CD14-mediated recognition of bacteria

CD14, commonly used as a marker for myeloid cells, is a membrane receptor for lipopolysaccharide (LPS) which helps cells, *i.e.*, mononuclear phagocytes to bind to LPS-binding proteins (LBP) for capture and transfer towards cell-surface CD14 receptor (355). Typically expressed on antigen presenting myeloid cells, *i.e.* monocytes, macrophages, DC and to a lesser extent, granulocytes, it acts through LPS-recognition causing TLR endocytosis and downstream signalling, and secretion of pro-inflammatory cytokines such as TNF-α and IL-6 (356). Interestingly, studies on CD14-/- mice showed little-to-no production of proinflammatory cytokines in response to LPS and *E.coli* O111 (357). Other novel findings regarding CD14 functions include their capability to act as a receptor for apoptotic cells on the surface of phagocytic cells (358,359) as well as their protective function from apoptosis (360).

Interestingly, CD14 either in its recombinant protein form or as secreted CD14 (sCD14), has been shown to bind to the surface of in vitro-activated human T cells (361), providing negative signals to onto these T cells via IL-1, IL-4 and IFN-γ inhibition (362), a finding which would, in part explain the decline in cytokine production seen by Barisa *et al* (229). Moreover, CD14 has been implicated in tuberculosis infection, through receptor-mediated uptake of non-opsonised bacteria by human microglia. This was shown through significant block of infection upon treatment of microglia with antibodies to CD14 or soluble CD14 (363).

The blocking of CD14 and complement may result in the lack of *E. coli*-induced cytokine and chemokine or growth factors typically found in whole blood (364). CD14-deficient mice have previously shown to produce significantly lower levels of TNF-α than their wild-type counterparts in an experiment involving LPS derived from Gram-negative bacteria, therefore signifying the importance of CD14 (365). Moreover, CD14 in combination with TLR-2 is known to recognise Gram-positive bacteria through the identifying of select lipids including lipoteichoic acid (LTA) (366). Furthermore, circulating soluble CD14 was found to be associated with the early intestinal colonization of *S. aureus* in infants (367) and *S. aureus* antigens have long been known to bind to CD14 for stimulating innate immune responses (368).

Implications of CD14 on T cells has been suggested on very few studies, with not much direct evidence being shown. For instance, T cell-monocyte complexes have previously been found in the peripheral blood and can be detected following immune perturbations including disease or vaccinations, suggesting a role for CD14 in T cell function (369). This is supported by evidence showing that depletion of CD14 was found to dramatically impair the expansion of γδT cells (370).

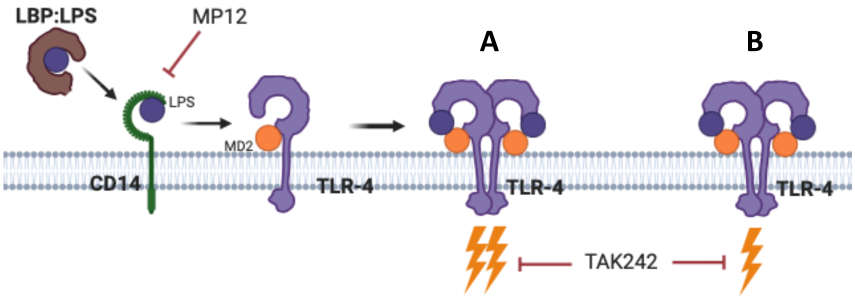

Figure 5.1- Interaction between LPS, CD14 and TLR-4
LPS, either in its soluble form or on bacterial cells interacts is transferred to CD14 via LBP. LPS can either interact directly with TLR-4 **(B)**, or the signalling can be enhanced via CD14. CD14 itself lacks an intracellular domain for signalling (signalling cascade depicted as lightning bolt) but associates with TLR-4 alongside MD2 to enhance TLR-4-dependent LPS signalling **(A)**. TAK242 acts to block downstream signalling of TLR-4, whereas MP12 blocks the binding site of LPS:CD14 interaction.

5.3. Methods and materials

5.3.1. Assessment of receptor involvement in the killing of bacteria

For the assessment of cell surface markers and receptors involvement in killing of bacteria, blocking agents to the appropriate molecules were used. These experiments required preparation of both cells and bacterial cultures for 24 hours before the intended experiment.

Preparation of Bacteria:

As such, a single colony of bacteria was transferred to liquid culture of LB (Sigma Aldrich) and left in a shaking incubator to grow overnight at 220 RPM, 37°C.

Preparation of blocking agents:

Blocking agents used in the killing experiments included: anti-TLR2 or its isotype control (Biolegend), anti-TLR4, anti-CD14, or their isotype control (BD Biosciences), peptide mimics for the CD14-LPS binding site: MP12 and a scrambled form of MP12; MP12-S (GenScript) and TAK-242 (ApexBio, USA). All blocking agents were prepared and stored according to manufacturer's instructions. For TAK-242, which was dissolved in DMSO, stock concentrations were further diluted in RPMI before addition to PBMCs.

Peptide name	**Sequence**
MP12	FHRWPTWPLPSP
MP12-S	FRPWLSHPPWTP

Table 5.1 - Amino acid sequence of MP12 and MP12-S peptides

Preparation of Cells:

In addition to this, the freshly isolated γδT cells were left to rest overnight with blocking agent of interest: anti-TLR-2, anti-TLR-4, anti-CD14, peptide mimics or TAK-242. Cells were rested in a 96-well plate at a concentration of $1x10^6$/ml at a volume of 100µl per well and prepared blocking agents were added to the appropriate wells at a concentration of 10µg/ml (anti-TLR-2, antil-TLR-4 and anti-CD14), 100nM (TAK-242) or 10 and 100µg/ml (MP12 and MP12-S). Plates with the prepared cells and blocking agents were incubated overnight.

5.3.2. Flow cytometry cell staining

5.3.2.1 Growth, maintenance, and PMA-stimulation of THP-1 cells

THP-1 cells were cultured in RPMI supplemented with 1% FBS and 1% penicillin/streptomycin at 37°C within a 5% CO_2 incubator. Cells were maintained at a cell density between 2.0×10^5 to 1.0×10^6 viable cells/mL.

THP-1 cells were stimulated with phorbol 12-myristiate-12 acetate (PMA, Sigma-Aldrich) at concentration at 10ng/ml for 24 hours. PMA was then removed, and cells were then rested for 24 hours in fresh media before THP-1 cells were detached from flasks.

5.3.2.2 Cell Preparation

Cells were prepared as stated in general methods and materials chapter.

5.3.2.3 Flow Cytometry staining

Cells were then stained with the following antibodies in the anchor marker panel as well as the additional panel listed:

Panel	Antibody marker	Fluorochrome	Clone	Manufacturer
Anchor marker panel	CD3	BV711	UCHT1	Biolegend
	αβTCR	PE	IP26	Biolegend
	Vδ1	FITC	B6	Biolegend
	Vδ2	PerCP	TS8.2	Thermo Fisher
	Blue live/dead	-	-	Thermo Fisher
4	CD14	APC	HCD14	Biolegend
	TLR-4	BV421	HTA125	Biolegend

Table 5.2 - Antibodies used to assess expression of innate immune receptors on γδT cell before and after expansion

Testing antibody	Corresponding isotype control
CD14 – APC, HCD14	APC Mouse IgG1, κ isotype control, MOPC-21
TLR-4 – BV421, HTA125	BV421 Mouse IgG2a, κ isotype control, MOPC-173

Table 5.3 - Isotype controls used to assess true CD14/TLR-4 expression

For staining of panel 4, antibodies were added to the cells and cells were then incubated for 30 minutes at 4°C in the dark before being washed twice at 400g for 5 minutes. Cells were resuspended in 200µl of staining buffer before being transferred to appropriately labelled FACS tubes.

5.3.3. RNA/cDNA preparation and Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)

5.3.3.1 RNA extraction

The trizol method was used for all RNA extractions. 1ml of trizol (Ambion) was added to samples for cell lysis before being mixed with 200µl of chloroform (Sigma Aldrich), vortexed vigorously and incubated at RT for 5 minutes. Samples were then centrifuged at 12000g for 15 minutes at 4°C. The top aqueous layer containing the RNA was transferred to a fresh sterile eppendorf containing 500µl isopropanol (Acros Organics) and vortexed. The mixture was incubated at RT for at least 1 hour before centrifuging at 12000g for 10 minutes at 4°C. The supernatant was discarded, and the total RNA pellet washed with 1ml of 75% ethanol and centrifuged at 7500g for 5 minutes at 4°C. After discarding the supernatant, the pellet was air dried and resuspended in 27µl of nuclease-free water (Sigma Aldrich).

5.3.3.2. Assessing RNA Quality/Quantity

RNA quality was measured using NanoDrop 2000c UV/IV Spectrophotometer (ThermoFisher). Total RNA was measured using the Qubit 2.0 Fluorometer (Invitrogen) via preparation of standards and samples using the Qubit RNA HS Assay Kit (Invitrogen).

5.3.3.3. DNasing

For DNasing process, the ambion Turbo DNase kit was used. 3μl of 10x DNAse turbo buffer and 2 units (1μl) of DNAse enzyme was added. Samples were incubated for 1 hour at 37°C in a heated block. 3μl of 10x DNAse stop solution was added and samples were incubated for 5 minutes at RT, resuspended during the incubation. Samples were then centrifuged for 3 minutes at 7500g at 4°C. The supernatant was transferred to a new tube and stored at -20°C.

5.3.3.4. cDNA synthesis

In a PCR tube, a volume equivalent to 2μg of RNA, 270ng of oligo dT (Bioline), 50ng of random hexamers (Bioline) and nuclease free water (total volume 10μl) was mixed and heated at 65°C for 5 minutes. The cDNA mix (4μl of bioscript buffer, 2mM dNTP mix, 1.7μl nuclease free water and 60U of reverse transcriptase bioscript (Bioline) was added and subjected to the following cycle:

- Primer annealing – 25°C for 10 minutes
- Reverse transcription – 42°C for 1 hour
- Inactivation – 75°C for 10 minutes

5.3.3.5. RT-qPCR using TaqMan gene expression assays

1-100ng cDNA at a volume of 4μL per 20μl reaction, alongside 1μL of 20x TaqMan Gene Expression Assay (Applied Biosystems) 10μL of 2X TaqMan Universal Master Mix (Applied Biosystems) and 5.0μL of RNase-free water were mixed and transferred to 96-well reaction plates (BioRad), sealed and centrifuged. Sample assays were done in duplicates and the reaction was performed using the BioRad CFX96™ Real-Time System with the following thermal cycle conditions:

- Hold stage – 50°C for 2 minutes
- Hold stage – 95°C for 10 minutes
- Cycle stage (40 cycles) – 95°C for 15 seconds, followed by 60°C for 1 minute.

5.3.3.6. Data analysis

Data was analysed using BioRad CFX manager and GraphPad Prism Software

5.4. Results

5.4.1. CD14 presence on γδT cells

We assessed the expression innate immune cell markers; TLR-4 and CD14, with expression of CD14 being further measured on isolated γδT cells by flow cytometry staining and RT-qPCR. Flow cytometry showed moderate levels of CD14 on γδT cells and was shown at both days 0 and after 14 days of expansion with zoledronate. We also observed moderate-expression of TLR-4 on γδT cells. Of note, the expression of CD14 and TLR-4 did not have a clear pattern of increase or decrease between day 0 and 14 days of stimulation and expansion with zoledronate, indicating natural fluctuations between donors. To assess whether the CD14 was intrinsically expressed or a form of s-CD14, CD14 expression on purified γδT cells was compared to PMA-stimulated THP-1 cells, as a positive control. CD14 expression on γδT cells was still apparent on purified γδT cells, although to much lesser degree than on PMA-stimulated THP-1 cells (figure 5.1B), making it unclear whether the source of CD14 was native to the γδT cell. The small expression of CD14 suggested that the CD14 was sourced from neighbouring cells within the PBMC culture, a notion which is supported by the fact that sCD14 has been found to be present on T lymphocytes (361). It is therefore present in the cellular microenvironment and can bind to neighbouring cells that would otherwise not express CD14. Through this mechanism, non-immune cells can enhance and even recruit the immune system to respond to LPS.

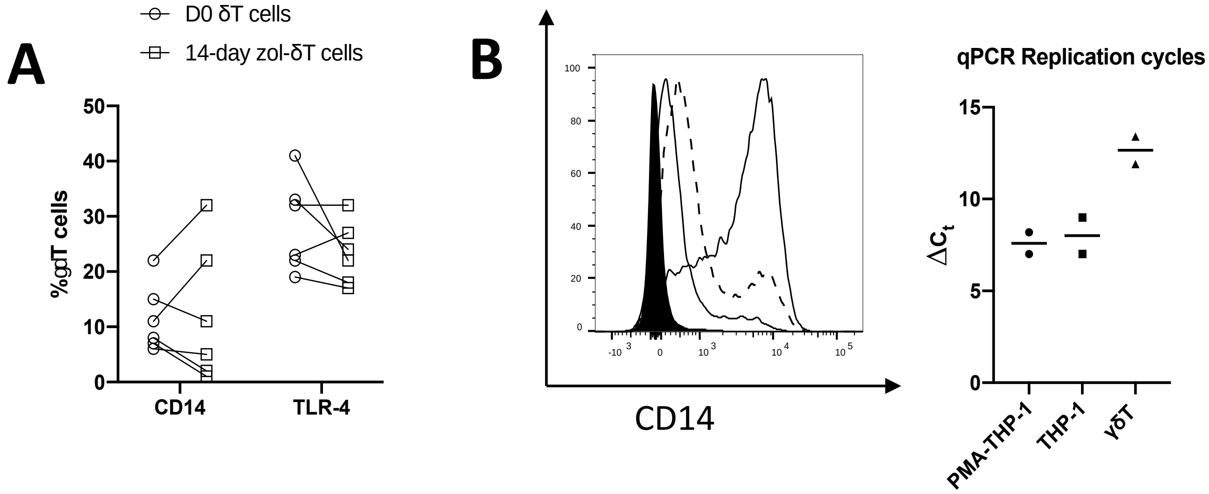

Figure 5.2 – Assessment of CD14 expression on γδT cells
(A) γδT cells within PBMCs were assessed for expression of CD14 and TLR-4 before and after zoledronate-stimulation and 14 days of expansion **(B)** Histograms denoting CD14 expression measured on isolated γδT cells after zoledronate-stimulation and 14 days of expansion (black solid line), THP-1 cells (dashed line) and PMA-stimulated THP-1 cells (grey-filled). Black solid line denotes isotype control. **(C)** RT-qPCR of CD14 was performed on isolated γδT cells after zoledronate-stimulation and 14 days of expansion, TLP-1 cells and PMA-stimulated THP-1 cells. ΔCt values were calculated for replication cycles of CD14 and GAPDH expression.

Thus we performed RT-qPCR on isolated γδT cells, to elude to the answer of the nature of CD14 expression on γδT cells – *i.e.* is CD14 protein produced within and expressed on the γδT cell or is it bound to the cell surface through secretion by neighbouring CD14+ cells? We utilised PMA-stimulated THP-1 cells as a positive control. Our results show high expression of CD14 in PMA-stimulated THP-1 cells, as expected, as signified by a low ΔCt value. Whereas the ΔCt value for CD14 expression of γδT cells was higher, indicating a larger difference between GAPDH internal control and CD14, thereby suggesting very low levels of CD14 RNA.

5.4.2. γδT cells utilise innate signals and receptors to efficiently kill both Gram-negative and Gram-positive bacteria

We then utilized blocking antibodies to the innate immune receptors TLR-2, TLR-4 and CD14 within killing assays to assess possible pathways and receptors involved in the antibacterial capacity of γδT cells displayed in the previous chapter. Due to observed expression of CD14 on both fresh and zoledronate-expanded γδT cells, as well as the small detection of CD14 RNA in γδT cells, we first assessed the impact of blocking CD14 on bacterial killing. Our results show a significant difference of an over 25% decrease in bacterial killing of both *E. coli* and *S. aureus* upon targeting, confirming its involvement. Screening of mimetic peptides for the CD14 binding site with LBP by Xu *et al*, highlighted MP12 as a peptide mimic of the CD14/LBP binding site (371). Thus, to further explore the effects of CD14-blockade on γδT cell-mediated bacterial killing, we applied the use of MP12, and a scrambled form as a control (MP12-S). However, there was no significant difference with addition of these peptides at both concentrations tested. Thus, our results show a role for CD14 but not the CD14/LBP binding site in the γδT cell killing of bacterial cells.

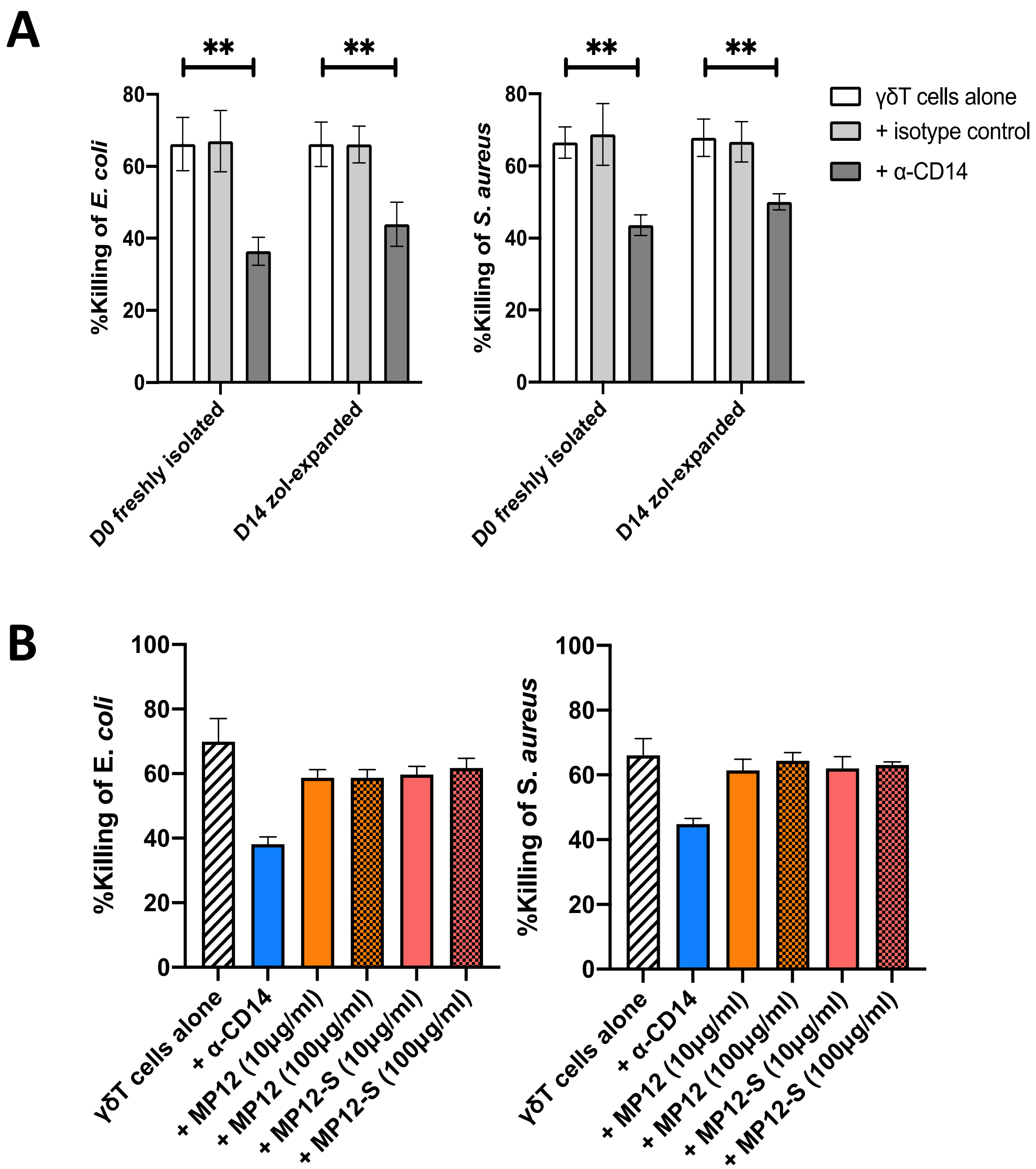

Figure 5.3 – CD14 blockade of γδT cells negatively impacts γδT cell killing of bacteria
γδT cells were isolated from freshly-isolated or 14-day zoledronate-expanded PBMC using the EasySep™ γδT negative Isolation kits and incubated with anti-CD14, isotope control (10µg/ml) or MP12 and MP12-S peptides (10µg/ml and 100µg/ml) overnight and tested for bacterial killing of *E. coli* (left) or *S. aureus* (right) by exposing the cells to bacteria for 30 minutes at an MOI of 5 CFU/ cell of bacteria. **(A)** Impact of anti-CD14 blockade on freshly-isolated or 14-day zoledronate-expanded γδT cell-mediated killing of bacteria (n=4) ** ($P < 0.005$) **(B)** Impact of anti-CD14, MP12 or MP12-S peptides on fresh γδT cell-mediated killing of bacteria (n=3)

We then further analysed the role of innate immune receptors on γδT cell-mediated killing by assessing the impact of TLR blockade. In a similar manner to the blockade of CD14, we utilised blocking antibodies to TLR-2 and TLR-4. Our results show a modest decrease in the killing of *E. coli* and *S. aureus*, although to not as significant of a change as CD14 blockade. We then also utilised TAK-242, a TLR-4 signalling inhibitor, to further deduce the role of TLR-4 in γδT cell killing of bacteria. Our data show a significant decline in bacterial killing when γδT cells were incubated with TAK-242, further confirming the role of this innate immune receptor in γδT cell-mediated recognition and cytotoxicity against *E. coli* and *S. aureus*.

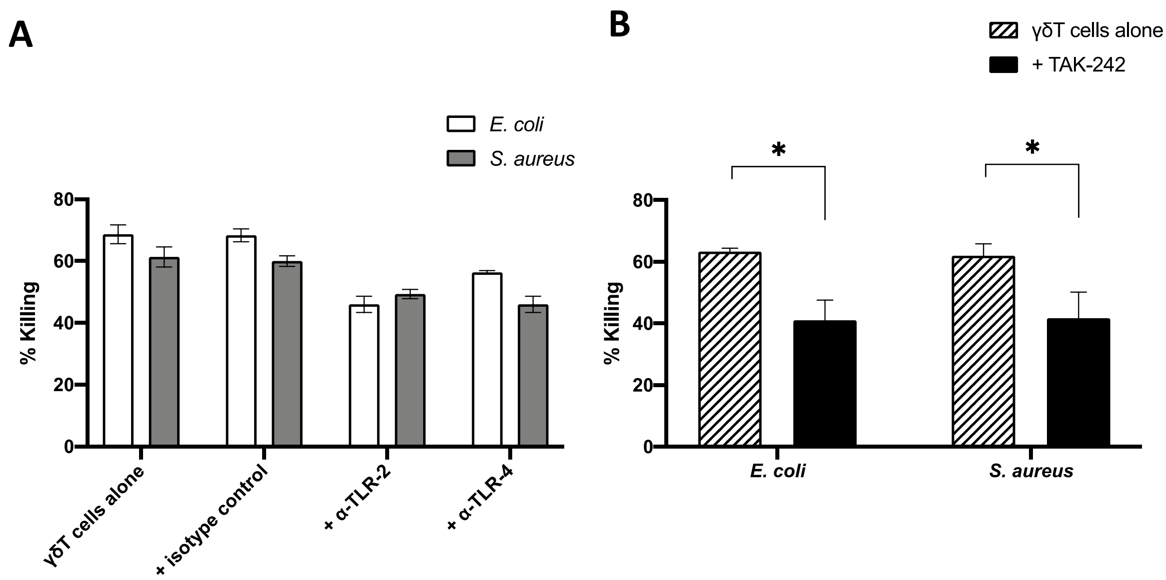

Figure 5.4 – TLR inhibition on γδT cells negatively impacts γδT cell killing of bacteria
γδT cells were isolated from freshly-isolated PBMC using the EasySep™ γδT negative Isolation kits and incubated with anti-TLR-2, anti-TLR-4 or isotope control (10µg/ml) or TAK-242 (100nM) overnight and tested for bacterial killing by exposing the cells to bacteria for 30 minutes at an MOI of 5 CFU/ cell of *E. coli* and *S. aureus*. **(A)** Impact of anti-TLR-2 or anti-TLR-4 blockade on fresh γδT cell-mediated killing of *E. coli* and *S. aureus* (n=4) **(B)** Impact of TAK-242 on fresh γδT cell-mediated killing of *E. coli* and *S. aureus* (n=3) * ($P < 0.05$)

Experimental studies on IPP levels required for γδT cell activation has found that high concentrations of IPP, usually produced by tumour cells are needed for γδTCR triggering (372). This suggests a role of antigen concentration for γδT cell triggering. Moreover, as previously discussed, immediate reactivity pattern of γδT cells can be further enhanced by interaction with innate immune receptors; TLRs and NKG2D (327,339). As such, we assessed the potential for triggering of multiple TLRs, possibly via different antigenic stimuli of the stimulating bacteria to lead to enhanced killing, via blocking of multiple receptors.

However, our results did not show a significant difference in bacterial killing by γδT cells with the use of CD14 blockade alone compared to combined CD14 and TLR-2 blockade, as well as no significant difference between CD14 blockade alone and combined CD14 and TLR-4 blockade.

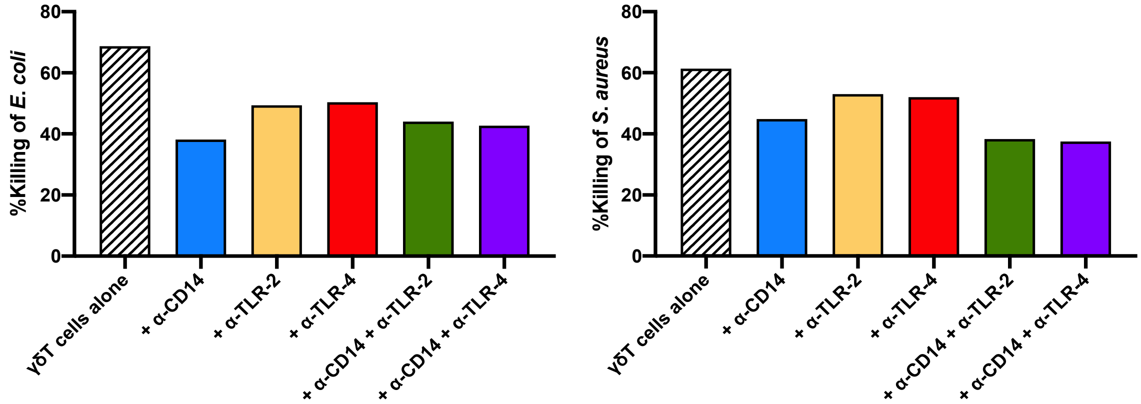

Figure 5.5 – Blocking of multiple innate receptors does not result in an enhanced decline of bacterial killing

γδT cells were isolated from freshly-isolated PBMC sing the EasySep™ γδT negative Isolation kits and tested for impact of cumulative effect of multiple blocking antibodies, each at concentrations of 10µg/ml on bacterial killing of *E. coli* (left) and *S. aureus* (right). γδT cells were incubated with either i) no antibody, ii) anti-CD14, iii) anti-TLR-2, iv) anti-TLR-4, v) anti-CD14 and anti-TLR-2 or vi) anti-CD14 and anti-TLR-4 overnight and tested for bacterial killing by exposing the cells to bacteria for 30 minutes at an MOI of 5 CFU/ cell (n=2)

5.5. Discussion

5.5.1. Investigating CD14 expression and its role in γδT cell antibacterial activity

γδT cells have exhibited behaviour similar to that of pAPCs, whereby they have been able to phagocytose and present antigen to other immune cells. The LPS receptor CD14, typically expressed on antigen presenting myeloid cells, *i.e.,* monocytes, macrophages and DC, acts through LPS-recognition causing downstream signalling and secretion of pro-inflammatory cytokines, and can therefore be an indicator of phagocytic activity of γδT cells. Thus, CD14's implication throughout immunity is vast and due to its presence on phagocytic cells, we explored its expression on γδT cells, with the intention of assessing its value within cytotoxic activity against bacteria

Data from Barisa *et al* has shown phagocytic capability of *E. coli*-expanded γδT cells and is supported by evidence of γδT cell-mediated phagocytosis of *P. falciparum (229,309)*. Accordingly, we then began to look at markers that both display this phenotypic switch and would also provide mechanistic explanation for the functional switch and enhanced bactericidal effect. As such, we turned our attention to the LPS receptor, CD14. Data in this chapter has identified variable expression of CD14 on γδT cells. We hypothesised that the expression of CD14 would increase upon expansion with zoledronate, in accordance with the acquisition of the phagocytic behaviour. However, our data on CD14 expression shows no clear pattern of change across expansion and in fact, shows a great deal of donor variability. Interestingly, recent data has shown BTN levels on CD14+ monocytes to impact γδT cell expansion rates, meaning it is likely that CD14 on monocytes of PBMC to impact γδT cell expansion rather than the other way round (373).

It is important to consider also, that CD14 can exist in either soluble or membrane-bound form, thus we assessed the presence of CD14 from isolated γδT cells in comparison to THP-1 and PMA-simulated THP-1 cells. Our results showed modest expression of CD14 on γδT cells. However, as the isolated γδT cells had been expanded within PBMC culture for 14 days, it is unclear whether the CD14 expression detected was a result of CD14 bound to the γδT cell surface prior to isolation, through secretion by neighbouring CD14+ cells within the PBMC culture. To answer this, we then performed RT-qPCR for CD14 expression of these isolated γδT cells, using THP-1 and PMA-stimulated THP-1 cells as controls. ▿Ct values were calculated from number of PCR replication cycles above threshold levels for CD14 vs GAPDH internal control. The ▿Ct of THP-1 and PMA-stimulated THP-1 cells indicated moderate and high expression of CD14 respectively Whereas the ▿Ct for γδT cells was higher in comparison, indicating a large difference between number of replication cycles need for GAPDH internal control and CD14 expression. This therefore indicates that the presence of CD14 on γδT cells to be very low and as this was only repeated twice, suggests but cannot fully confirm presence of detectable levels of CD14 RNA in γδT cells. This suggests that the source of CD14 could still be from the γδT cell exposure to neighbouring cells within the PBMC culture, prior to isolation, a notion which is supported by the fact that sCD14 has been found to be present on T lymphocytes (361). On the other hand, the use of RT-qPCR, fails to assess the possibility of the presence of a small subset of γδT cells within the total γδT cell population responsible for this CD14 expression, therefore displaying the RT-qPCR result as appearing very low for CD14 expression. Thus meaning, that γδT cells could in fact be responsible for their own CD14 expression.

Despite the lack of clarity on the source of CD14, our results are the first to show CD14 expression on γδT cells. Thus, we proceeded to assess the impact on CD14 on γδT cell-mediated killing of *E. coli* and *S. aureus*. Our results showed a clear role for CD14 in bacterial killing, implicated via the over 25% decline in killing upon CD14-blockade. These results are supported by the γδT cell phagocytic activity observed in various studies (229,309,310). Moreover, as CD14 expression has been shown to prevent cells from apoptosis (360), the CD14 expression on γδT cells may prevent apoptosis as a cause of stress signals from exposure to *E. coli* and *S. aureus*, leading to increased cytotoxic activity.

Thus, blocking of CD14 could impact the killing of bacteria by two mechanisms; preventing phagocytosis of bacteria and facilitating stress-induced apoptosis of γδT cells exposed to bacteria, which would subsequently lead to less bacterial killing. This is supported by data showing an increased phagocytic activity of leukocytes upon pre-exposure to LPS, a known trigger of CD14, an observation which decreased upon pre-treatment of anti-CD14, as well as anti-TLR-4 (350). Of importance to note, is that the same decline in bacterial killing we observed from pre-exposure to anti-CD14 was not observed from pre-exposure to the MP12 peptide (figure 5.2B). Thus, meaning the CD14-LBP binding site that MP12 is a peptide mimic for, is not involved in the CD14-mediated killing of bacterial cells. However, it may be that incubation of MP12 did not yield any effect on bacterial killing due to lack of presence of LPS and LBP. Our data can be explained by analysis of the CD14-LPS-inflammation pathway, whereby LBP-CD14 binding is located up-stream of the signalling pathway, meaning that blockade of this binding site by MP12 may not provide sufficient obstruction of signalling (374).

5.5.2. Exploring the role of other innate immune receptors in γδT cell antibacterial activity

However, as CD14 blockade resulted in decreased killing, but not a complete absence of γδT cell killing, it became apparent that a) additional receptors may be involved and b) other killing mechanisms are employed for cytotoxic activity against bacteria. This is supported by data suggesting that depending on the receptor triggered on a particular cell, results can vary. For instance, opsonised iRBCs failed to activate ADCC via CD16-binding on γδT cells, but instead triggered CD16-dependent antibody-dependent phagocytosis (309), meaning that triggering of a particular receptor can cause more than one type of inflammatory response.

Thus, we assessed TLR involvement, which also showed an impact of TLR-2 and TLR-4 on bacterial killing by γδT cells, the latter of which was confirmed by use of TAK-242. Also referred to as resatorvid, TAK-242 is a small-molecule inhibitor of TLR-4 signalling. It acts by selective binding to TLR-4 and interfering with TLR-4 interactions with adaptor molecules TIRAM and TRAM, subsequently hindering downstream signalling. to TLR-4 and interferes with interactions between TLR-4 and its adaptor molecules (375). Our findings are supported by various studies showing TLR-4 mediated recognition of *E. coli* and *S. aureus* (353), but also, by implications of TLR-4-mediated acidification rate of endosomes and endosomal maturation steps of phagocytosis (376,377). Importantly, we observed no enhanced decline of bacterial killing upon blockade of multiple innate immune receptors, indicating antigen concentration to not be a limiting factor in γδT cell mediated killing of bacteria.

Our data further underline the varying functional capacities of γδT cells in acting both as phagocytic killers and pAPCs and provides insight into the mechanisms employed by these cells when performing such functions.

5.6. Summary

Data from this chapter further underlines the varying functional capacities of γδT cells as innately bactericidal to both Gram-positive and Gram-negative bacteria, with no priming or expansion necessary for this function. Utilising blocking antibodies to TLRs and CD14, we show an impact of TLR-2, TLR-4 and CD14 on the killing of *E. coli* and *S. aureus* bacteria by freshly isolated γδT cells, with use of TAK-242 confirming the involvement of TLR-4. Thus, our data is the first of its kind, showing small, but visible levels of CD14 RNA from γδT cells, that translates into a functional involvement of this molecule when investigating the impact of blocking antibodies on bacterial killing.

We conclude that mainly innate signals and receptors are needed for the killing of bacteria by γδT cells as bactericidal effect observed by zoledronate-expanded γδT cells are likely an artefact of γδTCR-mediated pro-inflammatory activation of γδT cells. Further identification of the involvement of these molecules may enable the development of new classes of small and medium size molecules that can be used as alternative anti-microbials

6. Utilisation of bacterial-derived products for expansion and assessment of γδT cells

6.1. Aims and Objectives

Data from the previous chapters shows γδT cells as innately bactericidal to both Gram-positive and Gram-negative bacteria, and highlighted CD14 as well as TLRs to be involved in this process.

Thus, we aimed to further explore activation of γδT cells in the context of bacterial-mediated stimulation, without the use of bacteria. We therefore explored the use of bacteria-derived products such as LPS or OMVs sourced from *E. coli* bacteria on expansion of γδT cells and used these findings to assess whether:

1. Free LPS is able to stimulate γδT cells within PBMC for expansion, in a manner similar to zoledronate.
2. OMVs are able to stimulate γδT cells within PBMC for expansion, in a manner similar to zoledronate
3. If either product is successful, whether the resulting expanded γδT cells can kill bacteria as well as zol-expanded and freshly isolated γδT cells.
4. Blocking of TLRs and/or CD14 on bacteria derived product-expanded γδT cells can impact the killing of bacteria as shown by freshly-isolated and zoledronate-expanded γδT cells in the previous chapter.

6.2. Introduction

6.2.1. LPS and γδT cells

Various studies have shown an implication of LPS-response by γδT cells. As LPS is found on *E. coli* and various studies have shown influence of *E. coli* on γδT cells (164,313,378), it is therefore unsurprising that further evidence has been shown of LPS-mediated impact on γδT cells, much of this occurs in association with TLRs and is described in chapter 5.2. Mice that lacked γδT cells displayed increased lung damage in the form of inflammation and alveolar-capillary leak in response to LPS, indicating a direct impact of LPS on γδT cell response. Here it is shown that γδT cells may play a regulatory role to avoid excessive inflammation-mediated organ damage by inhibiting other cells (379). However other studies have shown stimulation of LPS to increase cytotoxic activity of γδT cells via proliferation, IFN-γ release and cytotoxic potential (380). Exacerbated inflammation response is further shown in a study by Zhang *et al* investigation LPS-induced sepsis in mice, whereby in γδT cells contributed to white matter injury that lead to subsequent abnormal motor function (381).

Indirect γδT cell response to LPS has been shown via further enhanced maturation of DCs that were stimulated by LPS (382). Moreover, there has been evidence to show that HMBPP-mediated activation of monocytes is enhanced by additional microbial-stimulation of LPS, indicating a dual LPS/HMPP-γδT cell effect on monocytes (158).

6.2.2. Outer Membrane Vesicles

Outer Membrane Vesicles (OMVs) are spherical, bilayered membrane nanoparticles formed from and released during growth of Gram-negative bacteria. Being derived from bacteria, they therefore contain many of the components found within the external surface of the source bacterium (383). For bacteria, OMVs serve to deliver DNA, RNA, proteins and toxins in order to promote morphological remodelling of the outer membrane during bacterial growth and in doing so, can also promote biofilm formation (384). Conversely, from an immunological perspective, OMVs function as a useful tool to stimulate immune cells against the bacteria, as they express a range of microbe-associated molecular patterns (MAMPs) consisting of antigens and TLR-activating components. As such, OMVs can be used to stimulate and enhance immune cell function.

OMVs derived from *Heliobacter pylori* were used for stimulation of human gastric epithelial cells have shown induction of the pro-inflammatory cytokine IL-8 (385) and additional studies have shown OMV-stimulation to facilitate PRR-mediated production of β-defensin and bacterial clearance (386). These findings were supported by several studies which have shown recognition of OMV-MAMPs by TLRs and evidence of bacterial protection in mice upon immunisation with OMVs (387,388). For instance, comparisons between wild-type, TLR-2 and TLR-4 deficient mice shows a role of TLR-4 in the immunogenicity of OMVs (389). We therefore utilised these nanostructures for use in our experimental conditions, as an alternative to zoledronate for stimulation of PBMC and γδT cell expansion, in a way of mimicking bacterial stimulation of γδT cells.

6.3. Methods and Materials

All ethical considerations were in accordance with those discussed in chapter 2. Freshly isolated PBMCs were subjected to the same isolation procedures as previously mentioned.

6.3.1. LPS-driven γδT cell expansion from PBMC

For LPS expansion, PBMCs were plated and stimulated with LPS from *E. coli* O111:B4 (Sigma Aldrich) at a concentration of 10μg of free LPS was added per 1 $x10^6$ cells/ml.

6.3.2. OMV-driven γδT cell expansion from PBMC

OMVs derived from *E. coli* were prepared by Jack Firth of UCL's department of Biochemical Engineering and gifted for use in our experiments. They were used to expand γδT cells as follows:

1ml of PBMCs ($1x10^6$/ml) from three healthy donors were incubated for 10-14 days with $2x10^{10}$ *E. coli* MG1655 Δpal ΔlpxM OMVs at a 20000:1 ratio, with IL-2 and media supplementation as previously described.

6.3.3. Flow cytometry staining

6.3.3.1 Cell Preparation

Cells were prepared as stated in general methods and materials chapter.

6.3.3.2 Flow Cytometry staining

Cells were then stained with the following antibodies in the anchor marker panel as well as antibodies in 1 of the 3 additional panels listed:

Panel	Antibody marker	Fluorochrome	Clone	Manufacturer
Anchor marker panel	CD3	BV711	UCHT1	Biolegend
	αβTCR	PE	IP26	Biolegend
	Vδ1	FITC	B6	Biolegend
	Vδ2	PerCP	TS8.2	Thermo Fisher
	Blue live/dead	-	-	Thermo Fisher
1*	IFN-γ	AF674	4S.B3	Biolegend
	TNF-α	AF700	MAb11	Biolegend
2	CD107α	APC	H4A3	Biolegend
	CD27	AF700	O323	Biolegend
	CD69	BV421	FN50	Biolegend
3	HLA-DR	BV421	L243	Biolegend
	CD86	AF700	L243	Biolegend

Table 6.1 - Antibodies used to assess γδT cell phenotype across OMV or zoledronate expansion

For staining of panels 2 and 3, antibodies were added to the cells and incubated for 30 minutes at 4°C in the dark before being washed twice at 400g for 5 minutes. Cells were resuspended in 200µl of staining buffer before being transferred to appropriately labelled FACS tubes.

*For panel 1, which included intracellular cytokine staining, antibodies within the anchor marker panel (except the live/dead antibody) were added to the cells and incubated for 30 minutes at 4°C in the dark before being washed twice at 400g for 5 minutes. Cells were then treated with monensin in media at a final concentration of 2µM/ml and incubated for 4-6 hours at 37°C 5% CO_2. Cells were then washed twice with 200µL stain buffer at 400g for 5 minutes and supernatant discarded, resuspended in 100µL of stain buffer and stained with live/dead antibody for 20 minutes at 4°C in the dark. Cells were then washed twice with 200µL PBS at 400g for 5 minutes and supernatant discarded. resuspended in 100µL fix/perm mix (Invitrogen) and incubated at 4°C for 1 hour or overnight, which was prepared in a ratio of 3 parts diluent and 1 part concentrate.

After this, cells were washed twice with perm buffer (Invitrogen) at 400g for 5 minutes and supernatant discarded. Cells were then stained with the appropriate antibodies in panel 1 and incubated for 20 minutes at 4°C in the dark before being washed twice with perm buffer at 400g for 5 minutes. Cells were resuspended in 200µl of staining buffer before being transferred to appropriately labelled FACS tubes.

All processing of FACS staining samples took place with a BD LSR II machine equipped with FACSDiva software and data analysed with FlowJo.

6.4. Results

6.4.1. LPS stimulation of PBMCs fails to expand γδT cells

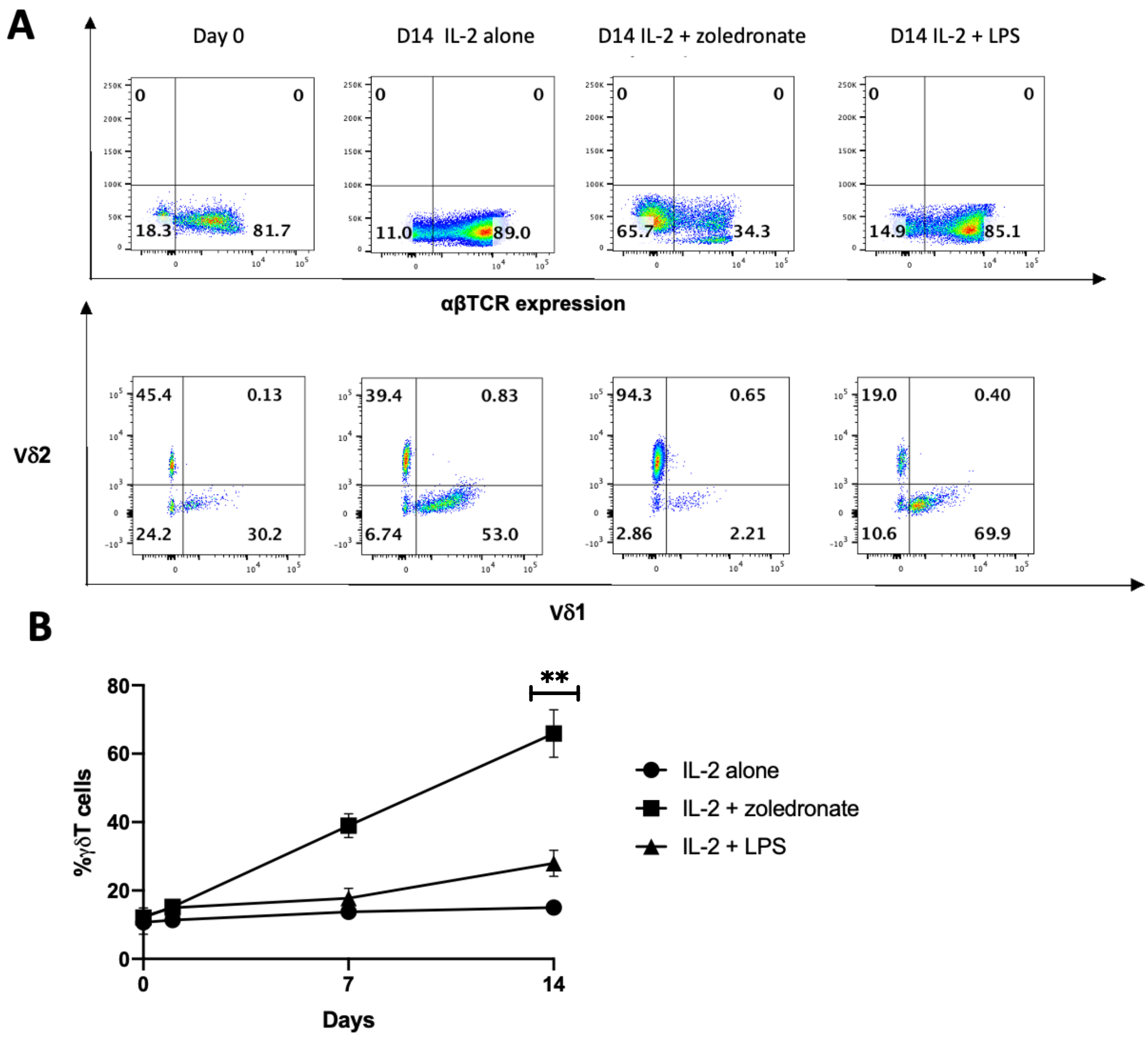

Figure 6.1 – LPS + IL-2 cannot expand γδT cells to comparable levels as zoledronate +IL-2
Freshly-isolated PBMC were stimulated with free LPS (10μg/ml) or zoledronate (5μm/ml) and left to expand in IL-2-supplemented media for 14 days with additional media and IL-2 supplementation every 2-3 days (**A**) The proportion of γδT cells (bottom left quadrant) vs αβT cells (bottom right quadrant). Data shown is a representative of n=3. (**B**) % γδT cells within CD3+ lymphocyte population (top) and viability of γδT cells after 14 days of expansion (bottom). Data shown is n=3. Error bars represent s.d. Statistical difference was found between the IL-2 + zoledronate and IL-2 alone/IL-2 + LPS groups (**) P > 0.005

Previous findings by our group showed the ability of *E. coli* to expand γδT cells (101). However, upon replication we were unable to yield the same result. As discussed in the previous chapter, these results were inconsistent, with varying degrees of expansion shown amongst donors and may have been attributed to unsuccessful UV irradiation of bacteria. We therefore aimed to assess the impact of LPS on expanding γδT cells instead, utilising free LPS from an *E. coli* source. Thus, we mimicked the same expansion conditions as we used for zoledronate-expanded γδT cells, this time adding in the extra condition of free LPS with IL-2. Our findings did not show free LPS to yield γδT cell expansions within PBMC to comparable levels as zoledronate, exhibiting 22% and 67% γδT cells after 14 days of expansion respectively. Interestingly, despite not exhibiting high levels of γδT cells after 14 days of expansion, the percentage of γδT cells from the overall CD3+ T cell population was consistently higher than PBMCs undergoing expansion with just IL-2 alone (16%). Moreover, the expanded γδT cells from LPS stimulation showed variation in their distribution of Vδ1/Vδ2 γδT cell subtype. Zoledronate expansion typically leads to high proportions of the Vδ2 subtype, whereas LPS stimulation expands more of the Vδ1 subtype. This is further indicated when comparing both zoledronate-expanded and LPS-expanded γδT cells with the γδT cells before expansion, which show 30% Vδ1 and 45% Vδ2 γδT cells respectively. These proportions then change to 2% Vδ1 and 94% Vδ2 after 14 days of zoledronate expansion and 69.9% Vδ1 and 19% Vδ2 after 14 days of LPS expansion.

6.4.2. OMVs are able to expand γδT cells in a manner similar to zoledronate capable of killing bacteria

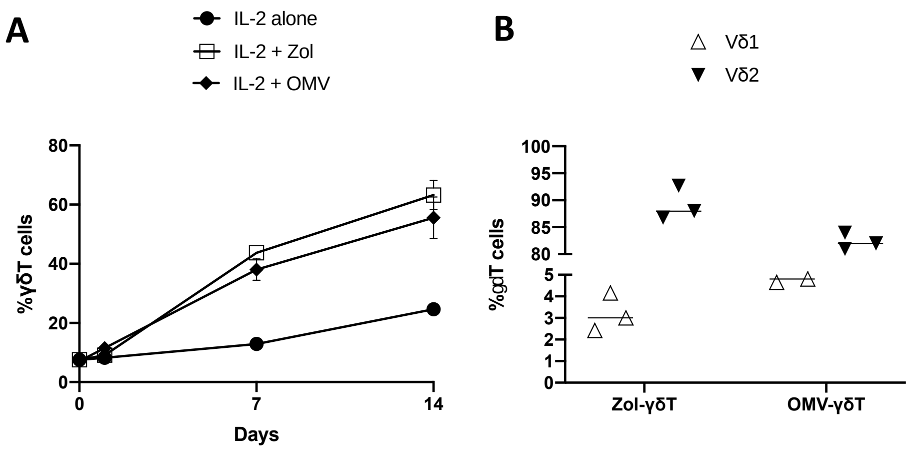

Figure 6.2 – OMVs are able to expand γδT cells to comparable levels as zoledronate
Freshly-isolated PBMC were stimulated with OMVs or zoledronate (5µm/ml) and left to expand in IL-2-supplemented media for 14 days with additional media and IL-2 supplementation every 2-3 days and **(A)** Percentage of γδT cells from CD3+ cells. Error bars represent s.d. **(B)** Subset distribution of expanded of γδT cells. Data shown for each stimulation condition is n=3. No statistical difference was found between the IL-2 + zoledronate and IL-2 +OMV groups.

Lastly, we utilised OMVs derived from *E. coli* as stimulants for expanding γδT cells. OMVs were used alongside zoledronate for stimulation of PBMCs and expansion of γδT cells from the same three donors. Our results showed OMVs to yield similar levels of γδT cells as zoledronate, indicating OMVs to express antigenic stimuli sufficient for the activation and expansion of γδT cells. Moreover, OMV-expansion of γδT cells leads to a similar subtype distribution as zoledronate expansion, with slightly higher levels of Vδ1 T cells observed.

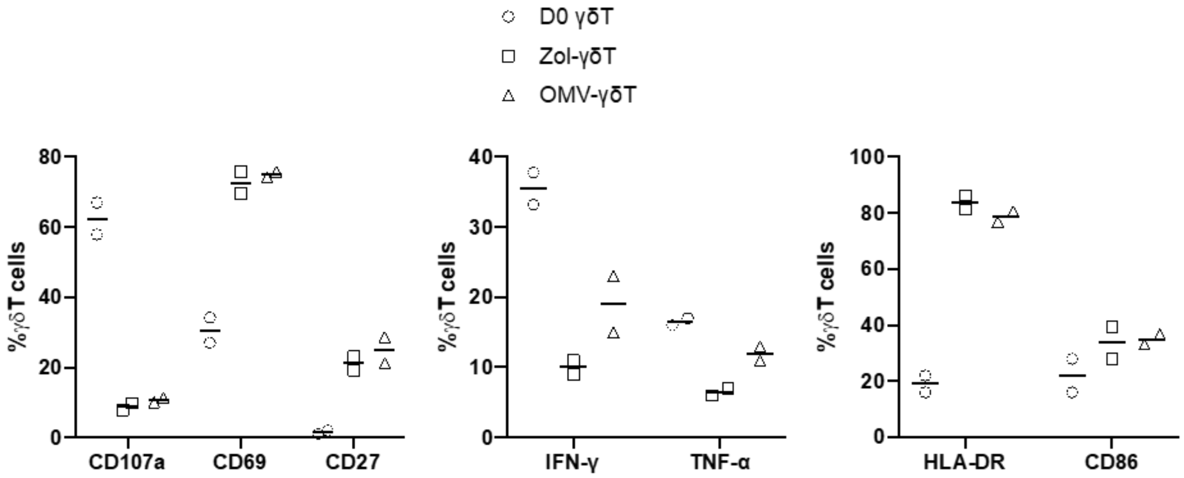

Figure 6.3 – OMVs-expanded γδT cells are phenotypically similar to zoledronate-expanded γδT cells
Freshly-isolated PBMC were stimulated with OMVs or zoledronate (5μm/ml) and left to expand in IL-2-supplemented media for 14 days with additional media and IL-2 supplementation every 2-3 days and were tested for cytokine expression before and after expansion. Data shown for each stimulation condition is n=2.

Moreover, upon expansion with OMVs, γδT cells displayed a phenotype similar to zoledronate-expanded γδT cells as descried in chapter 4. OMV-expanded γδT cells showed high expression of myeloid and helper cell markers HLA-DR and CD86 compared to D0 of expansion, as well as increased levels of CD27. However, expression of proinflammatory cytokines IFN-γ and TNF-α was decreased compared to day 0 γδT cells, albeit to a lesser degree than zoledronate-expanded γδT cells. However, due to a low sample number, the statistical significance of this observation is yet to be determined.

6.4.3. OMV-expanded γδT cells are capable of killing bacteria

Next, the OMV-expanded γδT cells were tested against the zoledronate-expanded γδT cells from the same donors for killing of *E. coli* and *S. aureus* bacteria. Indeed OMV-expanded γδT cells were able to kill bacteria to a similar degree as zoledronate-expanded γδT cells, as described previously. OMV-expanded γδT cells exhibited over 80% and 70% killing of *E. coli* and S. *aureus* respectively.

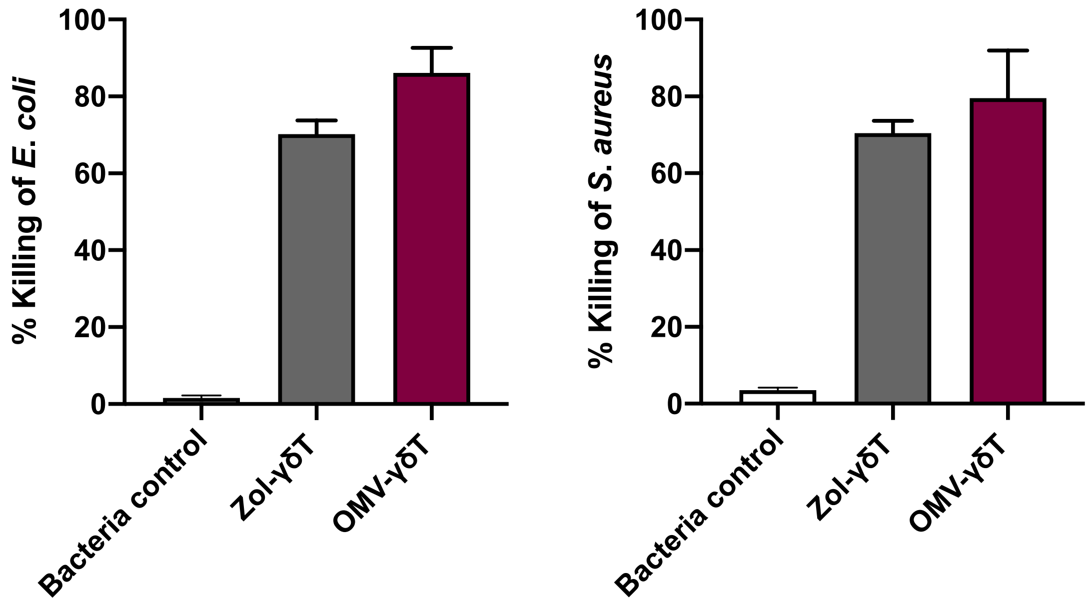

Figure 6.4 – OMVs-expanded γδT cells are able to kill *E. coli* and *S. aureus* bacteria
Freshly-isolated PBMC were stimulated with OMVs or zoledronate (5µm/ml) and left to expand in IL-2-supplemented media for 14 days with additional media and IL-2 supplementation every 2-3 days. γδT cells were isolated from respective OMVs or zoledronate PBMC expansions using the EasySep™ γδT negative Isolation kits at day 14 and tested for bacterial killing by exposing the cells to bacteria for 30 minutes at an MOI of 5 CFU/ cell. Bacterial control denotes to bacteria exposed to the same conditions as bacteria + cells, but without addition of cells of interest. Data shown is n=2.

To elucidate the mechanisms by which OMV-expanded γδT cells kill bacteria, we targeted innate immune receptors. Thus, OMV-expanded γδT cells were also tested for impact of CD14, and TLR-2 blockade on bacterial killing. A slight decline was observed with addition of anti-CD14 and anti-TLR-2, however, this decline was not statistically significant, thereby indicating that OMV-expanded γδT do not use innate immune receptors for bacterial killing.

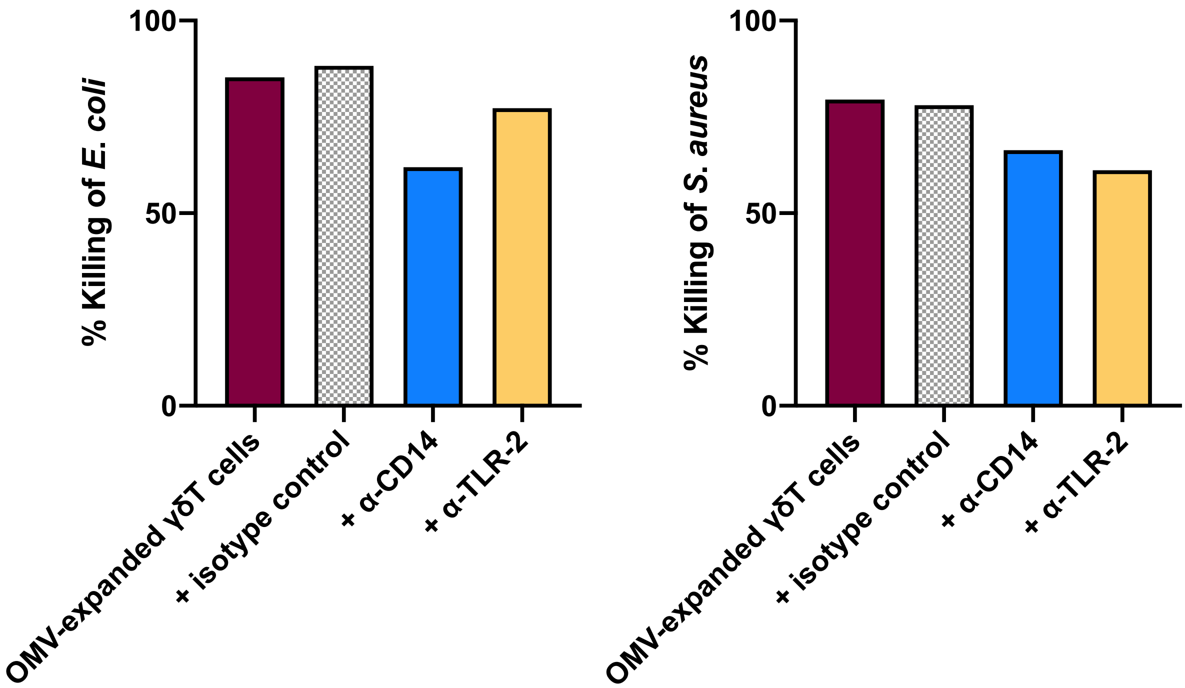

Figure 6.5 – Bacterial killing by OMV-expanded γδT cells is not affected by CD14 or TLR-2 inhibition

Freshly-isolated PBMC were stimulated with OMVs and IL-2 supplementation every 2-3 days. γδT cells were isolated from using the EasySep™ γδT negative Isolation kits and incubated overnight with and without the presence of anti-CD14, anti-TLR-2, or isotype control (10ug/ml) and tested for bacterial killing by exposing the cells to *E. coli* (left) or *S. aureus* (right) for 30 minutes at an MOI of 5 CFU/ cell. Data shown is n=2.

6.5. Discussion

6.5.1. Free LPS is unable to expand γδT cells within a PBMC setting

We employed the use of LPS from an *E. coli* source to expand γδT cells, in order to assess the effect of bacterial cell components to trigger γδT cell response, without the use of whole bacteria. Our findings showed an inability of free LPS to expand γδT cells to comparable levels of zoledronate stimulation. However, the γδT cell population was higher than with IL-2 stimulation alone, indicating a small increase in γδT cells. A study using LPS showed γδT cell expansion and response to be initiated in response to activation of macrophages by LPS (379). As there would been minimal levels of macrophages in the PBMC setting, this thereby provides a rationale for the small number of γδT cell expansion observed.

Of note, phenotypic analysis showed the γδT cells in response to LPS to consist of a larger Vδ1 population compared to zoledronate, which yielded largely Vδ2 cells. In critique of this data, we employed the use of free LPS for stimulation, rather than LPS-beads. Use of LPS-beads would have promoted cross-linking of the T cell receptor and may have led to enhanced γδT cell expansion. This is supported by the fact that LPS requires LBP for capture and transfer towards cell-surface CD14 receptor (355). Moreover, the low level of expansion exhibited by use of LPS, was formed from assessment of 14 days of expansion. However, as the expanded γδT cells were largely Vδ1 in subtype and Vδ1 T cells are known to be rapid responders, it is possible that the LPS stimulation would have yielded γδT cells to a higher level earlier on in the expansion timeline but were diminished by day 14.

Our experiments would have benefitted from different concentrations of free-LPS, with a direct comparison to LPS-beads, alongside use of LPS from different *E. coli* sources. For instance, use of LPS O55:B5 may have yielded a different outcome as this LPS has been shown to stimulate TLR-4 (and depending on type of LPS O55:B5 used, also TLR-2) on cells. Data from Dogan *et al* has shown a varying degree of hypothermia in rats exposed to different LPS sources, indicating that structural or biochemically differences in these LPS may cause different outcomes biologically (390). In addition, insight could be gained from additional time-points in-between the 14-day expansion, to assess when the preferential shift between Vδ1 and Vδ2 takes place within the PBMC setting for LPS-expanded γδT cells compared to zoledronate-expanded γδT cells.

6.5.2. Investigation of antibacterial capacities of OMV-expanded γδT cells

Evidence has shown that IPP-stimulation of γδT cells from bacterial extracts often do not reach the threshold of IPP needed for adequate activation of γδT cells (391). Use of zoledronate stimulation can overcome this challenge. However, γδT cells stimulated by zoledronate may present as an artificial form of γδT cell activation and expansion, thus observations observed with γδT cell expansion via zoledronate can be questioned for biological relevance. For this reason, and due to concerns over adequate UV-inactivation of bacteria within chapter 3, we implored the use of OMVs to stimulate and expand γδT cells within PBMC culture. Our results showed OMVs to be able to stimulate and expand γδT cells, to a similar degree as zoledronate stimulation. OMV-expanded γδT cells were also phenotypically similar to zoledronate-expanded γδT cells.

With this in mind, we then assessed the bacterial killing of these expanded γδT cells as representative of re-exposure to bacteria after prior infection. Our results show bacterial killing to comparable levels as fresh and zol-expanded γδT cells. As OMVs express bacterial MAMPs responsible for triggering of immune receptors, we proposed a possible priming effect within the expanded γδT cells which may yield an accelerated killing response of bacteria. Our results show a slight visible increase in the OMV-expanded γδT cells capable of killing of bacteria compared to zoledronate-expanded γδT cells from the same donors. However this was not statistically significant. Thus, it is unclear whether OMVs can be used to prime γδT cells for enhanced killing of bacteria compared to zoledronate, more replicates would be needed to further assess this.

CD14 and TLR-2 blockade of OMV-expanded γδT cells were also tested for their effect on bacterial killing. We hypothesised a similar observation as previous experiments. However, although we observed a decline in bacterial killing with use of anti-CD14 blocking antibody, this change was not statistically significant. This therefore proposes the idea that OMV-expansion of γδT cells is distinct to zol-expansion of γδT cells in terms of the resulting γδT cell phenotype and receptor expression, with the latter being able to kill bacteria in a different manner to zol-expanded and freshly isolated γδT cells. To assess this fully, future work would need to increase the number of donors to determine if statistical significance can be established. Moreover, exploring the γδT cells expansion and subsequent bacterial killing from utilising different OMV sources, with comparison between the OMV sources as well as to zoledronate would be insightful. For instance use of OMVs derived from known bacterial species which γδT cells have shown response to such as *P. aeruginosa* (156) in comparison to the *E. coli*-derived OMVs may show some differences in bacterial killing and/or the mechanisms involved.

6.6. Summary

In summary we show bacterial-derived products such as OMVs are able to expand γδT cells within in vitro PBMC settings, in a manner similar to zoledronate and that these γδT cells are capable of killing bacteria. Interestingly and in contrast to freshly isolated or zoledronate-expanded γδT cells, the γδT cell killing of bacteria from OMV-derived expansions was not impacted by blocking of CD14 or TLR-2. However, more replicates would be needed to fully test this.

Moreover, expansion was not extended to the use of free LPS, perhaps due to lack of LBP to assist in γδT cell interaction and receptor cross-linking, or source of LPS used.

7. Discussion

7.1. Zoledronate expansion of γδT cells within PBMC

Data from this thesis has shown important findings not only within the field of γδT cell expansion, but of γδT cell antimicrobial capacity and innate immune function. In general, γδT cells which exist as a minute population within circulating PBMC, present a challenge towards harnessing adequate numbers for experimental analysis. As it stands, γδT cells are capable of expansion in the PBMC setting through use of stimulation of zoledronate, which is the current gold-standard within both experimental and clinical use cases (273,392). Our lab has previously presented novel data showing the expansion of γδT cells in response to UV-irradiated *E. coli* achieving a similar expansion profile and phenotype as zoledronate-induced expansions.

Our findings have shown an essential role for UV-irradiated bacteria in the expansion of γδT cells within the PBMC setting. The addition of bacteria that had been improperly UV-irradiated led to unsuccessful γδT cell expansions and ultimately cell death. This is interesting when considering our data has shown γδT cells to be able to kill both *E. coli* and *S. aureus* bacteria, yet within PBMC culture, the γδT cells were not able to expand unless exposed to UV-inactivated and not live bacteria. We propose the main reason for this is due to impact of live bacteria on other cells within PBMC culture, which would have been essential for γδT cell expansion. In the previous model of expansion, executed by Barisa *et al*, UV-inactivated bacteria would be able to stimulate responses in other innate immune cells such as monocytes and αβT cells to be activated, without inducing cell death. These activated cells would release stress signals, in the form of phosphoantigens, as well as pro-inflammatory cytokines such as IL-12 and IFN-γ, which would signal expansion of γδT cells. Whereas *in vivo*, γδT cell activation can occur through the following known mechanisms: 1) as described above, through signalling from neighbouring cells, IPP activation from stressed cells and/or cytokine stimulation or 2) HMBPP or foreign phosphoantigen stimulation; both of which occur through triggering of the γδTCR or 3) through other innate receptors on the γδT cell surface. However, combining the facts that use of inactivated bacteria was involved in the stimulation performed previously, and live bacteria was not able to stimulate γδT cells to expand, it then becomes apparent that the expansion of γδT cells within *in vitro* PBMC culture from *E. coli* stimuli is not due to engagement of the γδTCR via *E. coli* surface antigens.

Thus, this prompted us to explore other mechanisms by which γδT cells react to bacteria, in doing so we aimed to assess how physiological representative the γδT cells acquired through zoledronate induced expansions were from unstimulated γδT cells. Due to their low cell numbers within PBMC, γδT cells in their naïve state are rarely studied within the γδT cell field but would be a better representation of *in vivo* infection. We observed γδT cells to acquire a unique phenotype of CD86+HLA-DR+CD27+ after 14 days of IL-2 and zoledronate expansion, whereas naïve unstimulated γδT cells express higher levels of pro-inflammatory cytokines, IFN-γ and TNF-α. It is possible that upon zol-stimulation the levels of cytokines spiked as other studies have shown (273,393) and then returned to a lower, than baseline level. Irrespective of this, it is clear that 14-day zoledronate-expanded γδT cells display a phenotype that is distinct from their naïve, unstimulated state and as suggested by Barisa *et al*, become phagocytic, helper-like cells capable of a rapid immune response upon re-stimulation (101).

7.2. Antimicrobial capacity of γδT cells of both freshly-isolated and zoledronate-expanded γδT cells

These findings proposed a unique contradictory sentiment whereby both the naïve γδT cells displayed qualities of enhanced cytotoxicity (higher levels of IFN-γ, TNF-α, CD107a) as well as the zoledronate-expanded γδT cells (confirmed through phagocytic potential). Indeed, both naïve and 14-day expanded γδT cells were able to kill both *E. coli* and *S. aureus* bacteria. However, although not statistically significant, a slightly higher potency to kill bacteria was observed in the freshly isolated γδT cells, which could be in part explained by donor-to-donor variation of zoledronate-expansion which would yield variation in γδT cell states post-expansion, providing an explanation for the donor-to-donor variation observed in the bacterial killing assays also.

This variation in killing also suggests a likelihood that the expanded γδT cells utilise a cytotoxic mechanism for killing that is separate to freshly isolated γδT cells. Moreover, prior to expansion, γδT cells of PBMC cells possess a higher proportion of Vδ1 subtype, which have been identified for their rapid proinflammatory capabilities (394). Thus, killing of bacteria by naive γδT cells from PBMC, utilises a larger proportion of these enhanced cytotoxic killers. Meanwhile zoledronate-induced expansions specifically expand the Vδ2 T cell subtype, which is subject to donor-donor variation and may stimulate and expand Vδ2 cells to a phagocytic phenotype with varying degrees of function. This is supported by findings which have shown IPP accumulation to polarise γδT cells into Tγδ17 cells, with aid of additional cytokines in the surrounding milieu (116). As such, donor-to-donor variation may also influence cytotoxic capacities depending on individual baseline cytokine levels. A larger sample size would be needed to address the significance of this observation.

Thus, our results stand to highlight the truly potent cytotoxic nature of γδT cells, whereby naïve or stimulated, they remain effective bacterial killers. Phenotypic differences between the two prompted us to explore the mechanism involved in this killing, it is possible that the killing occurred through two distinct mechanisms, or a common mechanism that occurs in γδT cells, irrespective of their state.

7.3. γδT express and show functional antimicrobial capacity of CD14 and other innate immune receptors

Phenotypic analysis showed unique expression of phagocytic marker CD14 on γδT cells, which varied between donors and did not indicate any clear pattern of expression between the two γδT cell states. The donor-to-donor variation suggests a heterogenous population of γδT cells whereby some individuals may express more CD14+ γδT cells. Our data is the first of its kind to show expression of CD14 on γδT cells, although acquisition of sCD14 has been shown on αβT cells (395,396) and upon assessment via use of anti-CD14 blocking antibodies within the bacterial killing assays, we also show novel data which implicates a clear role for CD14 in bacterial killing. However, it remains unclear how exactly CD14 plays a role in bacterial killing, whether it does so through phagocytosis or other cytotoxic mechanisms. Our use of MP12, did not provide evidence of LPS-related recognition, but can be critiqued though absence of LBP to assist in the MP12-binding to CD14. Moreover, it is possible that CD14-mediated recognition occurs in a manner not dependent on the LPS-LP binding site.

Indeed, it is possible that CD14 presence may play a different role at different stages of γδT cells expansion (day 0 vs day 14). A recent study showed that successful γδT cell expansion requires CD14+ monocytes to act as accessory cells (373). Moreover, T cell-monocyte complexes have previously been detected in peripheral blood following disease or vaccinations (397). Taken together, it is possible that CD14 from monocytes interact heavily with γδT cells during stimulation and expansion, with some potential transfer to the γδT cell surface. It's function thereof, is likely to lean into phagocytic capabilities as described by Barisa *et al*, with monocytes acting as influencing cells. Whereas low-level CD14 expression observed early on may behave as an innate immune receptor similar to TLRs for recognition of bacteria.

Thus, we have confirmed CD14 expression, and investigated its source, through two additional mechanisms; first, by flow cytometric analysis of isolated γδT cells and secondly; through RT-qPCR of 14-day zol-expanded γδT cells. Taken together, our findings suggested presence of minute levels of CD14 that suggests that the source is likely to be from γδT cell exposure to neighbouring cells within the PBMC culture, prior to isolation. Irrespective of source, the acquisition of CD14 expression, whether intrinsic or through neighbouring cells, relays a receptor which, upon stimulation can activate γδT cells toward a cytotoxic function.

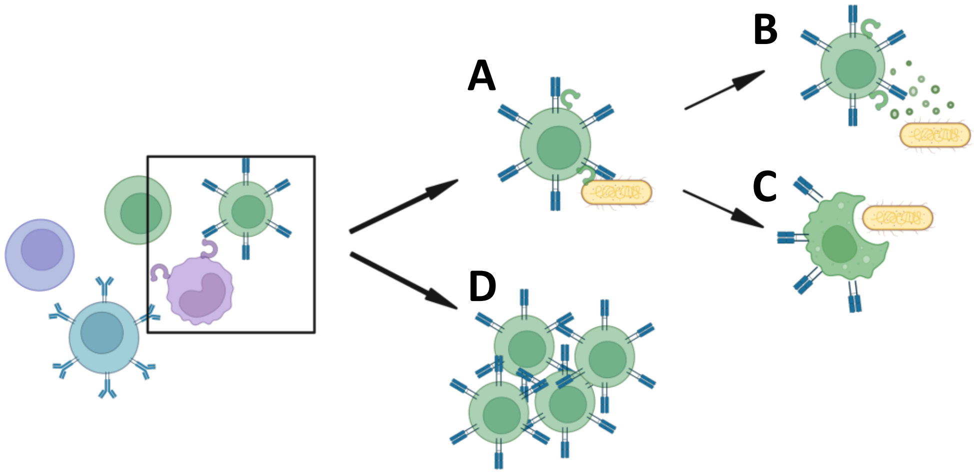

Figure 7.1 Model of impact of CD14 on γδT cells
Monocytes expressing CD14 (purple cells with purple receptors) can influence γδT cells (green cells) to expand upon stimulation **(D)**. Monocyte-driven or innate expression of CD14 aids with recognition and killing of bacteria **(A)** by either degranulation (**B)** or phagocytosis **(C)**. (Created on biorender.com)

Blocking of CD14 partially reduced the bacterial killing but did not completely block it. Thus, as our phenotypic analysis highlighted unique expression and bactericidal role of CD14 on γδT cells, we began to explore the role of other innate immune receptors. Through use of blocking antibodies to TLR-2 and TLR-4 and use of the TLR-4 signalling inhibitor, TAK-242, our work shows an involvement of TLRs in bacterial recognition and killing, thereby concluding bacterial killing as an innate function of γδT cells. The lack of a cumulative effect of blocking multiple innate receptors, combined with ability to kill both *S. aureus* and *E. coli*, indicates not only a lack of antigen specificity, yet for antigen stimulation and concentration to not be a defining limiting factor in the bacterial killing.

7.4. γδT cells express higher antimicrobial capacity in comparison to other lymphocytes

Within the context of infection, peripheral blood γδT cells, upon response to stimulation are responsible for cytotoxicity but importantly, signal to other immune cells such as neutrophils for successful inflammatory control (398). This is supported by our findings showing CD4+ and CD8+ to also exhibit killing of bacteria, (albeit to a decreased capacity than γδT cells). Thus, whilst other cells may be required for complete bacterial elimination, when triggered, γδT cells are capable of enhanced bacterial killing compared to other T cells. This may be caused by i) higher expression of TLRs ii) additional stimulation of CD14 compared to other T cells and/or iii) higher, more potent proinflammatory response than other T cells. *e.g.,* increased levels of degranulation.

Taking the COVID-19 pandemic into consideration, our findings are particularly important when considering the increased risk infectious diseases play on daily lives of even healthy individuals. Correct and specific identification of immune factors and cells involved in an optimal and enhanced immune response to pathogens is a key and crucial step into easily-accessible treatment for infections and is a particularly sought-after remedy due to the rise of antibiotic resistance. Here, we have identified of γδT cells as superior responders in comparison to other lymphocytes, making them ideal targets for treatment of immune enhancement.

7.5. Cytotoxicity of freshly-isolated γδT cells is limited to bacteria

Our data is the first to show killing by freshly isolated γδT cells, an observation that was only apparent towards the killing of *E. coli* and *S. aureus* but was not observed towards nalm-6 cancer cells. This is not surprising as recognition of cancer cells by γδT cells is usually through the γδTCR and without signals from the cellular milieu, fresh γδT cells may not receive enough stimulation to be activated against cancer cell targets. However, it is important to note that the latter findings were from two donors only and would benefit from a larger sample size. Moreover, these experiments utilised nalm-6 cells as the representative tumour cell target. Additional and more promising findings may be observed with use of alternative tumour cell lines such as MDA-MB-231 cell line or HeLa cells, due to evidence of γδT cytotoxicity towards an increasing number of adherent cell-lines and with future potential within the breast cancer field (306).

Thus the choice of cancer cell-line is important in the assessment of γδT cell cytotoxicity as clear differences in killing capabilities can be seen between different cancer types (399). We suggest that the killing of cancer cells by freshly isolated γδT cells can therefore not be ruled out as it is possible that zoledronate expanded γδT cells are capable of killing different cancer types to fresh γδT cells.

7.6. OMVS are able to expand γδT cells into cells capable of killing bacteria

As our findings clearly showed a role for γδT cell bacterial response, we revisited the capacity of bacterial stimulation and expansion of γδT cells through the use OMVs. Ability of OMVs to stimulate and expand γδT cells as well as show bacterial killing to comparable levels as fresh and zol-expanded γδT cells, supports the notion that bacterial stimulation can indeed expand γδT T cells. Interestingly, γδT cells expanded with the *E. coli* derived OMVs did not display any excessive killing of *E. coli*, indicating that the cytotoxicity observed was not accelerated through prior bacterial exposure or priming. Moreover, we did not observe a significant change in bacterial killing upon inhibition of CD14 or TLR-2, indicating that OMV-expanded γδT cells may utilise a different receptor mechanism for bacterial killing than freshly isolated or zol-expanded γδT cells.

Indeed, it is possible that the OMVs may express or release unique *E. coli*-specific phosphoantigens that could activate γδT cells via the γδTCR and promote an adaptive immune response. Interestingly, transcriptomic analysis of γδT cells has shown pathogen-driven differentiation to innate or adaptive phenotypes (325). However, thus far, very few γδT cell reactive non-mycobacterial phosphoantigens have been discovered (378). Further analysis of OMVs and their capabilities of γδT cell expansion and subsequent cytotoxicity of the γδT cells may shed light to alternative bacterial phosphoantigens capable of γδT cell response *in vivo*. Use of OMVs therefore serve as a promising candidate for γδT cell expansions due to their likely ability to stimulate γδT cells through more than one receptor.

8. Conclusion

In summary our data has shown novel findings exhibiting γδT cells as innately bactericidal to both Gram-negative and Gram-positive bacteria using *E. coli* and *S. aureus* as respective representatives, with no priming or expansion necessary for this function. We show that innate signals and receptors are needed for the killing of bacteria by γδT cells but are not the only receptors involved in bacterial activity, implicating the varying functional capacities of γδT cells in acting both as phagocytic killers and pAPCs.

Thus, within this thesis we have shown:

1) Requirement of successful UV inactivation of bacteria is necessary for γδT cell expansion.
    i) Intact bacteria are not able to expand γδT cells within our *in vitro* PBMC model.
    ii) Free LPS is also not able to expand γδT cells successfully.

2) An impact of zoledronate on γδT cells, expressing a phenotype vastly different after 14 days of expansion in comparison to their fresh counterparts, giving rise to HLA-DR and CD86 expression with a decreased expression of TNF-α and IFN-γ compared to freshly isolated γδT cells.

3) Ability of γδT cells to kill *E. coli* and *S. aureus* bacteria – a finding that was consistent in both 14-day zoledronate-expanded γδT cells and freshly isolated γδT cells.
    i) Enhanced donor-to-donor variation within the zoledronate-expanded γδT cell killing of bacteria
    ii) Enhanced killing among freshly isolated γδT cells compared to freshly isolated CD4+T, CD8+T or B cells.
    iii) Killing of bacteria by freshly isolated γδT cells was not extended to cancer cells.

4) Low levels of CD14 expression on both freshly isolated and 14-day zoledronate-expanded γδT cells, as confirmed by both flow cytometry and RT-qPCR.

5) Involvement of TLR-2, TLR-4 and CD14 in the killing of *E. coli* and *S. aureus* bacteria by both freshly isolated γδT cells and 14-day zoledronate-expanded γδT cells.
   i) Enhanced donor-variation within the zoledronate-expanded γδT cell killing of bacteria.
   ii) Blocking of these receptors did not completely prevent bacterial killing, suggesting a role for other receptors and/or combined effect of multiple receptors in bacterial killing.

6) Ability of *E. coli*-derived OMVs to expand γδT cells and for those γδT cells to also kill bacteria.
   i) Blocking of the innate receptors CD14 and TLR-2 did not prevent bacterial killing.

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9. Future Studies

γδT cells are increasingly gaining popularity for their innate-like properties and their ability to carry out cytotoxic functionalities in the absence of APCs and co-stimulation. This provides a new paradigm shift towards the development of novel allogeneic medicines and modalities. Novel new-found insights in the space of γδT cell-based therapeutics extends to overcoming previously difficult to control concerns of antibiotic resistance towards bacterial infections.

This thesis has set out to investigate and provide a deeper understanding of γδT cell mechanisms in responding towards extraneous bacterial challenges. However, further experimentations and topics need to be addressed in order to advance the potential of γδT cell-based therapeutics in this space.

9.1. Consolidation of findings

Our data has shown novel findings of i) expression of CD14 on γδT cells and ii) a role of CD14 in γδT cell-mediated bacterial killing. Additional investigation of bacterial killing should be employed by use of microscopy imaging of killing, through fluorescent labelling of bacteria, γδT cells and receptors of interest, we can directly observe γδT cell killing of bacteria as well as provide visual information on how receptors are involved in the killing. Moreover, the mechanism of killing should be explored further through the following techniques:

i) Inhibition of phagocytosis via cytochalasin D, an inhibitor of actin polymerization
ii) Inhibition of degranulation via use of inhibitors such as monensin
iii) Measurement of cytotoxicity using ^{51}chromium (^{51}Cr) release assay

9.2. Exploring γδT cell cytotoxicity against different expansion assays

The derivation of PBMC from whole blood to the isolation of γδT cells involves several steps and protocols which should not be overlooked upon studying the outcome of γδT cell function towards target cells or stimulatory challenges. Alternatives means of expansions should be considered and tested in adjacent to the current gold-standard of zoledronate-induced expansions. This allows for a wider and more flexible breadth of expanding γδT cells without dependency on traditional methods that may provide restrictions in the phenotype or quality of the final product produced.

Additional important points of investigation also include the assessment of alternative interleukins, composition of the complete media used throughout the 14 days of expansion. For instance, evidence has shown IL-15 to promote cytotoxic behaviour of γδT cells (288) Moreover, an impact on cytokine milieu of PBMC has been shown to assist in the development of γδ17 cells, which are known for their potent IL-17A pro-inflammatory responses. Thus, exploring and expanding different γδT cell subtypes (*e.g.,* γδT1, γδT2, γδT17 or γδTregs) as well as subsets (*e.g.,* Vδ1, Vδ2, Vδ3) are of particular interest. Data has shown Vδ1 T cells to be more potently cytotoxic and capable of more rapid inflammatory responses than Vδ2 T cells, thereby making them interest subtypes of investigation (91,400). However, this is limited by access to Vδ2 T cells, as within PBMC, Vδ2 levels are low. High Vδ2 levels are located in hard-to-access mucosal sites. Thus more insight on how to specifically expand Vδ2 cells from PBMC would be useful.

9.3. Exploring γδT cell cytotoxicity against a greater selection of bacteria

A greater selection of Gram-positive and Gram-negative bacteria is of great important in assessing γδT cell functions. The response of γδT cells towards different strains of the same bacteria should also be investigated for a more thorough analysis of how different stimulatory challenges and antigens may result in unique outcomes. For instance, use of *E. coli* strains O157:H3 and E2348/69 (EHEC-associated and EPEC-associated respectively) or *E. coli* NCTC 13400/13451/13452 (multidrug resistant) and *S. aureus* strains NCTC 13142/13435 (methicillin-resistant) (401–404).

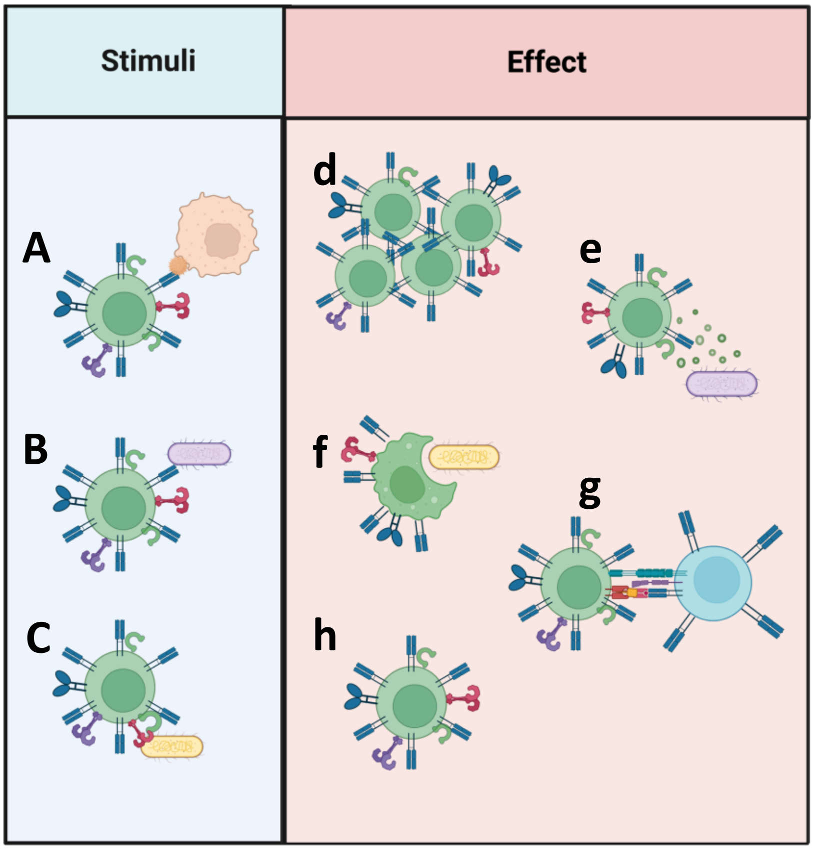

Figure 9.1 - Type of γδT cell reactivity varies depending on stimuli
γδT cells can react to internal stimuli via stress signals on infected or cancerous host cells **(A)** or via external signals from microbial ligands to either the γδTCR **(B)** or other innate immune receptors **(C)**. In turn γδT cells can react by expansion **(d)**, release of pro-inflammatory mediators (cytokines) and/or degranulation **(e)**, phagocytosis **(f)** and antigen presentation to αβT cells **(g)**, or if correct stimuli are not received, may result in no response **(h)** *i.e.,* non-γδT cell reactivity to a particular bacterium. (Created on biorender.com)

Additionally, other bacterial species have been identified as γδT cell targets and should also be explored. These include *Shigella* and *Salmonella* due to their close relativity to *E. coli*, as well as *Mycobacterium* species, for which data already exist to suggest a role of γδT cells (171,300), but experiments have not utilised freshly isolated γδT cells. For instance, Bacillus Calmette–Guérin (BCG), the attenuated form of the bovine *M. tuberculosis,* and used in humans for vaccination against TB, is an easily attainable, lab-safe bacterium alongside *Mycobacterium avium*, an environmental bacteria species. Both of these bacterial species would be promising candidates of interest to assess freshly isolated γδT cell reactivity to mycobacteria.

In addition to this, it would be insightful to expand the pathogen repertoire to include viruses and parasites. Data to support γδT cell activity against HIV and *P. falciparum* already exist (173,309,312,405). Thus we could further explore this activity in the context of innate immune receptor blockade, and specifically, use of freshly isolated γδT cells. Alternatively, we could assess γδT cell activity against other viral or parasitic targets *e.g. influenza A*, for which there is promising evidence (406) and would be particularly useful when designing seasonal vaccines.

9.4. Effect of restimulation on cytotoxic mechanisms of γδT cells

For assessing γδT cell cytotoxicity via killing assays, a greater weight could be added to the timepoints studied in conjunction with the re-stimulation of γδT cells. This allows for the assessment of the longevity of cytotoxic activity provided by the expanded γδT cells. Findings should be confirmed and coupled with assays in adding a richer understanding beyond the percentage of killed target cells.

Moreover, it is possible that the killing mechanism as well as capacity is affected by restimulation of γδT cells. Thus, to investigate this, testing of bacterial killing of γδT cells freshly isolated from PBMC, vs expanded for 14 days with zoledronate and expanded for 14 days with zoledronate then re-stimulated, would be advantageous. Flow cytometric analysis of phenotype across the three cell conditions would yield information on up-regulation or down-regulation of receptors or cytokines of interest.

Moreover, re-evaluation of bacterial inactivation assays and optimisation of such would allow us to use and perfect bacteria for stimulation. After which, *E. coli*-expanded γδT cells could be tested for killing of bacteria in comparison to zoledronate-expanded, freshly isolated or *S. aureus*-expanded, as well as re-stimulated cells. Assessment of phenotype alongside cytotoxicity would help to shed light on expression profiles of reacting γδT cells and if memory phenotype markers are present on the γδT cells, as has been shown by various studies (97,282,283,300). Recent studies have shown an adaptive-like quality of Vδ1 T cells. Interestingly, this adaptive-like response has been in response to pathogen stimulation via HCMV and *P. falciparum*, showing an antigen-specific stimulation that can result in transition from naïve to effector cell populations that can result in either innate or adaptive qualities (325). It would thus be useful to test a range of pathogens as γδT cells may utilise innate immune receptors for some bacteria species and adaptive (as well as memory) responses to other bacteria species.

9.5. Investigating γδT cell interactions with other cells

γδT cells are known to wield a great selection or receptors, each with unique functions and alternative outcomes. To further understand how these cells behave and how they might provide a positive impact in this space, additional assays beyond direct killing should be explored. Future assays could be developed to explore if such γδT cells communicate to other cells in a dual or multi co-culture assay for ultimately providing an increased / rapid response, increased killing of target cells, or providing signals to other cell types for new purposes, *i.e.* the number of γδT cells antigen presenting versus the number of those killing.

Of particular interest is to assess the effect of antigen exposure (through use of OMVs, zoledronate, or inactivated bacteria) to γδT cells alone, before exposing those γδT cells to PBMC culture. Through confocal microscopy, assessments can be made on how γδT cells present antigen to αβT cells upon activation. Whilst in parallel, exploration of the same stimuli co-cultured with monocytes or DCs can be used to compare and contrast antigen presentation, as well as explore interactions between activated monocytes and T cells (both αβ and γδT) in terms of CD14+ influence on γδT cell phenotypic behaviour.

These findings would help to elucidate non-cytotoxic helper functions of γδT cells that have not been thoroughly investigated to date.

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