The role of innate lymphocyte metabolism in sepsis

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BY

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Alhanouf Ibrahim Hamad Al-Harbi

For My Mom, Dad

and

My Daughters Sadeem and Deema

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Abstract

Sepsis is the result of a dysregulated systemic immune response to microbial infection that leads to organ failure and death in about 30% of affected individuals. The antimicrobial response is biphasic with an overwhelming inflammatory response preceding a period of profound immune suppression. We hypothesized that altered metabolism in immune cells during sepsis may affect their functions and contribute to the clinical phenotypes of the disease. Natural killer (NK) cells and Vδ2^{+} γδ T cells from patients in late stages of sepsis and healthy controls were assessed for functional and metabolic readouts. Total peripheral blood NK cells were found at normal frequencies in the sepsis patients, whereas the CD56^{bright} subset was found at significantly lower frequencies compared to those from healthy donors. The expression of the activation marker CD69, the transferrin receptor CD71, and the amino acid transporter CD98 at baseline and in response to cytokine stimulation, were similar on NK cells from sepsis patients compared to controls. The frequencies of Vδ2 T cells were lower in sepsis patients compared to controls. Although Vδ2 T cells from sepsis patients had reduced CD69 expression, they had significantly higher basal expression of CD98 compared to healthy donors. Vδ2 T cells from sepsis patients and controls subjects activated with cytokines or the antigenic ligand (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) upregulated CD69 and CD71 expression. Stimulated NK cells and Vδ2 T cells from sepsis patients produced interferon-γ (IFN-γ) at lower frequencies compared to the same cells from healthy donors. Furthermore, the metabolic regulator mTORC1 exhibited reduced phosphorylation in NK cells from sepsis patients possibly explaining the functional defects in the production of IFN-γ. Mitochondrial investigation indicated that NK cells and Vδ2 T cells from sepsis patients had similar mitochondrial structure and function (mitochondrial mass, ATP synthase and reactive oxygen species production) to those from healthy donors. Thus, NK cells and Vδ2 T cells from sepsis patients display similar functional and metabolic profiles to those from healthy donors, except for a selective defect in mTORC1 activity in NK cells and IFN-γ production in NK

cells and Vδ2 T cells. A thorough investigation into Vδ2 T cell metabolism and function showed that HMB-PP or zoledronate can be used to expand and generate high purity Vδ2 T cell lines from peripheral blood mononuclear cells of healthy donors. Further activation of expanded Vδ2 T cells with HMB-PP or PMA/ionomycin resulted in increased IFN-γ production, whereas stimulation with zoledronate, IL-12/IL-15, or anti-CD3/CD28 mAb failed to induce IFN-γ, IL-4 or IL-17 production. Hence, zoledronate might be optimal for expanding Vδ2 T cells, whereas HMB-PP is optimal for inducing cytokine production. Metabolic analysis using Seahorse technology demonstrated that resting and activated Vδ2 T cells from healthy donors predominantly use glycolysis to generate energy. HMB-PP stimulated Vδ2 T cells had higher levels of glycolysis, with increased rates of basal glycolysis and glycolytic capacity compared to resting Vδ2 T cells. In addition, HMB-PP stimulated and zoledronate stimulated Vδ2 T cells consumed oxygen at higher rates than compared to resting Vδ2 T cells, indicating that activation induces oxidative phosphorylation. Our data support that Vδ2 T cells undergo metabolic changes in response to activation. These finding provide the first evidence that Vδ2 T cells may be targeted for the treatment of sepsis and that that the therapeutic activities of Vδ2 T cells may be regulated by modulating their metabolism.

Publications

Dysregulated T helper type 1 (Th1) and Th17 responses in elderly hospitalised patients with infection and sepsis.

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Irish Society for Immunology, September 13-14, 2018, Dublin, Ireland: ***Metabolic changes in innate lymphocytes in patients with sepsis*- Alhanouf Al-Harbi**, JD Coakley, Thomas Ryan, Derek G. Doherty, Clair M. Gardiner.

Courses

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Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 1

Chapter 2

Abbreviations

2-NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4- yl)Amino)-2
2DG	2 deoxy-D-glucose
ADCC	Antibody dependent cell cytotoxicity
APC	Antigen presenting cell
BM	Bone marrow
BrHPP	Bromohydrin pyrophosphate
B- and T-lymphocytes	(BTLA)
Attenuator receptors	
BSA	Bovine serum albumin
CMV	Cytomegalovirus
cRPMI	Complete RPMI 1640 Glutamax medium
CTLs	Cytotoxic T lymphocyte, CD8+T cell
DCs	Dendritic Cells
DMSO	Dimethylsulphoxide
EBAO	Ethidium bromide/acridine orange
ECAR	Extracellular acidification rate
ETC	Mitochondrial electron transport chain
FCS	Foetal calf serum
FSC	Forward scatter Gastric
G6P	Glucose-6-phosphate
GAPD	Glyceraldehyde 3-phosphate dehydrogenase
GrzB	Granzyme B
HMB-PP	(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
HSC	Hematopoietic stem cells
iDCs	Immature dendritic cells
IFN-γ	Interferon-γ
IH2	Innate helper 2 cells
IL-2	Interleukin-2
ILCs	Innate lymphoid cells

ION	Ionomycin
IPP	Isopentenyl pyrophosphate
ITAMs/ITIM	Immunoreceptor tyrosine-based activation (or inhibitory) motifs
IFN	Interferon
KIR	Killer cell immunoglobulin like receptor
LTi	Lymphoid tissue inducer
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MICA/MICB	MHC class I-related chains A or B
mTORC	Mammalian target of rapamycin complex
NK cells	Natural Killer cells
NADPH	Nicotinamide adenine dinucleotide
NKG2D	Killer cell lectin-like receptor subfamily K
OCAR	Oxygen consumption rate
OxPhos	Oxidative phosphorylation
pAg	phpshoantigen
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed cell death-1
pS6	S6 ribosomal protein
PRP	Pattern recognition receptors
RORα	Retinoic acid receptor-related orphan receptor-α
ROS	Reactive oxygen species
PPP	Pentose phosphate pathway
SSC	Side scatter
SREBP	Sterol regulatory element binding protein
TCA	Tricarboxylic Acid Cycle
TGF-β	Transforming growth factor beta
TCR	T cell receptor

T_H	T helper
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor- α
Treg	Regulatory T cells

Chapter 1- Introduction

1. Introduction

1. 1 Sepsis

Sepsis is a medical condition characterized by life-threatening organ dysfunction which most frequently occurs as a result of a dysregulated host immune response to infection (Singer et al., 2016). Sepsis is one of the leading causes of death worldwide, with mortality rates of up to 30%. It is recorded that in USA more than 260,000 people die of sepsis every year. Though it can affect people of any age. It most commonly occurs in older adults, pregnant women, children aged less than one year, and people suffering from diseases such as diabetes, kidney and lung diseases or cancer (Bone et al., 1992). The symptoms of sepsis are similar to those of other conditions of infection/inflammation including: fever, chills, rash, rapid breathing and heart rate, confusion and disorientation, making the diagnosis of sepsis difficult.

Septic shock is a subset of sepsis in which profound circulatory, cellular and metabolic abnormalities are linked to a greater than 40% mortality (Singer et al., 2016). Septic shock is characterized by multiple organ failure, low blood pressure, heart weakness and cellular abnormalities which substantially increase mortality (Carvalho, 2019). Failure of innate and adaptive immunity occurs once patients develop septic shock (Lukaszewicz et al., 2009; Venet et al., 2013). This is associated with increased apoptosis of immune cells, reduced pathogen killing and impaired production of pro-inflammatory cytokines (Forel et al., 2012; Grimaldi et al., 2011; Hotchkiss et al., 2005; Inoue et al.,

2013). Antigen presentation is associated with increased expression of inhibitory receptors on T cells and proliferation of T regulatory (Treg) cells (Faivre et al., 2012; Grimaldi et al., 2011; Venet et al., 2004). Impairment of both pro- and anti-inflammatory immune responses play roles in the pathogenesis of septic shock (Angus & der Poll, 2013).

The pathogenesis of sepsis can be described as a race toward death between a dysregulated systemic immune response and a microbial infection or trauma that leads to organ failure (Cao et al., 2019). Epidemiological studies have revealed that sepsis is most commonly caused by bacteria but may be also caused by fungi, viruses or parasites or trauma in a sterile environment. Most cases of septic shock are caused by Gram-positive bacteria (Martin, 2012), followed by endotoxin producing Gram-negative bacteria. However, several studies have revealed that a common cause of septic shock is fungal infection (Dellinger et al., 2013). Many times, septic infection occurs due to mixed infection. The most common causing agents of sepsis are *Staphylococcus aureus* and *Escherichia coli*. A high mortality rate is associated with *Pseudomonas aeruginosa* infection.

The antimicrobial response in patients with sepsis is biphasic with an overwhelming inflammatory response preceding a period of profound immune suppression (Hotchkiss, 2014). The pro-inflammatory and anti-inflammatory immune responses play a critical role in promoting and preventing the development of infection and organ dysfunctions (de Pablo et al., 2014). In order to improve the outcome of patients with sepsis, a further

investigation of the immunological phases of sepsis is required to develop immunotherapies to combat the disease (Cheng et al., 2015).

1. 2 Innate and adaptive immunity in healthy individuals

The innate immune system is the first line of defence against pathogens and tumours. It is activated upon binding of conserved components of pathogens, known as pathogen associated molecular patterns (PAMP) to a variety of pattern recognition receptors (PRR) expressed by effector cells of the immune system, leading to their activation. These innate immune effector cells include monocytes, macrophages and neutrophils, which kill bacteria and fungi by phagocytosis; eosinophils, basophils and mast cells which attack worms and parasites by releasing toxic mediators; and natural killer (NK) cells which kill infected and malignant host cells. Pathogen recognition can also result in the release of a variety of soluble mediators, such as anti-microbial peptides, cytokines, complement and proteins that opsonise pathogen for destruction by the above-mentioned immune effector cells. Innate immune responses are rapid and antigen-non-specific (Murphy et al., 2010). The adaptive immune system is activated upon the recognition of antigen from a pathogen or a vaccination. Pathogens are captured by dendritic cells and brought to the lymphoid tissues where their proteins are digested into peptides, bound to major histocompatibility complex (MHC) molecules and presented to T cells, leading to their activation. Pathogens passing through the lymphoid tissues are also directly recognised by B cells. The antigen receptors on T cells and B cells display hypervariability allowing for the activation of pathogen-specific defence, which takes several days to month, but it persists throughout life and can be

recalled upon subsequent encounter of the same pathogen. T cells and B cells together provide antigen-specific defence. T cells kill pathogen-infected cells and selectively recruit and activate the effector cells of the innate immune system via the secretion of cytokines, whereas B cells produce antibodies which neutralise pathogens and opsonise them for destruction by the same effector cells. Both innate and adaptive immune system interact with each other leading to defence against pathogens.

1. 3 Immunology of sepsis

The immune response in sepsis patients involves an initial hyperinflammatory phase characterised by uncontrolled activation of innate immune cells and the production of high levels of pro-inflammatory cytokines such as tumour necrosis-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), IL-8, interferon-γ (IFN-γ) and granulocyte macrophage colony stimulating factor (GM-CSF) (Gouel-Chéron et al., 2012; Wu et al., 2009). This hyperinflammatory stage is followed by a hypo-inflammatory phenotype of immunosuppressive phase (Hotchkiss et al., 2013) characterised by leukocyte apoptosis and the release of immunoregulatory cytokines (Cao et al., 2019). Cells of both innate and adaptive immune systems including neutrophils, monocytes, macrophages, B cells, T cells, NK cells and DCs, may become depleted and impaired in their function (Fig 1.1) (de Pablo et al., 2014, Cao et al., 2019; Hotchkiss et al., 2013). The immunosuppressive phase of sepsis, also known as a compensatory anti-inflammatory response syndrome, may include deficiencies of phagocytosis, antigen processing and presentation, apoptosis of DC, and functional alterations in T and B lymphocytes, NK cells, neutrophils and monocytes (Biron et al., 2015, Toro et al., 2013). These changes are associated with a low expression of MHC class II molecules, and increased production of anti-

inflammatory cytokines such as IL-4, IL-10, IL-27, TGF- β and TNF-α soluble receptor. Many factors are involved in the progression and development of this immunosuppressive stage of sepsis, including the virulence of pathogen, age and genetic factors of the host (Hotchkiss, 2014, Toro et al., 2013, Boomer et al., 2011,Hotchkiss & Nicholson, 2006; Hotchkiss et al., 2016; Otto et al., 2011; Torgersen et al., 2009).

T lymphocytes play vital roles in the immunological response to sepsis. Previous studies have determined a marked decline of conventional αβ T cells ($CD4^+$ and $CD8^+$ T cells) in septic patients. Surviving αβ T cells display a phenotype shift from pro-inflammatory Th1 phenotype to anti-inflammatory Th2 phenotype characterized by the reduction in pro-inflammatory cytokines and an increase in the production of inhibitory cytokines as well as variation of surface molecules, such as PD-1 and PDL-1 (Boomer et al., 2011). Sepsis-induced immunosuppression involves apoptotic depletion of T cells, increased expression of negative costimulatory molecules, increased regulatory T (Treg) cell expression and T cell exhaustion (Fig 1.2) (Boomer et al., 2011; Cao et al., 2019).

A previous study from our lab has demonstrated that patients with bacterial sepsis exhibit a persistent failure of T cell activation which was associated with diminished $CD8^+$ T cell and CD4 Th17 cell numbers (Coakley et al., 2019). Treg cells produce IL-10 and TGF-β which suppress Th1, Th2 and Th17 cell differentiation, prevent immune reactivity against self and harmless antigens and resolve immune responses once pathogens are removed from the body. Many studies have revealed that Treg cells and IL-10 production characterises the immunosuppressive phase of sepsis (Faivre et al.,

2012; Grimaldi et al., 2011; Venet et al., 2004). Therapies proposing to enhance the functions of these cells may represent an essential and new trend.

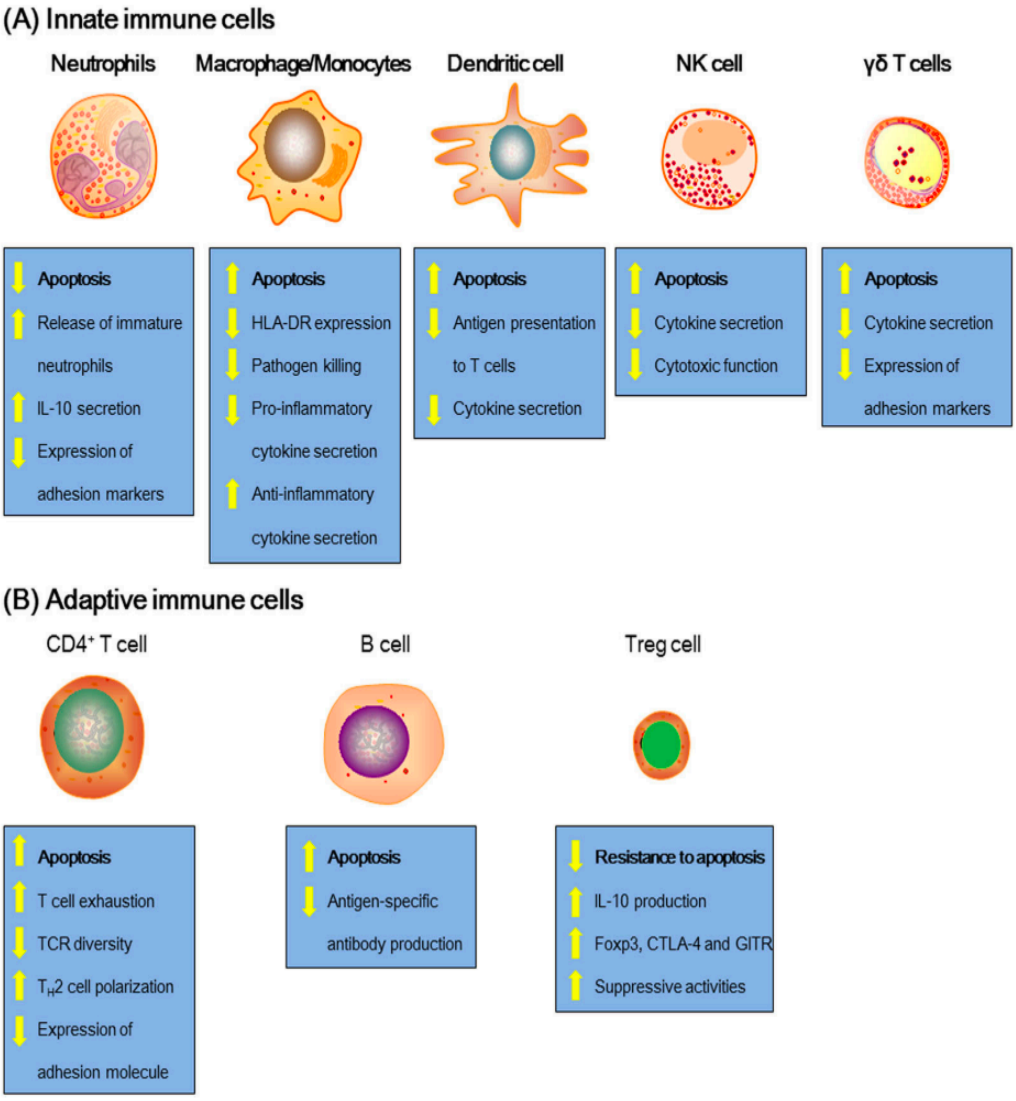

Figure 1. 1 An overview of changes innate and adaptive immune cells in sepsis.

Sepsis-induced immune paralysis is identified by immunological defects that impair host immunity. Leukocyte loss, often resulting in diminished capacity to fight and eliminate pathogens, is a primary feature of immune suppression during sepsis. Altered immune cell function induced by uncontrolled apoptosis is a major cause of profound immunosuppression. Leukocyte apoptosis, including that of innate immune cells and adaptive immune cells, is associated with a higher risk of secondary infections and poor

outcome in various diseases. As shown here, sepsis rapidly triggers apoptosis in macrophages/monocytes, dendritic cells, NK cells, γδ T cells, CD4+ T cells, and B cells. However, apoptosis of neutrophils is delayed, and Treg cells are more resistant to sepsis-induced apoptosis. Immune cell depletion due to apoptosis is the primary mechanism of sepsis-induced immune suppression (Cao et al., 2019).

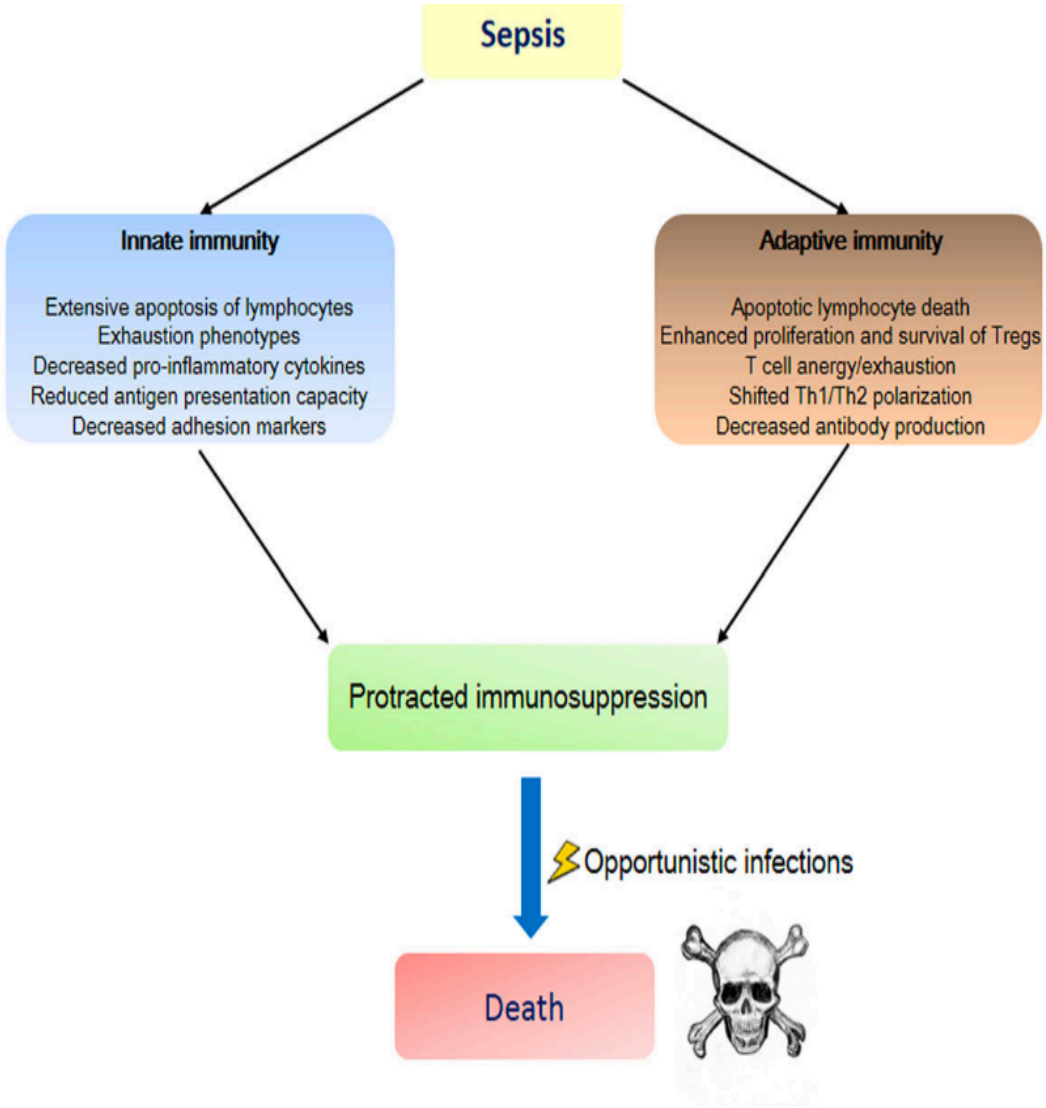

Figure 1. 2 Modification in innate and adaptive immunity in the pathophysiology of sepsis.

Early activation of innate immunity is the first line of defence against infection and plays a central role in the initiation of adaptive immunity. However, in sepsis, excessive immune responses lead to several alterations in innate and adaptive immunity that contribute to protracted immunosuppression and increase the risk for opportunistic infection (Cao et al., 2019).

1. 3. 1 Immune response in sepsis and metabolism involvement

There has been growing evidence to establish that metabolic shift is critical in the change of hyperinflammatory to hypo-inflammatory phase in sepsis. Innate immune cells must carry out their role of clearing a pathogenic insult throughout the hyper-inflammatory phase of sepsis. This process requires high amounts of energy. Glycolysis is a major source of energy for immune cells during acute stress, such as hyper-inflammation. Another efficient source for energy is oxidative phosphorylation (OxPhos), but it is slower than glycolysis. Therefore, innate immune cells that fight pathogen preferentially utilize glycolysis to meet the high energy life-threatening microbial invasions. That leads to OxPhos inhibition in immune cells under stress due to infection. Evidence confirms that a hypo-metabolic state displaces the hyper-metabolic anabolic state of sepsis in cell, animal, and human models. It has also been observed that immune cells in hypo-inflammatory phase using fatty acids as an energy source rather than glucose. This low energy phenotype may be a survival need for the immune cells in sepsis as if cells were to continue proliferating despite inadequate amounts of ATP, apoptosis would be significantly upregulated (Vachharajani & McCall, 2019).

"Immunometabolism" is a novel and essential area of immunology and although there is a current need for detail investigations, it provides a new direction for research on sepsis.

1. 4 Natural Killer cells

1. 4. 1 What are Natural Killer Cells?

Natural Killer (NK) cells, which belong to the category of "innate lymphoid cells", are cells that launch a nonspecific and rapid defence against pathogens and tumours (Barrow et al., 2018; Jost & Altfeld, 2013; Morvan & Lanier, 2016). They can kill cancer cells or cells infected with viruses. Approximately 5-15% of peripheral lymphocytes are NK cells, but they are found in higher numbers in the liver, spleen, lung and bone marrow. NK cells mediate antimicrobial responses as a result of their ability to identify pathogen-associated molecular patterns (PAMP) and cell-surface receptors that signify infection or tumour transformation. NK cells can combat infection directly by producing cytotoxic mediators such as granzymes and perforin. The also release pro-inflammatory cytokines, such as IFN-γ, which promote the activation of other innate and adaptive immune cells such as dendritic cells, monocytes, macrophages, and neutrophils and T cells (Colonna, 2017, de Pablo et al., 2014, Walzer et al., 2005). They are derived from hematopoietic stem cells in the bone marrow, however, they do not possess the rearranging antigen receptor genes found in T cells and B cells of the adaptive immune system. Their heavy dependence on a lack of MHC class I expression by target cells is framed as the "missing self" hypothesis, as discussed below. The development of NK cells and their subsets, their phenotypes and functions, and roles in the pathogenesis of sepsis are introduced in the upcoming sections.

1. 4. 2 NK Cell Subsets

NK cells are frequently defined as cells that express CD56 but lack expression of CD3 (Angelo et al., 2015). CD56 is one of the main surface markers that defines human NK cells in their mature stages. Two subsets of NK cells, distinguished by the intensity of CD56 expression, are found in blood and tissues. $CD56^{bright}$ NK cells account for ~10% of total human NK cells in the circulation and express low levels of FcγRIII (CD16), whereas $CD56^{dim}$ cells have a higher expression of CD16 (Hanna & Mandelboim, 2007). $CD56^{dim}$ NK cells are more cytolytic and account for 90% of human NK cells in peripheral blood. CD16 is responsible for antibody mediated cytotoxicity by binding the Fc portion of IgG. In contrast, $CD56^{bright}$ NK cells most notably produce cytokines, chemokines and IFN-γ (Cooper et al., 2001; Fehniger et al., 2003; Keating et al., 2016). Based on the expression of CD16 and CD56, NK cells have been classified under three phenotypes - $CD16^{+/-}CD56^{bright}$ (mainly found in the tissues), $CD16^{+}CD56^{dim}$ (mainly circulating) and $CD16^{+}CD56^{null}$ which are mainly associated with infections and pathological conditions. These subsets of cells also have other essential markers. For example, $CD56^{bright}$ NK cells co-express CD94 (Mahapatra et al., 2017). $CD56^{dim}$ NK cell subsets possess killer immunoglobulin-like receptors (KIR). Through development, NK cells gradually lose expression of CD56, acquiring $CD56^{dim}$ phenotypes.

Other markers that are expressed on activated NK cells include CD25, CD69 and human leukocyte antigens (HLA) class II (HLA-DR). Although the functions of the activated NK cells are similar across species, there are differences in the expression of the membrane

markers. For example, in mice, there is a lack of KIR, but they express lectin-like Ly49 receptors, which perform similar functions.

1. 4. 3 Receptors that activate and regulate NK cells

NK cells express a wide range of stimulatory and inhibitory receptors that regulate their activation status against target cells. Table 1.1 show markers and receptor of human NK cells (Montaldo et al., 2013). NK cells have two main groups of inhibitory receptors, including KIR and NKG2 receptors.

NKG2 receptors are a group of C-type lectin receptors. NKG2A, NKG2C, and NKG2E, heterodimerize with CD94 forming inhibitory receptors, while NKG2D associates with DAP10 to form an activating receptor. CD94 binds to the non-classical HLA molecule HLA-E on target cells (Sullivan et al., 2007). NKG2A, NKG2C and NKG2E are contain immune-tyrosine based activating motifs (ITAMs) in their cytoplasmic tails which deliver inhibitory signals upon ligation of CD94 (Lieto et al., 2006). NKG2D has very little similarity to the other NKG2 receptors. It does not dimerise with CD94, however, it is expressed as a homodimer on the surface of the NK cells. Additionally, it interacts with the stress induced molecules MICA and MICB, and ULBPs 1, 2 and 3 (Eleme et al., 2004).

KIR is a selective receptor which interact with human leukocytes antigens A , B and C (HLA-A, HLA-B and HLA-C) which present peptides derived from cytoplasm of target cells to $CD8^+$ T cells (Thielens et al., 2012). The inhibitory KIRs contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) placed on long cytoplasmic tails, whereas the

activating KIRs associate with the device protein DAP12, that itself contains ITAMs (Thielens et al., 2012).

Other NK cell stimulatory receptors include the natural cytotoxicity receptors (NCR); NKp46, NKp30 and NKp44 (Kruse et al., 2014). These receptors recognise a broad range of ligands, including microbial-derived ligands and ligand on host cells.

One of the key functions of NK cells is antibody dependent cell cytotoxicity (ADCC). NK cells express Fc receptors which bind to the Fc portions of immunoglobulin (Ig) molecules, which coat the surface of target cells (Morel et al., 1999; Smyth et al., 2005; Wang et al., 2015). These Fc receptors have ITAM domains that induce signal transduction leading to target cell lysis by the NK cells (Boomer et al., 2011). Thus, the innate immune functions of NK cells can be triggered in conjunction with the adaptive immune system. Stimulation of ADCC is being used for cancer therapy. Monoclonal antibodies that target tumour antigens, such as anti-GD2 mAb can stimulate ADCC of melanoblastoma and neuroblastoma cells. For treating HER2 positive breast cancer, trastuzumab has been found to act via NK cell mediated ADCC (Kohrt et al., 2015; Wang et al., 2015).

Table 1. 1 Markers and receptors of human NK cells (Sivori et al., 2019).

Table. 1. Human NK cell receptors and their ligands

Molecule	Ligand	$CD56^{bright}$	$CD56^{dim}$
Non-HLA-specific receptors			
Coreceptors			
CD59	LFA-2 (CD2)	All PB NK cells	
NTB-A (CD352)	NTB-A (CD352)	All PB NK cells	
NKp80	AICL	All PB NK cells	
DNAM-1 (CD226)	Nectin-2 (CD112), PVR (CD155)	All PB NK cells	
2B4 (CD244)	CD48	All PB NK cells	
Inhibitory			
PD-1 (CD279)	PD-L1 (CD274), PD-L2 (CD273)	–	Subsets
Siglec-7 (CD328)	Ganglioside DSGb5	Most of PB NK cells	
IRP60 (CD300a)	α-Herpes virus, Pseudorabide virus, Phosphatidylserine, Phosphatidylethnolamine	All PB NKs	
Tactile (CD96)	PVR (CD155)	All PB NK cells	
IL1R8	IL-37	PB NK cells	
TIGIT	PVR (CD155)	PB NK cells	
TIM-3	Gal-9, PtdSer, HMGB1, CEACAM1	Subset of NK cells	
Activating			
NKp30 (CD337)	B7-H6, BAG6/BAT3	++	+
NKp44 (CD336)	21spe-MLL5 - Nidogen-1	Activated NK cells	
NKp46 (CD335)	CFP (properdin), viral HA and HN, PfEMP1	++	+
NKG2D (CD314)	MIC-A, MIC-B, ULPBs	All PB NK cells	
FcγRIII (CD16)	IgG	–/+	+/++
HLA-specific receptors			
Inhibitory			
NKG2A/KLRD1 (CD159a/CD94)	HLA-E	+	Subsets
KIR2DL1 (CD158a)	HLA-C2	–	Subsets
KIR2DL2/3 (CD158b)	HLA-C1, few HLA-B[b]	–	Subsets
(CD158d)[a]	HLA-G	–	Subsets
KIR2DL5 (CD158f)	???	–	Subsets
KIR3DL1 (CD158e1)	HLA-A-Bw4, HLA-B-Bw4	–	Subsets
KIR3DL2 (CD158k)	HLA-A*03 and *11	–	Subsets
ILT2/LIR-1 (CD85J)	Different MHC-I alleles	.	Subsets
LAG-3 (CD223)	MHC-II	Activated NK cells	
Activating			
KIR2DS1 (CD158h)	HLA-C2	–	Subsets
KIR2DS2/3 (CD158j)	???	–	Subsets
KIR2DL4 (CD158d)[a]	HLA-G	–	Subsets
KIR2DS4 (CD158i)	HLA-A*11 and some HLA-C	–	Subsets
KIR2DS5 (CD158f)	???	–	Subsets
KIR3DS1 (CD158e1)	HLA-Bw4, HLA-F	–	Subsets
NKG2C (CD159a)	HLA-E	–	Subsets

Table 1 continued

Molecule	Ligand	$CD56^{bright}$	$CD56^{dim}$
Homing receptors			
2° lymphoid tissues			
CCR7 (CD197)	CCL19, CCL21	+	−
CXCR3 (CD183)	CXCL9, CXCL10, CXCL11	++	Subsets
L-Selectin (CD62L)	GLyCAM-1, MadCAM-1	++	Subsets
Inflammation sites			
CXCR1 (CD181)	CXCL8 (IL-8)	−	+
CXCR2 (CD182)	IL-8-RB	−	+
CX3CR1	Fraktalkine	−	+
ChemR23	Chemerin	−	+
Others			
CXCR4 (CD184)	CXCL2	Subsets of NK cells	
CCR5 (CD195)	RANTES, MIP1α and MIP1β	Subsets of NK cells	
S1P5	S1P	−	+
c-Kit (CD117)	SCF (KL)	+	−

[a]KIR2DL4 has been shown to have both inhibitory and activating functions[164]
[b]KIR2DL2/L3 weakly recognize also HLA-C2 alleles and few HLA-B alleles that bear the HLA-C1 epitope (e.g., HLA-B*4601 and HLA-B*7301)

1. 4. 3. 1 Cytokines that activate NK cells

NK cell functions are affected by various cytokines, including IL-2, IL-12, IL-15, IL-18, IL-35 and type I interferons (IFNs, IFN-α and β). Distinguished at first as NK cell stimulatory factor IL-12, produced by macrophages and DC, promotes cytotoxicity and IFN-γ production by NK cells (Chan et al., 1992). IL-2, IL-15, IL-18, IL-12, IL-35, IFN-α and β derived from macrophages and DC can also induce NK cells activation leading to an increase in cytotoxicity and IFN-γ production (Chace et al., 1997; Lucas et al. , 2007, Wu et al., 2015). Furthermore, combinations of IL-12 and IL-2 or IL-12 and IL-15 can synergise to activate NK cell effector functions (Liu et al., 2000; Zhao & French, 2012). IL-18 can boost IFN-γ production by NK cells by stabilizing IFN-γ mRNA transcripts through the MAPK p38 pathway (Mavropoulos et al., 2005). IL-10 and TGF-β are immunosuppressive cytokines that can inhibit and downregulate NK cell activities and functions (Rook et al., 1986). TGF-β downregulates the expression of NKG2D on the NK cell surface (Crane et al., 2010).

1. 4. 3. 2 The missing-self mechanism

The expression of MHC class I molecules on the surface of the target cells helps the NK cells to distinguish between “self” and “non self”. Inhibitory receptors bearing ITIM motifs on the NK cell surface recognize certain MHC-I molecules and if they are downregulated (the missing-self), as frequently occurs in virus-infected and tumour cells, NK cell inhibition is abrogated leading to cell activation due to the presence of activating ligands (Lanier, 2005). The final output is dependent on the balance between

stimulatory and inhibitory signals. Tumour cells and viruses often downregulate MHC-I to prevent peptide antigen presentation of antigens to conventional $CD8^+$ T cells.

1. 4. 4 NK Cell functions

1. 4. 4. 1 NK cell cytotoxicity

Cytotoxic lymphocytes, including NK cells, kill cells in three stages - recognition of the target cell, contact with the target cell resulting in the formation of an immunological synapse, and death of the target cell (Rajasekaran et al., 2016). NK cells can release cytolytic molecules into target cell leading to caspase-mediated apoptosis (Khosravi-Far & Esposti, 2004). The cytolytic proteins are present within small granules that travel to the site of contact between the NK cells and the target cell. One cytolytic molecule, perforin binds to the target cell membrane and polymerises to form a pore through which other cytolytic molecules enter the target cell and induce apoptosis by activating caspases (Osińska et al., 2014). Granzymes are a group of serine protease that cleave many substrates inside the target cells and activate apoptotic pathways (Smyth et al., 2001). NK cells can also kill infected host cells or tumour cells by ligating death receptors on these cells. Fas ligand (FasL) is a death ligand expressed by NK cells. It is a membrane pound protein which moderates the death-inducing signalling complex (DISC) activation in target cells through binding their Fas receptors (Warren & Smyth, 1999). TNF-related apoptosis-inducing ligand (TRAIL) is also produced by NK cells and can bind to specific death receptor on target cells and activates a caspase-8 dependent apoptotic pathway.

1. 4. 4. 2 Cytokine Production by NK cells

Many essential pro-inflammatory cytokines are produced by NK cells, in response to infection or contact with cancer cells. Peripheral NK cells predominantly produce Th1 related cytokines, including IFN-γ and TNF-α (Cook et al., 2014; Vivier et al., 2011). These cytokines control the activities of multiple cells of the innate and adaptive immune systems, including macrophages, neutrophils, dendritic cells, T cells and B cells (Blanchard et al., 1991; Van den Bosch et al., 1995). They act through receptors on other cells by activating specific signalling pathways that culminate in the activation of transcription factors resulting in gene expression.

IFN-γ is an important cytokine produced by NK cells, in particular the $CD56^{bright}$ subset. IFN-γ receptor engagement activates the Jak-Stat signal pathway, which involves sequential recruitment of members of the Janus kinases and the signal transducer and activator of transcription proteins (STATs), to control the transcription of target genes. IFN-γ induces activation of the antimicrobial defences of macrophages, and antigen presentation via the upregulation of MHC molecules, IFN-γ also regulates B cell functions by modulation the production of immunoglobulin (Schroder, Hertzog, Ravasi, & Hume, 2004).

Another major pro-inflammatory cytokine that NK cells produce is TNF-α. It has many functions which involve balancing of the inflammatory response induced by the activation of transcription factors, such as activator protein 1 (AP-1) and NF-κB,

controlling cell expansion and differentiation and actuation of apoptotic and necroptotic cascades (Wajant et al., 2003; Pfizenmaier, & Scheurich, 2003).

Some anti-inflammatory cytokines can also be released by NK cells in response to activation. These include IL-10 and TGF-β (Mehrotra et al., 1998; Weiss & Attisano, 2013), which repress the activation of transcription factors and gene expression that result in pro-inflammatory cytokine secretion. TGF-β operates via Smad dependent and independent signal transduction pathways to suppress T-bet and the expression of IFN-γ (Weiss & Attisano, 2013). TGF-β has also been shown to inhibit the mammalian target of rapamycin (mTOR) signalling complex of NK cells (Viel et al., 2016; Zaiatz-Bittencourt et al., 2018).

1. 4. 5 NK Cells in Sepsis

Some studies have reported increases in NK cell numbers and IFN-γ levels in sepsis, many studies have also reported a decline in these parameters. A clinical study indicated a reduction in of NK cells absolute number and a lower amount of IFN-γ production in patients with sepsis, severe septic and septic shock compared with healthy controls (Forel et al., 2012). There is a decrease in NK cell numbers in the initial 14 days during sepsis (Holub et al., 2000). This change was also noticed as early as 24 hours after sepsis onset (Boomer et al., 2012). This is associated with immunosuppression and impaired immunity against infection in the host. This phenomenon is not restricted only to NK cells, but to other immune cells (Hotchkiss et al., 2013; Muenzer et al., 2010). Chiche *et*

al. (2011) reported reactivation of cytomegalovirus in septic patients, citing the reason to be impaired IFN-γ production (Chiche et al., 2011).

In septic shock, the patients are considered to be more prone to "compensatory anti-inflammatory response syndrome (CARS)". Monserrat and colleagues studied the changes in NK cells during the first 28 days in ICU in 52 patients with septic shock. They found that the survivors of septic shock had lower numbers of NK cells in their circulation than healthy controls, suggesting that these cells are activated earlier in septic patients (Monserrat et al., 2009). Thus, the amount of NK cells during the initial inflammatory response can help predict whether the septic shock can be fatal (de Pablo et al., 2012).

1. 5 What are γδ T Cells?

γδ T cells are a subset of T cells that account for 1-5% of peripheral T cells but are found in large numbers in the intestinal mucosa. γδ T cells express TCR γ and δ chains along with the CD3 molecule (Carding & Egan, 2002). The TCRs of γδ T cells recognise a variety of non-protein antigens, including glycolipids and pyrophosphates, and stress-inducible molecules expressed by virus-infected and tumour cells. The activation of γδ T cells can also occur via PAMPs, and by superantigens (Hedges et al., 2005). Cytokine production and cytotoxic activity are key effector functions of γδ T cells. γδ T cells are also occupied in crosstalk with other immune cells, such as dendritic cells, macrophages, B cells and neutrophils (Bhan et al., 2016, Jameson et al., 2003).

1. 5. 1 Human γδ T cell subsets

In humans, γδ T cells have three main subsets, based on their δ chain expression Vδ1, Vδ2, Vδ3 T cells. Various subsets of Vδ1 T cells recognize virus-infected cells expressing MICA and MICB (Groh et al., 1998), self-glycolipids presented by CD1c and CD1d (Bai et al., 2012; Spada et al., 2000), and the algal protein phycoerythrin (Zeng et al. 2012). The TCR of Vδ2 T cells recognizes phosphoantigen (pAg), intermediates of isoprenoid metabolism, a family of syntheses including the Ecoli and mycobacteria-derived (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), and supplementary natural molecules generated by various microorganisms and plants, generated by the DOXP/MEP (1-deoxy-d-xylulose-5-phosphate/2-C-methyl-d-erythritol- 4-phosphate) pathway, present in some bacteria and protists (Eberl et al., 2003; Morita et al., 2007), and isopentenyl pyrophosphate (IPP) found in humans, which are produced in response to tumour transformation and aminobisphosphonate drugs (Fig. 1.3) (Gober et al., 2003; Kabelitz, et al., 2004; Moser & Eberl, 2007; Wang et al., 2011; Weschet al., 2001). The antigen-presenting molecule for pAgs has been shown to be butyrophilin 3A (Harly et al., 2012; Rhodes et al., 2015; Sandstrom et al., 2014). Subsets of Vδ3 T cells can recognize HLA-A2 (Allison et al., 2001) and CD1d (Mangan et al., 2013; Petrasca et al., 2018). In addition to the TCR, Vδ1, Vδ2 and Vδ3 T cells can be activated via ligation of other stimulatory receptors, including NKG2C, NKG2D, NKp30, toll-like receptors, and the β-glucan receptor, dectin 1 (Fausther-Bovendo et al., 2008; Hudspeth et al., 2012; Kuroda et al., 2012; Maher et al., 2015; Wesch et al., 2011).

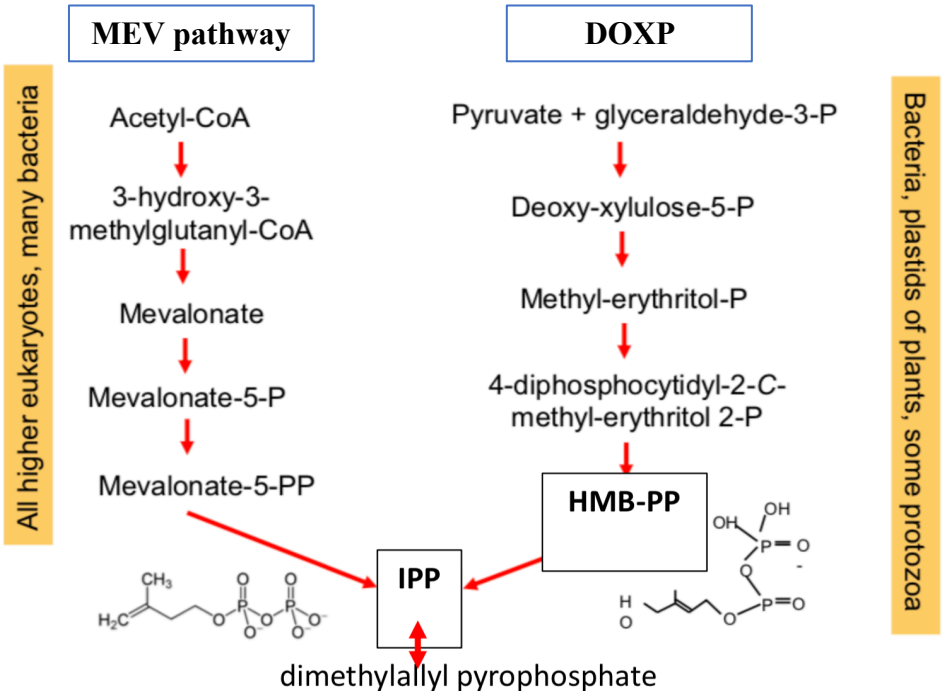

Figure 1. 3 Mevalonate and 1-deoxy-D-xylulose-5-phosphate pathways for isoprenoid biosynthesis.

The Mevalonate (MEV) pathway exists in eukaryotes, in numerous pathogenic and non-pathogenic bacteria (including many Gram-positive bacteria). In contrast, the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway is detected in most bacteria and protozoa. The DOXP pathway, HMBPP might not lead to IPP directly, but rather to its isomer, dimethylallyl pyrophosphate, (DMAPP). By contrast, in the MEV pathway, mevalonate-5-pyrophosphate directly leads to IPP.

1.5.2 Functions of γδ T cells

Activated Vδ1, Vδ2 and Vδ3 T cells produce cytokines, chemokines, antiviral and antimicrobial factors, which stimulate other immune cells such as monocytes, neutrophils, DCs, B cells and other T cells (Chien et al., 2014; Eberl et al., 2009; Lawand et al., 2017; Petrasca & Doherty, 2014). Vδ1, Vδ2 T and Vδ3 T cells subsets can induce DC maturation to cytokine producing antigen-presenting cells (APC). Furthermore, Vδ2 and Vδ3 T cells can promote maturation and antibody production and release by B cells (Mangan et al., 2013; Petrasca et al., 2018). Most notably, Vδ2 T cells can promote tumour cell apoptosis and inhibit tumour cells proliferation (Joalland et al., 2018; Sengupta, 2019).

1. 5. 3 Vδ2 T Cells

Vδ2 T cells are the most abundant subset of γδ T cells, representing 1-5% in healthy human peripheral blood (Caccamo et al., 2006). During infection, they can expand locally and/or systemically to up to 50% of all peripheral T cells (Morita et al., 2007). As a result, they become activated and can kill infected cells and release pro-inflammatory cytokines such as IFN-γ, TNF-α, GM-CSF, IL-4, IL-10, and IL-17. Furthermore, they play a vital role in linking innate and adaptive immunity by promoting the differentiation of different subsets of antigen presenting cells (APCs) (Tyler et al., 2015). Vδ2 T cells account for 50-95% of γδ T cells in the peripheral blood of healthy individuals (Hinz et al., 1997; Morita et al , 2000; Sandstrom et al., 2014). Most Vδ2 T cells co-express the Vγ9 chain, forming Vγ9Vδ2 heterodimers (Venturi et al., 2008). This TCR chain

combination either be configured before birth, or alternatively may be selected by antigen (Willcox et al., 2018).

Being the major proportion of circulating γδ T cells in the human blood, the $Vγ9^{+}Vδ2^{+}$ T cells generally effect both innate and adaptive responses. Broadly, the γδ T cells are regarded as built up of two populations of cells $Vδ2^{+}$ and $Vδ2^{-}$ (Halary et al., 2005). Figure 1.4 summarizes these subdivisions, based on Davey et al. (2018). Based on the functions, the $Vγ9^{-}$ subset is more prone to having adaptive responses to immune stimulation, whereas the $Vγ9^{+}Vδ2^{+}$display more innate functions and means of defensive responses (Davey et al., 2018).

There are other markers which classify these $Vγ9^{+}Vδ2^{+}$ T cells , for example, CD27 and CD45RA (Pang et al., 2012). Based on these markers, the $Vγ9^{+}Vδ2^{+}$subsets can further be divided into subsets based on functions. Initially, there are naïve $Vγ9^{+}Vδ2^{+}$ which bear both the markers and constitute the major proportion of cells in lymph nodes. On stimulating with phosphoantigens, they lose CD45RA and become largely known as "central memory" T cells and secrete reduced amounts of IFN-γ (Dieli et al., 2003). Then, these cells gradually convert to both $CD45RA^{-}$ and $CD27^{-}$ on IL-2 and phospho stimulation becoming effector memory cells, now bearing many chemokine receptors like CCR2, CXCR3, CCR5 and CCR6. This has also been achieved with IL-15 stimulation and bearing CD45RA (Caccamo et al., 2005).

The classification of Vγ9^{+}Vδ2^{+} T cells into these subsets have been instrumental in the clinical field, where they have been used as markers for predicting treatments in patients with cancer (Nicol et al., 2011a). The functions of Vγ9^{+}Vδ2^{+} T cells have also been correlated with expression of some markers like CD56 and CD16 (Alexander et al., 2009). There is also a homing receptor-cutaneous lymphocyte associated antigen (CLA), that is present on many Vγ9^{+}Vδ2^{+} T cells. Their expression of the previously mentioned markers and degree of release of cytokines have been correlated with skin diseases (Laggner et al., 2011). Thus, the literature reviewed above tells us that the subsets of cells defined by the presence or absence of the markers have different functional implications and also may have different trajectories across development. It is essential to study and explore more subsets and other cell surface markers, and correlate them with more human diseases.

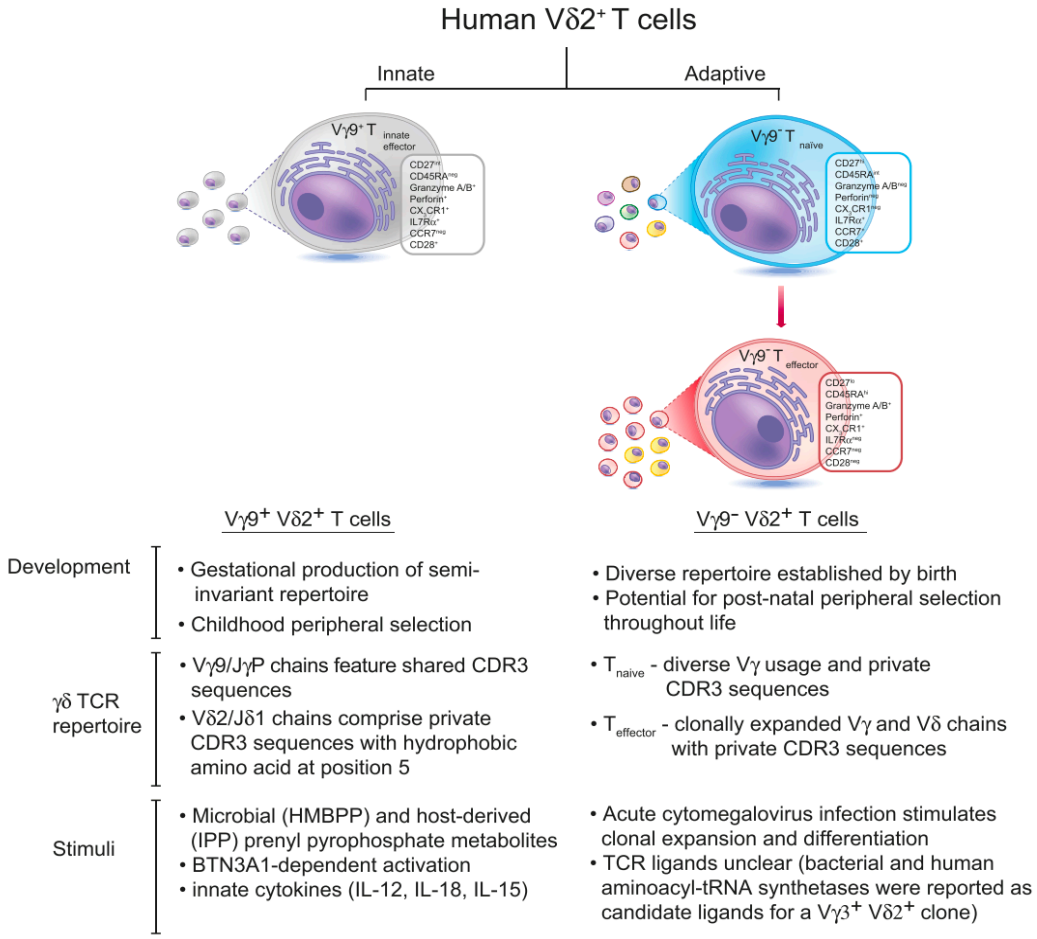

Figure 1. 4 Subsets of Vδ2 T cells.

The predominant Vγ9+Vδ2+ subset is produced during gestation, expresses Vγ9 chains with public CDR3 sequences, and undergoes peripheral selection and polyclonal development during childhood to become pAg-reactive "innate-like" effector cells. In contrast, Vγ9negVδ2+ T cells display Vδ2 paired with various Vγ chains showing private CDR3 sequences, and these cells distribute in the peripheral blood as naïve T cells until they meet a specific antigenic challenge (which can include, but is likely not limited to, CMV infection). Antigen-specific Vγ9negVδ2+ T cells undergo clonal extension and modify into effector T cells, alike to Vδ1+ T cells. The antigens identified by Vγ9negVδ2+ T cells remain unknown, although bacterial and human aminoacyl-tRNA synthetases have been identified as candidate antigens for a single Vγ3+Vδ2+ T cell clone (Davey et al., 2018).

1. 5. 3. 1 Chemotaxis of Vδ2 T cells

Vδ2 T cells can migrate rapidly from the blood to sites of infection and tumours in response to chemokines released at these sites (Futagbi et al., 2007; Qin et al., 2011; Zhou, Kang et al., 2012). They express CCR5, which directs them towards tumours where the ligands for the CCR5 receptor are expressed (Hannani et al., 2012). They also express CXCR3 (Dagna et al., 2002). Additionally, selectins, lymphocyte function antigen (LFA-1) and other adhesion molecules serve to recruit Vδ2 T cells from the blood into the tissues (Thomas et al., 2001). Once in the tissues, these cells can directly kill tumour cells and bacteria.

1. 5. 3. 2 Activation of Vδ2 T cells

The TCR, co-stimulatory molecules and receptors that are typically found on NK cells have a crucial role in Vδ2 T cell activation and regulation. Vδ2 T cells have a unique TCR-dependent recognition of non-peptide ligands (phosphoantigens) on cells in stressful conditions (Xiang & Tu, 2017). Vδ2 T cells respond to pyrophosphate intermediates of the biosynthetic pathways of isoprenoids. The most potent activator of Vδ2 T cells is HMB-PP, a pyrophosphate intermediate of the non-mevalonate pathway of isoprenoid synthesis, found mainly in bacteria. Upon phosphoantigen stimulation, Vδ2 T cells upregulate CD16 expression and ligation of CD16 with IgG results in the production of TNF-α (Lafont et al., 2001).

Butyrophilin 3A (BTN3A/ CD277) is the long-sought unconventional 'presenting' molecule for HMB-PP and IPP. Binding of phosphoantigens to the intracellular B30.2 (PRY-SPRY) domain of BTN3A results in a conformational change resulting in an

extracellular ligand which binds to the Vγ9Vδ2 TCR leading to Vδ2 T cell activation (Fig. 1.5) (Harly et al., 2012; Sandstrom et al., 2014). This intracellular identification of HMB-PP and IPP is similar to cases of B30.2-mediated innate immune responses mediated by proteins such as TRIM5a and TRIM21 (D'Cruz et al., 2013; Rhodes, De Bono, & Trowsdale, 2005; Tyler et al., 2015).

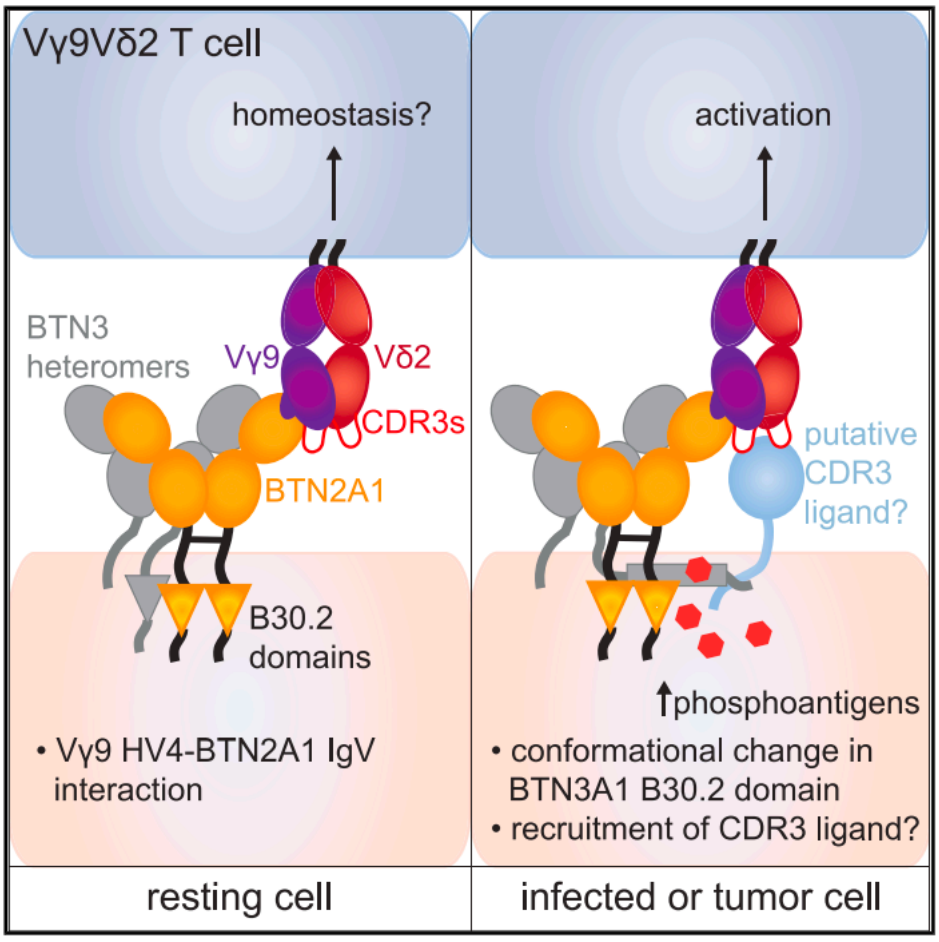

Figure 1. 5 Butyrophilin-2A1 Directly Binds Germline-Encoded Regions of the Vγ9Vδ2 TCR and Is Essential for Phosphoantigen Sensing.

Butyrophilin 2A1 (BTN2A1) links to BTN3A1 on the cell surface and attaches directly to germline-encoded regions of the Vg9 chain of the Vg9Vd2 TCR. Consequently, BTN2A1 cooperates with BTN3A1 to potentiate Vg9Vd2 T cell identification, operating an essential role in phosphoantigen sensing (Karunakaran et al., 2020).

In addition to TCR ligation, Vδ2 T cells can be activated by cytokines and by ligation of stimulatory receptors. Most Vδ2 T cells express NKG2D which is typically found on NK cells. The activation of Vδ2 T cells by ligation of NKG2D with anti-NKG2D antibody or the ligands MICA, MICB or ULBPs leads to the production of IFN-γ and TNF-α and cytolytic degranulation resulting in target cell killing (Rincon-Orozco et al., 2005). Many Vδ2 T cells express NKG2C, a stimulatory receptor which dimerises with CD94, allowing them to recognise target cells expressing HLA-E (Wischhusen et al., 2005).

Vδ2 T cells also express co-stimulatory receptors including CD28 and TNF receptor superfamily members (Xiang & Tu, 2017), which promote the survival and proliferation of Vδ2 T cells as result of improving IL-2 production (Ribot et al., 2012). Toll-like receptors (TLRs) including TLR2, TLR3, TLR4, TLR5 and TLR9 are also found on Vδ2 T cells. Ligation of TLR can co-stimulate TCR-mediated Vδ2 T cell activation and the release of IFN-γ, TNF-α and GM-CSF (Beetz et al., 2008; Nedellec et al., 2010).

Cytokines receptors are also important for activation and regulation Vδ2 T cells function, development, proliferation, differentiation and survival (Xiang & Tu, 2017). Vδ2 T cells can be expanded by stimulating them with IL-2 and IL-15 (García et al., 1998; Hayashi & Altman, 2007). Other studies have demonstrated that Vδ2 T cells stimulation with IL-12 and IL-18 induce the differentiation of IFN-γ^+ Vδ2 T cells (Tsuda et al., 2011), whereas other cytokines, such as IL-23 and/or IL-1β (Caccamo et al., 2011; Moens et al., 2011; Ness-Schwickerath et al., 2010) or IL-21 (Bansal et al., 2012; Caccamo et al., 2012) can induce Vδ2 T cell differentiation into effector cells that release Th2 or Th17

cytokines. The combination of IL-15 and TGF-β can stimulate Vδ2 T cells to differentiate into FoxP3 expressing regulatory T cells (Casetti et al., 2009).

Inhibitory receptors including B- and T-lymphocytes Attenuator (BTLA) receptors and PD-1 can negatively regulate Vδ2 T cells responses. (Gertner-Dardenne et al., 2013). Resting Vδ2 T cells do not express PD-1, whereas activated Vδ2 T cells upregulated PD-1 expression (Iwasaki et al., 2011). PD-1 and PD-L1 interaction can decrease the cytotoxicity and production of IFN-γ by Vδ2 T cells (Iwasaki et al., 2011). Figure 1.6 shows an overview of markers and receptors involved in regulating human Vδ2 T cells activity (Xiang & Tu, 2017).

1. 5. 3. 3 Functions of Vδ2 T cells

Activated Vδ2 T cells display a variety of effector functions including direct cytotoxicity of infected and tumour transformed cells, rapid and potent cytokine secretion, and cell contact-dependent differentiation and activation of monocytes, neutrophils, dendritic cells, T cells and B cells (Chien et al., 2014; Dunne et al., 2010; Kabelitz & He, 2012; Morita et al., 2007; Petrasca & Doherty, 2014; Vantourout & Hayday, 2013). Vδ2 T cells are also capable of presenting peptide antigens to conventional $CD4^+$ and $CD8^+$ T cells, leading to their activation. Vδ2 T cells have functional plasticity to differentiate into effector cells with cytokine profiles characteristic of Th1, Th2, Th17 and Treg cells.

1. 5. 3. 1 Cytotoxicity by Vδ2 T cells

Activated Vδ2 T cells can kill target cells by releasing granzymes, perforins and other anti-microbial molecules such as granulysin (Todaro et al., 2009; Wrobel et al., 2007). Perforin is a membrane-disrupting molecule, and most granzymes are involved in eradicating infected and cancer cells (Chowdhury & Lieberman, 2008). Vδ2 T cells produce granulysin which is a cytolytic and proinflammatory molecule (Krensky & Clayberger, 2009) that can kill extracellular Mycobacterium tuberculosis in tuberculosis and Plasmodium falciparum in malaria directly (Dieli et al., 2001; Farouk et al., 2004). Intracellular M. tuberculosis as well can be eradicated by both granulysin and perforin (Dieli et al., 2001).

1. 5. 3. 2 Cytokine production by Vδ2 T cells

Activation of Vδ2 T cells by phosphoantigens leads to the rapid secretion of cytokines (Correia et al., 2009; Gomes et al., 2010; Nedellec et al., 2010). HMB-PP-activated Vδ2 T cells predominantly produce Th1 cytokines, such as IFN-γ and TNF-α (Dunne et al, 2010), but they can also be induced to produce IL-13, IL-4 and IL-5, IL-17 and IL-10 (Caccamo et al., 2006; Ness-Schwickerath et al., 2010; Petrasca & Doherty, 2014; Vermijlen et al., 2007; Wesch et al., 2001). Dunne et al. (2010) described the cytokine profile of Vδ2 T cells after activation with IPP or HMB-PP. They found that they produced the proinflammatory cytokines of IFN-γ and TNF-α, but not IL-10, IL-13 and IL-17. They also showed that IL-2 and IL-15 accelerated the cytokine responses of Vδ2 T cells to the phosphoantigens. Cytokine production by Vδ2 T cells was independent of the expression of CD4 or CD8 on these cells. A minor subset of Vδ2 T cells which expresses

CD27 and CXCR5 has been shown to release IL-4 and IL-10 (Caccamo, Battistini, et al., 2006).

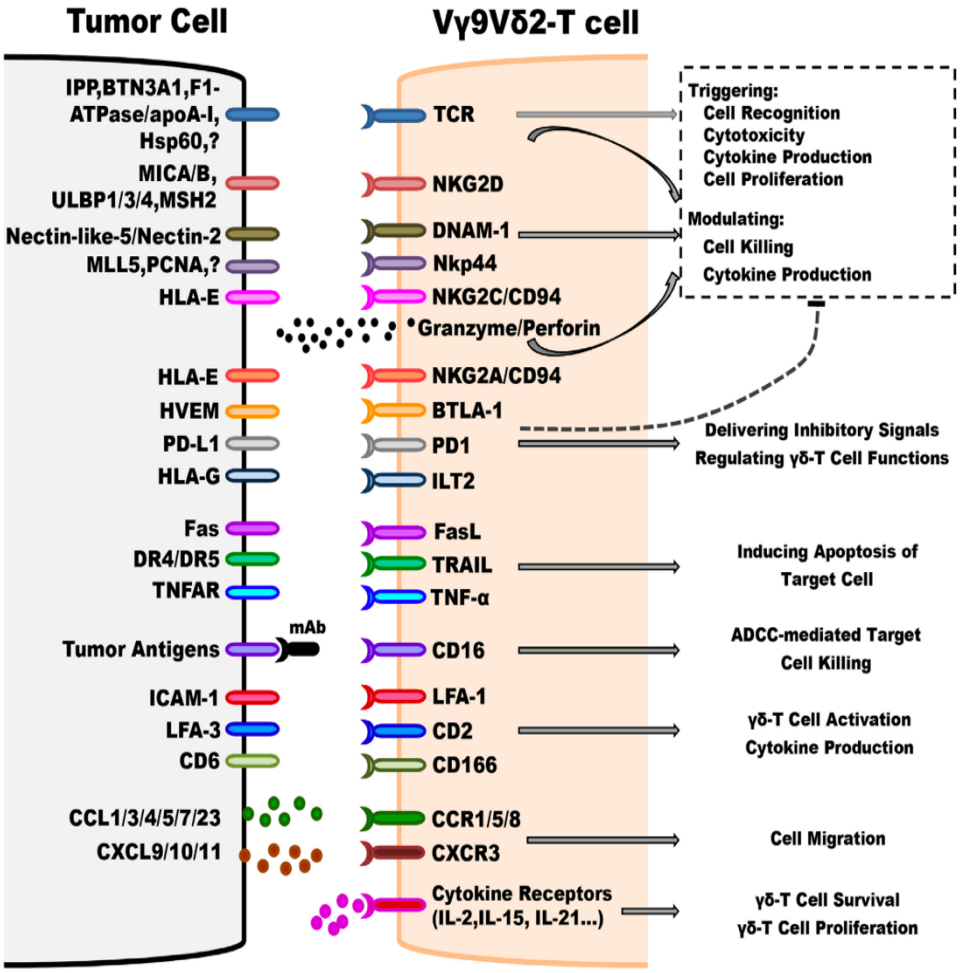

Figure 1. 6 an overview of markers and receptor that are involved in regulating human Vδ2 T cells activity.

Vδ2 T cells can distinguish between tumorous and normal cells using the T cell receptor (TCR) and other innate receptors. The Vγ9Vδ2 TCR senses isopentenyl pyrophosphate (IPP) presented by butyrophilin 3A1 (BTN3A1) whereas NKG2D senses stress signals (such as MICA/B, ULBP1-4 and MSH2) displayed on target cells. The γδ TCR is the predominant factor that can trigger cell activation without any contribution of other co-stimulators. Following activation, Vδ2 T cells kill tumor cells by releasing effector molecules, such as granzymes and perforin, or by ligating death receptors Fas with FasL, DR4/DR5 with TNF-related apoptosis-inducing ligand (TRAIL), TNF-α receptor with TNF-α pathways, or antibody-bound tumour cells with CD16. The activation threshold is finely regulated by inhibitory receptors, such as NKG2A/CD94. Moreover, adhesion

patterns, such as lymphocyte function-associated antigen 1 (LFA-1)/intercellular adhesion molecule-1 (ICAM-1), are also involved in regulating the antitumoral activity of Vδ2 T cells. The chemokine receptors, including CCR5, control the ability of Vδ2 T cell to migrate to the tumor site. The survival and proliferation of Vδ2 T cells are mostly modulated by different cytokines, such as IL-2 and IL-15 (Xiang & Tu, 2017).

1. 5. 3. 3 Activation of other cells by Vδ2 T cells

Vδ2 T cells can help in maturation of the dendritic cells and B cells (Dunne et al., 2010; Ismaili et al., 2002; Leslie et al., 2002; Petrasca & Doherty, 2014). Vδ2 T cell-matured DC were able to stimulate alloreactive T cells to proliferate and produce IFN-γ. Vδ2 T cell-matured B cells induced alloreactive T cell proliferation, but not IFN-γ production (Petrasca & Doherty, 2014). Vδ2 T cell-matured B cells are also capable of producing the antibody isotypes IgG, IgA, IgM and IgE (Petrasca & Doherty 2014). Activated Vδ2 T cells can induce neutrophils differentiation into APCs for $CD4^+$ and $CD8^+$ T cells via GM-CSF, IFN-γ and TNF-α production (Davey et al., 2014).

1. 5. 3 . 4 Antigen Presentation by Vδ2 T cells

Antigen presentation is the capability to process antigens in a way that they are recognisable by T cells. Vδ2 T cells can act as APCs because of their ability to take up foreign antigens by endocytosis and phagocytosis and present antigenic peptides on MHC molecules to conventional T cells (Meuter et al., 2010; Wu et al., 2009). Importantly, Vδ2 T cells can prime and activate naïve $CD4^+$ and $CD8^+$ T cells (Brandes et al., 2009). Vδ2 T cells can also cross present antigens, taken up by endocytic pathways, on class I MHC molecules to CD8 T cells (Brandes et al., 2009). They are capable of

presenting a wide variety of antigens including well defined proteins, debris from damaged cells and bacterial cells. Since Vδ2 T cells also have cytotoxic capacities they are uniquely capable of lysing cells, which can be detrimental to the health of the individual, and then presenting antigens derived from them to other T cells (Khan et al., 2014).

1. 5. 4 Vδ2 T Cells in Sepsis

Andreu-Ballister *et al.* (2013) reported a major decline in numbers of γδ T cells in sepsis patients as septic process worsens. This finding was also reported by Matsushima *et al.* (2004). Galley et al. (2015) showed that γδ T cells from sepsis patients did not proliferate as well as γδ T cells from control subjects in vitro (Galley et al., 2015). Thus, sepsis is associated with a numerical and functional impairment in γδ T cells. Liao *et al.* (2017) studied a cohort of 107 patients, of which almost 50% were critical with septic shock syndrome. They found that the frequencies of γδ T cell were significantly lower than in healthy individuals. There was also a notable decrease in CD69, NKG2D and PD-1 expression by these cells. An intriguing finding was that γδ T cells from sepsis patients displayed signs of activation but when further stimulated with activators and cytokines, their responsiveness and the change from baseline was lower. This implies that there is a significant shift, in the properties of this subset of T cells in patients with sepsis (Liao et al., 2017).

Less is known about the Vδ2 T cell subset of γδ T cells in sepsis patients. Toro et al. (2013) showed that there is a significant reduction in the frequencies of Vδ2 subset of γδ

T cells in the patients with severe sepsis compared to healthy individuals (Toro et al., 2013). Due to the paucity of knowledge on Vδ2 T cells in sepsis, we investigated the numbers, phenotypes and functions of Vδ2 T cells in sepsis in the present study.

1. 5. 5 Vδ2 T Cells and Immunotherapy

Vδ2 T cells possess unique features of innate and adaptive immunity and functional plasticity, making them ideal candidates to develop immunotherapies for cancer and infection. They can be rapidly activated using conserved antigens without the need for MHC presentation and antigen processing. Furthermore, they can be easily expanded using aminobisphosphonates such as zoledronate, and synthetic phosphoantigens such as bromohydrin pyrophosphate (BrHPP) (Nussbaumer & Koslowski, 2019; Tanaka et al., 2018). They are currently being tested for the treatment of cancer. There are two strategies used to exploit Vδ2 T cells for cancer immunotherapy. The first one is *in vivo* stimulation and expansion of Vδ2 T cells, either by aminobisphosphonates, or synthetic phosphoantigens. Aminobisphosphonates, target the mevalonate pathways, leading to IPP accumulation in cancer cells which in turn induce Vδ2 T cells activation. In contrast, phosphoantigens directly activate Vδ2 T cells. *In vivo* expansion of Vδ2 T cells leads to induction of effector functions such as IFN-γ production (Nussbaumer & Koslowski, 2019). The second strategy is *ex vivo* expansion of autologous or allogenic Vδ2 T cells using phosphoantigens or aminobisphosphonates, followed by adoptive transfer back into patients (Alnaggar et al., 2019; Nussbaumer & Koslowski, 2019; Wada et al., 2014). Clinical trials have shown that adaptive transfer of γδ T cells is well tolerated (table 1.2) (Kobayashi et al., 2011; Nussbaumer & Koslowski, 2019). Autologous Vδ2 T cells,

expanded with zoledronate, have been used in gastric cancer patients, leading to a significant decline in tumor cell numbers (Wada et al., 2014). Using a different strategy, expanded allogeneic Vδ2 T cells from PBMC from healthy donors have been used in cholangiocarcinoma patients who displayed chronic mediastinal lymph node metastasis following liver transplantation. By the end of therapy, not only was there no evidence of adverse effects of cell infusion, but also they experienced a full response with no detectable peritoneal lymph node metastasis (Alnaggar et al., 2019). A comprehensive list of clinical trials using Vδ2 T cells is shown in Table 1.2. These clinical studies indicate that Vδ2 T cells have a unique biology and that Vδ2 T cells immunotherapy appears to be safe and promising. Therefore, it is possible that immunotherapies using Vδ2 T cells will benefit patients with sepsis.

Table 1. 2 Overview of clinical trials of adoptive transferred autologous expanded of Vδ2 T cells ex vivo (Nussbaumer & Koslowski, 2019).

Year	Disease	Treatment	n	OR	CR	Reference
2007	RCC	Vδ2 T cells +zoledronate +IL-2	7	3/7	0/7	(Kobayashi et al., 2007)
2008	RCC	Vδ2 T cells BrHPP +IL-2	10	0/10	0/10	(Bennouna et al., 2008)
2009	MM	Vδ2 T cells + BrHPP +IL-2	6	0/6	0/6	(Abe et al., 2009)
2010	NSCLC	Vδ2 T cells + zoledronate+IL-2	10	0/10	0/10	(Nakajima et al., 2010)
2011	RCC	Vδ2 T cells +zoledronate +IL-2	11	1/11	1/11	(Kobayashi et al., 2011)
2011	Melanoma Colon cancer Breast cancer Cervical cancer Ovarian cancer Gastrointestinal cancer	Vδ2 T cells +zoledronate +IL-2	18	1/12	1/12	(Nicol et al., 2011b)
2011	NSCLC	Vδ2 T cells +zoledronate +IL-2	15	3/12	0/12	(Sakamoto et al., 2011)
2013	Colon cancer	Vδ2 T cells	6	0/12	0/6	(Izumi et al., 2013)
2014	NSCLC	Vδ2 T cells	15	0/6	0/12	(Kakimi et al., 2014)
2014	Gastric cancer	Vδ2 T cells +zoledronate	7	0/12		(Wada et al., 2014)

BrHPP, bromohydrin pyrophosphate; CR; complete response; IL, interleukin; MM, multiple myeloma; NSCLC, non-small cell lung cancer; OR, objective response; RCC, renal cell cancer.

1. 6 Immunometabolism

Immune cells of the body have various functions ranging from defence against pathogens and tumours to tolerance towards harmless antigens. This implies that the cells need resources to aid them in these functions, and the requirements can change according to the needs of the hour. Immunometabolism is the area of immunology that is concerned with the energy requirements of cells of the immune system and how it contributes to their functions (Keating et al., 2016; O'Neill et al., 2016).

Energy metabolism must function to sustain a balance between energy generation and consumption and provide cells with elementary building blocks for the synthesis of macromolecules. In addition, immune cell function and differentiation can be directly regulated by cellular metabolism. Glucose, glutamine, fatty acids and amino acids are nutrients that immune cells utilize to generate ATP for energy and to supply precursors for the synthesis of nucleotides, amino acids and lipids. For instance, mitochondrial OxPhos, a process which requires oxygen, is used by some lymphocytes in order to efficiently generate ATP. Effector lymphocytes, however, predominantly use aerobic glycolysis, a process in which glucose is converted to lactate in the presence of oxygen. This is the most common metabolic signature that is used by highly proliferative cells. Aerobic glycolysis can also directly affect the functions of effector lymphocytes. Many factors such as nutrient and oxygen availability and cytokine stimulation can upregulate immune cell metabolism and functions (Loftus & Finlay, 2016).

1. 6. 1 Metabolic profile and nutrients

Different categories of immune cells have distinct functions and use different metabolic pathways for energy generation. They also have distinct nutrient and energy requirements.

Naïve T and effector T cells have different metabolic rates and demands. Naïve T cells have low metabolic rates, for example, use small amounts of nutrients, such as glucose, glutamine and fatty acid as fuels to metabolise using OxPhos to generate energy (Buck et al., 2015). Following stimulation, T cells get activated and undergo many physical changes including proliferation and differentiation leading to an increase in their metabolic rates. This involves a switch to anaerobic processes like glycolysis that aid in providing energy and cellular mediators for growth, proliferation and the induction of cytokines. This in turns requires more nutrient to use as fuel for bioenergetic and biosynthetic pathways (Buck et al., 2015; Geiger et al., 2016; Ma et al., 2017; Ma et al., 2018; Sinclair et al., 2013).

Memory T cells, however, have lower biosynthetic requirements and revert to a latent state. As a result, they return to use oxidative metabolism to generate energy. An intracellular fuel is preserved by memory T cells in the form of glycogen and tricylglycerides which supply them with metabolic security and versatility fundamental to maintain lifespan and rapid response that are key to memory T cell functions (Buck et al., 2015; Ma et al., 2018). On the other hand, regulatory T cells utilise fatty acids as

fuel for OxPhos as a means of obtaining energy for sustenance (Zeng & Chi, 2015) and they can shift to use of glycolytic metabolism to provide the requirements for growth and proliferation (Gerriets et al., 2016; Zeng et al., 2013).

B cells produce antibodies that play a crucial role in humoral immunity. Upon activation, B cells increase glycolysis rates and thereby need to increase the uptake of glucose to fuel the production of energy for antibody production (Doughty et al., 2006; Dufort et al., 2014).

Glycolysis is the breakdown of glucose to generate energy in the cell. It is an anaerobic process, which means that in all the steps of this process, there is no oxygen required for the conversion of glucose to pyruvic acid. Many immune cells use glycolysis as the primary metabolic pathway for generating ATP. The end product of this pathway, pyruvate, is produced as a result of multiple steps converting the glucose into essential intermediates and resulting in production of reduced nicotinamide adenine dinucleotide (NADH) and ATP. The disadvantage of this process is the extremely low amount of ATP produced, as compared to aerobic processes. However, the advantage of this process is that it is fast and can occur in the presence or absence of oxygen (Lunt & Vander Heiden, 2011). Lymphocytes can transition to glycolysis when they get activated. Hence, this is an important process in the face of inflammatory situations, due to the speed of the process that aids in quickly synthesizing macromolecules for proliferation and survival (Pfeiffer et al., 2001). Under normal conditions, depending on the availability of oxygen, the pyruvate can be broken down into acetyl CoA and then

forwarded to the Tricarboxylic Acid (TCA) Cycle for further ATP generation. If oxygen levels are low, then pyruvate is converted into lactate. The intermediates of glycolysis can be diverted to the pentose phosphate pathway for producing many essential metabolites (Lunt & Vander Heiden, 2011). This process and cellular metabolism are regulated by mammalian target of rapamycin complex 1 (mTORC1) (Linke et al., 2017; Loftus & Finlay, 2016). mTORC1 can recognize nutrients and respond to extracellular and intracellular signals, such as hormones and growth factors. Upon recognition, mTORC1 modifies protein translation inside the cells (Fig. 1.7) (Weichhart et al., 2015).

Glucose is used by lymphocytes such as NK cells, M1 macrophages, mature DC, neutrophils, and effector T and B cells upon activation. It is the main source of fuel for glycolysis to meet their demands to differentiate, proliferate and produce cytokine which in turns increase the rates of aerobic glycolysis (Donnelly et al., 2014; Keating et al., 2016; Kelly & O'Neill, 2016; Michalek et al., 2011). In addition, in NK cells, glucose supports high rates of mitochondrial respiration being carried out by way of activating the citrate-malate shuttle, rather than the TCA cycle to drive OxPhos (Assmann et al., 2017; Loftus et al., 2018). Glycolysis requires glucose transport through transporter receptors in lymphocytes. T cells and NK cells upregulate the glucose receptor Glut1 upon activation (Keating et al., 2016; Rathmell et al., 2000).

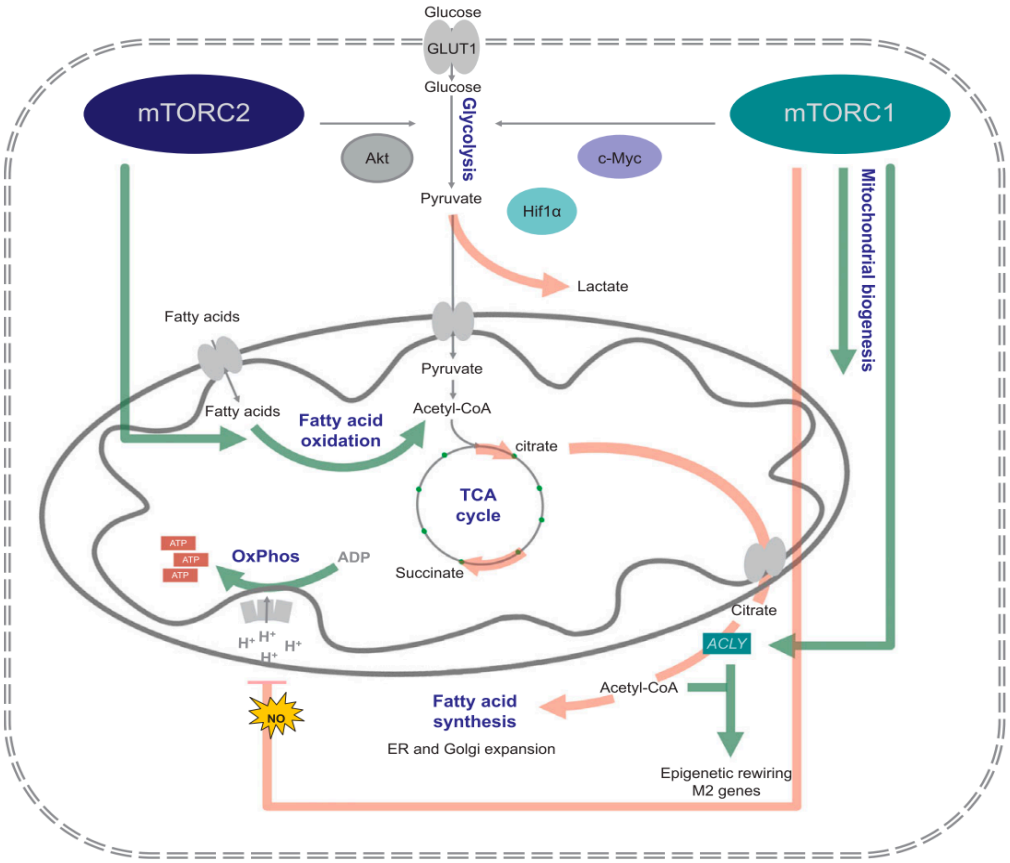

Figure 1. 7 Metabolic control by mTORC.

In innate immune cells mTOR-mediated metabolism can either support the response of inflammatory DCs and M1 polarized macrophages (red highlighted lines), as it happens for example during bacterial infection, or it can prime the immunometabolism of resident and M2 polarized macrophages (green highlighted lines). Both mTOR complex 1 and mTORC2 promote glucose uptake via the Glut1. mTORC1 fosters inflammatory polarization via activation of HIF1a and c-Myc mediated glycolytic gene expression. It also promotes the production of toxic NO, which poisons the electron transport chain. This feeds back into increased lactate production and FAS. In other cases, mTORC1 promotes FAS via SREBPs, although this has not been formally shown in macrophages. FAS- derived FA are utilized to rapidly expand the Golgi apparatus and ER. This enables the cells to produce huge amounts of inflammatory cytokines. In a homeostatic setting, mTORC1 also promotes glucose uptake, mitochondrial biogenesis and thus OXPHOS. IL-4 promotes mTORC1 mediated ACLY to achieve epigenetic rewiring using citrate-derived acetyl-CoA and FAO via mTORC2 (Linke et al., 2017).

Amino acids have a role to play in controlling various signalling pathways that are crucial for the function of immune cells (Walls et al., 2016). For instance, the transcription factor c-MYC regulates NK cells glycolytic flux (Finlay et al., 2012). c-MYC expression in NK cells is controlled by amino acid availability (Loftus et al., 2018). Specifically, the amino acid transporter (SLC7a5) for glutamine uptake is upregulated on NK cells upon cytokine activation of c-MYC. In addition, the expression of other nutrient receptors on activated NK cells such as CD71 (the transferrin receptor), CD98 (L-amino acid transport SLC7a5, LAT1), glutamine transporter (SLC1a5) and glucose transporter (GLut1) are increased and upregulated where they can influence immune signalling (Jensen et al., 2017; Keating et al., 2016; Keppel et al., 2015; Marçais et al., 2014; Salzberger et al., 2018).

The TCA cycle is a major metabolic pathways used by most immune cells, especially those that need a high amount of energy to circulate for long times and survive like memory $CD8^+$ T cells (O'Sullivan et al., 2014). Otherwise, known as Krebs cycle, this is the route for the acetyl CoA getting oxidised and converted into CO_2 and also generating energy in the form of $FADH_2$ and NADH. Subsequently by OxPhos, there is generation of ATP via the electron transport chain.

Fatty acid metabolism has been mainly observed in cells that are not directly inflammatory, rather those cells that are regulatory in nature or are involved in memory formation, like the memory T cells. However, the metabolism of fats does modulate the

immune responses of the cells. This kind of metabolism is mostly visible in memory T cells and Treg cells, but is downregulated in the effector T cells, once they become activated (Wang et al., 2011). In essence, fatty acid oxidation serves to promote tolerance in the T cells thereby allowing them to give a sustained response and no inflammation (van der Windt et al., 2013). This in turn helps in their survival (van der Windt et al., 2012). Fatty acid synthesis, on the other hand, induces proinflammatory immune responses. Stimulated inflammatory macrophages undergo an increase in the production of fatty acids synthesising enzymes. (Feingold et al., 2012; Posokhova et al., 2011).

Altogether, it seems that various immune cells have different nutrient requirements that can fuel their differentiation and functions. These are the key pathways (Table 1.3) and we will focus in more detail on the mitochondrial ones.

Table 1. 3 Overview of immune cells metabolic pathways.

Cell	Metabolic pathway
Naïve T cell	OxPhos
T_{eff} cell	Glycolysis, fatty acid synthesis, amino acid metabolism
T_{reg} cell	TCA cycle, FAO
Memory T cell	TCA cycle, FAO
M1 macrophage	Glycolysis, TCA cycle, PPP, fatty acid synthesis, amino acid metabolism
M2 macrophage	TCA cycle, FAO, amino acid metabolism
Activated B cell	Glycolysis, fatty acid synthesis
Activated DC	Glycolysis, fatty acid synthesis
Neutrophils	Glycolysis

1. 6. 2 Mitochondria

Mitochondria are essential organelles inside the cell and the hub of the metabolic processes occurring inside the cell. They undergo changes according to the environmental and metabolic needs of the cell. Mitochondria are crucial players in any demanding metabolic situation. Upon activation of lymphocytes, an increase in mitochondrial mass can be detected by flow cytometry. However, this technique only gives us an overview of mitochondrial mass, but does not provide information on mitochondrial structure and can function (Dhingra & Kirshenbaum, 2014; Gardiner, 2019). Mitochondria can convert their structure from the ovoid structure (fissed) to elongated tubular networks (fused) fused according to cellular requirements which in turn can modify not only energy production but also their effector functions (Pagliuso et al, 2018). Formation of memory T cells is also marked by a number of changes in the mitochondria, including a change in the structure from a more punctate form to a single big mass of mitochondria (fused). This change is suggested to aid in more OxPhos (Buck et al., 2016). However, more diffuse mitochondria structures are detected in effector T cells which are linked to less OxPhos (Buck et al., 2016).

Mitochondrial membrane potential (MMP) and Mitochondrial ROS (mROS) are key outputs that can be measured by flow cytometry (Zorova et al., 2018). mROS is generated whilst there's "leakage" of electrons from the mitochondrial electron transport chain (ETC) that could partially reduce oxygen to superoxide (Li et al., 2013). Superoxide dismutase converts superoxide to hydrogen peroxide. Both superoxide and hydrogen peroxide are considered mROS. The former can harm DNA, mitochondrial

integrity, and proteins unless scavenged quickly with the aid of antioxidants. In assessment, hydrogen peroxide is thought to function as an vital signaling molecule within the mitochondria (Schieber & Chandel, 2014; Veal et al., 2007). ATP synthase is an enzyme within the mitochondria that generates ATP. It requires a proton gradient to power its activity.

1. 7 Hypothesis and objectives

We hypothesized that altered cellular metabolism in NK cells and Vδ2 T cells, in patients with sepsis, may affect their functions and contribute to the clinical phenotypes observed. We also hypothesized that characterise the metabolic profile altered cellular metabolism in Vδ2 T cells upon treatment with HMB-PP, IL-12/15 or zoledronic acid, may differentially affect their functions. We proposed the following objectives:

1) To evaluate if the frequencies, phenotypes and functions of circulating NK cells and Vδ2 T cells and their expression of markers of metabolism are altered in patients with sepsis compared to healthy control subjects in order to determine if these cells contribute to disease.

2) To compare the capacities of HMB-PP, zoledronate and IL-12 + IL-15 to induce proliferation, expansion and effector function of Vδ2 T cells *in vitro*.

3) To identify the metabolic profile of resting and activated Vδ2 T cells using Seahorse technology.

Chapter 2- Materials and methods

2. 1 Materials

Equipment, laboratory consumables, reagents and kits used in this study are listed in tables 2.1- 2.3.

Table 2. 1 Equipment

Equipment	Model	Company
Balance	Adventure Pro	Ohaus, NJ, USA
Blood pack	MSE 6500L	Macopharma
Centrifuge	Eppendorf 5415 D	Eppendorf,Hamburg, Germany
Haemocytometer	Neubaur improved	Germany
CO_2 Incubator		
Flow cytometer	FACS Canto II	BD Bioscinces
Flow cytometer	Fortessa	Becton Dickinson, Oxford
FlowJo software		TreeStar
Laminar air flow class II safety cabinet	Clean Air MSC BSS6-2	Thermo Fisher scientific, MA, USA
Laminar air flow class II safety cabinet	MSC 1.2	Thermo Electron LED Gmbh, Germany
Magnet	LS Magnet	Miltenyi Biotech, Bergisch Gladbach, Germany
Magnet stand	MACS Multi stand	Miltenyi Biotech, Bergisch Gladbach, Germany
Magnet	The big Easy EasySep	Stemcell technologies, france
Microscope	Inverted; NAO 30	Olympus Corporation, Japan
Multichannel pipette	Finn pipette F2	Thermo Fisher Scientific, MA, USA
PH meter	Benchtop PH20-01	Hanna instruments, RI, USA
Pipettors p20, p100, p200, p1000	Eppendorf Research plus	Thermo Fisher Scientific, MA, USA
Pipette filler	Motorised, Fisher brand	Thermo Fisher Scientific, MA, USA

Tissue culture dishes (6, 12, 24 and 48 well)		Thermo Fisher Scientific, MA, USA
UV microscope	E200 Eclipse	Nikon, USA
Vortex	Vortex Genie	Scientific industries,NY, USA
Water Bath	YCW-010E	Germany Industries corp; Taiwan
XFp 8-well microplates		Agilent Technologies

Table 2. 2 General Reagents and Kits

Reagents	Supplier
1M HEPES	Gibco Invitrogen
Anti-CD28 (mAb)	BD Bioscinces
Anti-CD3 monoclonal antibody (mAb)	BD Bioscinces
Anti-PE Microbeads	Miltenyi Biotec
Anti-TCR γδ Microbead Kit human	Miltenyi Biotec
Celltak®	BD Pharmingen
Dimethyl sulfoxide (DMSO)	Sigma-Adrich
Dulbecco's phosphate buffered saline (PBS)	Gibco
(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP)	Sigma Aldrich
Ethylenediaminetetraaceteic acid (EDTA)	Sigma-Adrich
Fixation Buffer	BD Bioscinces
Foetal Bovine Serum (FCS)	Labtech International
Bovine Serum Albumin	Sigma-Adrich
Golgi Plug	BD Bioscinces
IL-12	Miltenyi Biotec
IL-15	Miltenyi Biotec
IL-2	BD Bioscinces
Ionomycin	Sigma-Aldrich
L-Kynurenine	Sigma
Lymphoprep ™	StemCells Technology
MitoTEMPO	Sigma
Penicillin -streptomycin	Gibco Invitrogen
Phorbol-12-myristate-13 acetate (PMA)	Sigma-Adrich
Prem/Wash Buffer	BD Bioscinces

Rotenone	Fluorochem letd
Seahorse Media	Agilent Technologies
Sodium -azide	Sigma-Aldrich
Zoledronic acid Mylana 4 mg/5ml	Pharmacy
T cell activation/expansion kit	Miltenyi Biotec

Table 2. 3 Fluorochrome-conjugated monoclonal antibodies for flow cytometry

Antibody	Function	Staining	Fluorochrome	Clone	supplier
CD3	T cells markers	Extracellular	PerCP Pacific Blue	HIT3a	BD Bioscinces BioLegend
CD56	NK markers	Extracellular	PE-CY7	HCD56	BD Bioscinces BioLegend
TCR-Vδ2	Phenotype markers	Extracellular	PE FITC BV510	B6	BD Bioscinces BioLegend
CD71	Transferrin receptor	Extracellular	APC	Cy1g4	BioLegend
CD98	L-amino acid Transporters	Extracellular	PE	-	BD Bioscinces
CD69	Activation marker	Extracellular	PerCP/Cyanine5.5 FITC	FN50 L78	BD Bioscinces
Live/Dead Stain	Live/ Dead marker	Extracellular	Near-IR	-	Thermo Fisher
Granzyme B (GnzB)	Mediation of cytotoxicity	Intracellular	BV510	-	BD Bioscinces
IFN-γ	Cytokine	Intracellular	FITC Percp/cy5.5	B27 B27	BioLegend

			PE-Cy7	4S.B3	
IL-17	Cytokine	Intracellular	PerCP/Cy5.5	-	BioLegend
Mitotracker	Mitochondrial Mass	Intracellular	Green FM	-	Cell Signaling Technology
ATP5B	ATP synthase, H+ Transporting	Intracellular	Alex Flour 647	-	Abcam
P-S6bRibosomal Protein (S235/236)	ribosomal protein S6 mTORC1	Intracellular	PE	-	Cell Signaling

2. 2 Methods

2. 2. 1 Subjects

The Ethics Committee of St. James's and Tallaght Hospitals provided ethical approval for analysis of blood samples from patients with sepsis. Thirty two patients were recruited. All were consenting adults who were admitted to ICU at St. James's Hospital. The mean age of the patients was 73.5 years and the age range was 27-87. The Ethics Committee of Trinity College Dublin provided ethical approval for analysis of age-matched healthy volunteers blood samples. All healthy donors (N = 29) for this study were consenting adults. Due to the high age of the patients, we were unable to obtain age-matched control subjects (mean age = 45.5; age range =27-68). Demographics and clinical characteristics of the subjects are shown in table 2. 4.

2. 2. 2 Isolation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-anticoagulated venous blood from sepsis patients and healthy subjects by density gradient centrifugation over Lymphoprep. 15 ml of Lymphoprep was transferred to a 50 ml Falcon tube and 35 mL of diluted blood in a 1:2 ratio with PBS containing 1% FCS was added to the tube and centrifuged for 30 minutes at 400 g with the brake off (acceleration and deceleration set to zero). The buffy coat layer was then carefully harvested and washed twice with PBS containing 1% FCS and centrifuged for 10 minutes at 450 g to remove any remaining Lymphoprep. The final PBMC pellets were resuspended in complete RPMI 1640 Glutamax medium (cRPMI: RPMI 1640 medium

containing 10% heat-inactivated FCS, 25 mM HEPES, 2 mM glutamine, 50 mg/ml streptomycin and 50 U/ml penicillin).

2. 2. 3 Enumeration of PBMC

Cells were counted and their viability was determined by staining them with ethidium bromide and acridine orange (EB/AO). Ethidium bromide is an intercalating agent that binds to DNA and fluoresces orange under UV light. Acridine orange is a fluorescent cationic dye that can interact with DNA and RNA, and when bound to DNA and exposed to UV light, it appears green. In this way, viable cells fluoresce green and dead cells appear orange. A 100 X solution containing 15 mg AO, 50 mg EB, 1 mL 95% ethanol and 49 mL dH_2O was made and diluted 1 in 100 in PBS. 10 µL of cells were added to 190 µL 1 X EB/AO solution. Then, 10 μL of EB/AO-stained cells was placed into the well of a haemocytometer slide. Live cells were counted in two large squares (1 mm x 1 mm). Each of these squares accommodates 0.1 mm^3 of liquid so the number of cells in 1 ml was calculated, taking into account the 1/20 dilution.

2. 2. 4 Cryopreservation and reconstitution of cells

After enumeration, the cells were pelleted by centrifugation for 7 minutes at 450 g with brake and acceleration at maximum. Fresh freezing mixture, consisting of 90 % FCS and 10 % dimethyl sulphoxide (DMSO) was made and the cell pellet was resuspended in 2 ml of freezing mixture. This cell mixture was rapidly transferred to cryovials and placed in a Mr. Frosty container in an -80^{0}C freezer. For long-term storage, cells were then transferred to liquid nitrogen after 24 hours. Cells were reconstituted for cell stimulation experiments when required by rapid thawing under running water. The vial

was removed from the hot water before it had completely thawed and cells were transferred to a universal tube. Complete RPMI was added dropwise, while shaking the tube to mix continuously. This was then topped up with RPMI to 20 mL. Tubes were centrifuged for 7 minutes at 450 g with brake and acceleration at maximum. The supernatants were discarded and cells resuspended in 1 ml of complete RPMI solution.

Table 2. 4 Demographic and Clinical characteristics of study subjects

Clinical Data	Immunophenotype Study	
	Control	***Septic***
Number	29	32
Age Median	45.5	73.5
APACHE score	N/A	19[16-24.5] p<0.0001
SAPS score	N/A	48 [37.75-54.5]
SOFA score on admission	N/A	7 [5.75-10] p<0.0001
SOFA score on day of first sample	N/A	7 [5-8.25]
Time to 1st sample from admission (days)	N/A	1.5 [0.75-2]
ICU duration (days)	N/A	14.5 [8.75-33.25]
Mortality in ICU	N/A	11 (34.4%)
Mortality in Hospital	N/A	13 (40.6%)
Inotropic Support	N/A	30 (93.75%)
Days on inotropes	N/A	7 [3-13]
Invasive ventilation	N/A	28 (87.5)
Days on invasive ventilation	N/A	14.5 [5-29.25]
P/F ratio mmHg	N/A	170 [135.75-240.5]
Muscle Relaxant infusion	N/A	11 (34.4)
Acute Kidney Injury	0	26

			(81.25)
Renal Replacement Therapy		0	16 (50)
Concomitant cardiac failure		0	6 (18.75)
Stress dose steroids		0	6 (18.75)
Source of Sepsis	Respiratory	N/A	16 (50)
	Abdominal	N/A	11 (34.4)
	Skin	N/A	4 (12.5)
	Urine	N/A	0 (0)
	Osteomyelitis	N/A	0 (0)
	Mediastinitis	N/A	1 (3.1)
Type of organism	Gram- ve organism	N/A	9 (28)
	Gram+ ve organism	N/A	9 (28)
	Fungal	N/A	1 (3)
	Empiric treatment	N/A	13 (41)
Secondary Infections		N/A	17 (53.1)
Lactate on admission		N/A	2.86 [2.27-4.09]

Data and parameters are presented as medians and interquartile ranges [Q1-Q3]. n, number of patients; APACHE score; disease severity based on current physiologic measurements, age & previous health conditions. The score can help in the assessment of patients to determine the level & degree of diagnostic & therapeutic intervention, Acute Physiologic Assessment and Chronic Health Evaluation; SOFA score, Sequential Organ Failure Assessment score; N/A, Not Applicable; P/F ratio of arterial oxygen partial pressure to fractional inspired oxygen.

2. 3 Flow cytometry

2. 3. 1 Principles of flow cytometry

Flow cytometry is a powerful tool for the analysis of multiple parameters of individual cells within heterogeneous populations. The cell sample is injected into a flowing stream containing sheath fluid and is passed through a laser beam at the rate of thousands of cells per second. The cells scatter and refract light which is captured as each cell passes through. The magnitude of light scattered in the forward direction at low angles, is proportional to the cell size. Light scattering at larger angles, such as to the side, is influenced by granularity and structural complexity inside the cell. Light is quantified by three detectors which convert light intensity into voltage. The use of fluorochrome-conjugated monoclonal antibodies is one of the most common ways to study cellular characteristics using flow cytometry. The antibodies will bind to specific molecules on the cell. When a laser of a particular wavelength excites the fluorophore, a fluorescent signal is emitted and detected. A series of filters and mirrors is in place so that the particular wavelengths are delivered to the appropriate detectors. This information is then translated into a voltage pulse proportional to the amount of fluorescence emitted and can be presented graphically as distinct populations (see Fig. 2.1).

The FACS Canto-ll (Becton Dickinson, UK) flow cytometer is equipped with three lasers which emit light at different wavelengths. The violet laser emits at 405 nm, the blue laser emits light at 488 nm and the red laser emits at 640 nm. Collectively, the 3 lasers are capable of detecting 10 distinct parameters, including forward scatter and side scatter), and 8 fluorochromes. A table of fluorochromes used is depicted below (Table

2.5). Tandem dyes, which are created by combining a two fluorochromes to alter the emission spectra, increase the numbers of stains that can be used. FL channels are unique to each flow cytometer and they indicate which fluorochromes can be used together. Only one fluorochrome can be used from each FL channel. FL3 and FL5 can be difficult to resolve from each other due to the emission spectra.

Table 2. 5 Fluorochromes used in this study and their specifications

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

2. 3. 2 Cell surface labelling of cells for flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAb) were first titrated to determine optimum amounts for use. A cocktail containing the optimised amounts of the relevant antibodies in flow cytometry buffer (PBS supplemented with 2% FCS) was prepared in advance of staining the cells. 0.1-0.25x10^6 PBMC were transferred to labelled flow cytometry tubes. Cells were stained for 20 minutes in dark at 4°C with saturating concentrations of titered antibodies (Table 2.4) in flow cytometry buffer in a flow cytometry tube. A live/dead cell stain was included in each panel. The cells were washed with flow cytometry buffer. Cells were acquired using a FACS Canto or LSR Fortessa flow cytometer and analysed by FlowJo software (Treestar). NK cells were defined as $CD3^-$ $CD56^+$ lymphocytes and Vδ2 T cells were defined as $CD3^+$ $Vδ2^+$ lymphocytes as shown in the gating strategy (Fig. 2.1).

2. 3. 3 Activation of PBMC

PBMC were cultured at 5×10^6 cells/ml in round-bottom 96 well plates or flat-bottom 96 well plates in complete RPMI 1640 Glutamax medium. Cells were cultured in medium alone or stimulated with various combinations of IL-12 (30 ng/ml), IL-15 (100 ng/ml), IL-2 (50 U/ml), (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) (10 nM), zoledronic acid (5 µM) or anti-CD3 mAb (1 mg/ml) with anti-CD28 mAb (1 mg/ml). Cells were then incubated at 37°C, 5% CO_2 for 18 hours. In the last 4 hours of the stimulation, GolgiPlug was added to wells in order to prevent cytokine secretion to enable us to do intracellular staining. Following stimulation cells were washed in flow cytometry buffer

(2% FCS in PBS) and centrifuged at 1400 rpm for 3 minutes, before staining them with mAbs for analysis by flow cytometry.

2. 3. 4 Intracellular cytokine staining for flow cytometry

Following the completion of cell surface staining, the cells were resuspended in 100 µl/tube of fixation buffer (Cytofix/Cytoperm), covered with aluminum foil and incubated at 4°C. After 20 minutes, the fixed cells were permeabilised by washing them with Perm/Wash**,** which contains saponin, diluted (1:10 with sterile water) and centrifuged at 400 g. Cells were resuspended in Perm/Wash buffer and stained with antibodies specific for IFN-γ, granzyme B, IL-17, pS6 and/or ATP5B in the dark at 4°C for 30 minutes. Cells were washed twice in Perm/Wash buffer and centrifuged at 1400 rpm for 3 minutes. The cells were then ready for analysis by flow cytometry.

2. 3. 5 Mitotracker staining

Mitotracker Green FM (ThermoFisher) is a dye that is used to measure mitochondrial mass. PBMC were stained with MitoTracker Green FM (100 nM) in cRPMI medium for 30 minutes at 37°, 5% CO_2. Cells were then washed in FACS buffer and stained for extracellular surface markers. Mitochondrial mass of individual cell types was then analysed by flow cytometry.

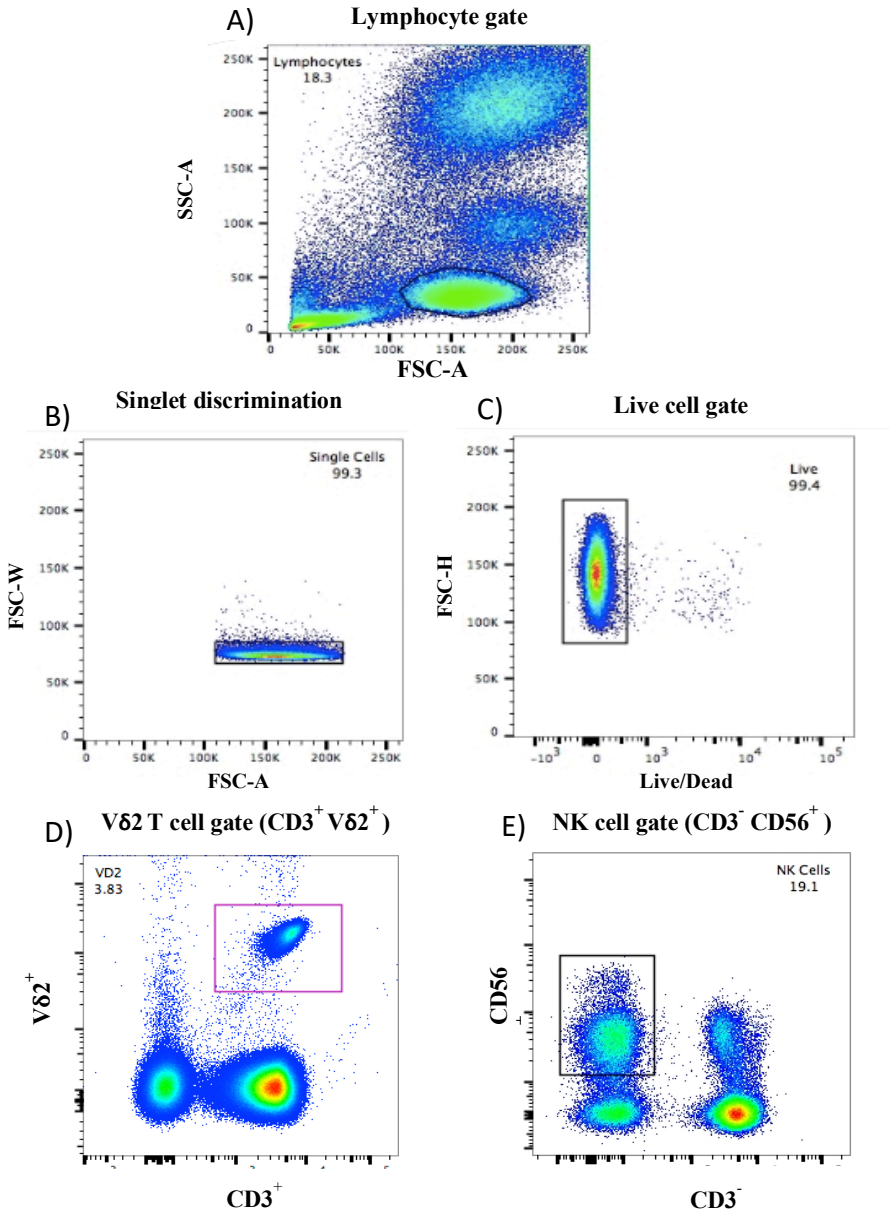

Figure 2. 1 Gating strategy for flow cytometric analysis of Vδ2 T cells and NK cells.

PBMC were stained with mAbs specific for CD3, CD56 and Vδ2 TCR and analysed by flow cytometry. (A) Lymphocytes were initially selected based on their forward scatter area vs side scatter area profile. (B) Single cells were discriminated from doublets by gating based on forward scatter width vs forward scatter area. (C) Live/dead staining was used to identify living cells and exclude dead cells. (D) Vδ2 T cells were defined as being $CD3^+$ $Vδ2^+$. (E) NK cells were defined as being $CD3^-$ $CD56^+$.

2. 3. 6 MitoSOX

MitoSOX Red dye was used to determine the levels of mitochondrial superoxide as a measurement of mitochondrial reactive oxygen species (mROS). PBMC were stained with MitoSOX (1.5 μM) in RPMI for 15 minutes at 37°C. As a positive control, PBMC were simultaneously treated with rotenone (20 μM), which increases ROS production. As a negative control, cells were treated with MitoTEMPO (25 μM), which decrease ROS production. MitoTEMPO, is a superoxide scavenger mimicking superoxide dismutase from mitochondria (Ni et al., 2016). It accumulates in the mitochondria eliminating mitochondrial ROS. Cells were then washed in FACS buffer and stained for extracellular surface markers.

2. 3. 7 CellROX

CellROX Deep Red enables the detection of ROS in live cells by flow cytometry. PBMC were stained with CellROX (1 μM) in RPMI for 30 minutes at 37°C. Tert-Butyl hydroperoxide (TBHP, an inducer of ROS) (1 mM) was used as a positive control (increases ROS production) and n-acetylcysteine (NAC, an antioxidant) (4 mM) was used as a negative control (decrease ROS). Cells were treated with TBHP or NAC before staining with CellROX. Cells were then washed in FACS buffer and stained for extracellular surface markers. The production of ROS by individual cell types was then analysed by flow cytometry.

2. 3. 8 Analysis of amino acid uptake by cells

PBMC was used to measure Kynurenine uptake, in particular system L amino acid transport through SLC7A5 to identify cell activation as described in (Sinclair et al., 2018). Following the completion of cell surface staining, the cells were resuspended in 200 µl warmed HBSS medium and incubated in a water bath at 37 °C. Then, 100 µl of HBSS or 100 µl of leucine (5 mM, specificity controls such as System L blocked samples) was added to appropriate samples. Next, 100 µl of kynurenine (400uM) was added to samples. 100 µl of HBSS was added to make up final volume 400 µl. After 4 minutes, the uptake was stopped by adding 125 µl PFA (final concentration 2 %)for 20 minutes at room temperature in the dark. After 20 minutes of fixation, the cells were washed with FACS buffer in and centrifuged at 1400 rpm for 3 minutes. Then, cells were acquired by flow cytometry.

2. 3. 9 Magnetic bead enrichment of Vδ2 T cells

Vδ2 T cells were enriched from PBMC by positive selection magnetic bead cell sorting (Miltenyi Biotec). Magnetic-activated cell sorting (MACS) is a method for separating cell populations depending on their surface phenotypes, and uses superparamagnetic nanoparticles (50 nm diameter) and columns. The procedure allows cells to be separated by incubating them with magnetic nanoparticles coated with antibodies directed against desired surface antigens, thus allowing the desired cells expressing the antigen to attach to the magnetic nanoparticles. The cell solution is then transferred to a column placed in a strong magnetic field. Thus, the cells attached to the nanoparticles stay in the column, while those that do not the express antigen flow through.

Vδ2 T cells were also enriched using the anti-human TCR γδ microbead kit. Up to 10^7 PBMC were re-suspended in 40 µl cold MACS Buffer (sterile PBS containing 0.5 % BSA and 2 mM EDTA) and 10 µl of anti - γδ TCR hapten-antibody was added. Amounts were scaled up when larger numbers of PBMC were used. The mixture was incubated for 10 minutes shaking at 4°C. Then 30 µl of MACS buffer and 20 µl of anti-hapten FITC-conjugated magnetic bead particles were added and the mixture incubated for 15 minutes shaking at 4°C. When PBMC isolated from buffy coat packs, which typically yield >10^8 cells, were used, the amounts were scaled up 5-fold, in order to enrich for γδ T cells but not to purify them completely, so that it still contains $CD14^+$ cells (monocytes) which have been shown to be required to present HMB-PP to the Vδ2 T cells (Miyagawa *et al*, 2001). The magnetically-labelled cells were washed in MACS buffer (3 ml per 1 x 10^7 cells) and centrifuged at 300 g for 10 min and the cell pellets were re-suspended in buffer (5 ml per 1 x 10^7 cells).

Vδ2 T cells were also enriched using an anti-PE microbead kit. Up to 10^7 PBMC were re-suspended and stained with the primary PE-conjugated Vδ2 TCR antibody for 20 min. Cells were then re-suspended in in 100 µl cold MACS Buffer (sterile PBS containing 0.5 % BSA and 2 mM EDTA) and 10 µl of PE-conjugate was added. Amounts were scaled up when larger numbers of PBMC were used. The mixture was incubated for 10 minutes shaking at 4°C. Then 1-2 mL of MACS buffer were added and the magnetically-labeled cells were washed in MACS buffer (3 ml per 1 x 10^7 cells) and centrifuged at 300 g for 10 min and the cell pellets were re-suspended in buffer (80 µl per 1 x 10^7 cells) and 20 µl of anti-PE microbead particles were added and the mixture incubated for 15 minutes

shaking at 4°C. The magnetically-labelled cells were washed in MACS buffer (3 ml per 1 x 10^7 cells) and centrifuged at 300 g for 10 min and the cell pellets were re-suspended in buffer (5 ml per 1 x 10^7 cells).

Magnetic bead-labelled cells, prepared by either method described above, were next separated using column placed in a magnet. Large MS columns were used for positive selection of up to 10^8 magnetically labeled cells from up to 2×10^9 total cells. The columns were attached to large MACS magnets. The columns were primed by passing 3 ml of cold buffer through the column. A MACS pre-separation filter was used to prevent cell clumps from clogging the column. After the cell suspension was passed through the column, the pre-separation filter was removed and the column was washed three times with 3 ml of MACS buffer. yδ T cell negative populations were eluted from the column, while the column containing yδ T cells was removed from the magnet and flushed with 5 ml of buffer and collected in a separate tube and re-suspended in cRPMI. Cell surface phenotyping and purity were determined by staining with mAb against CD3 and Vδ2 and examining by flow cytometry.

2. 3. 10 Cell sorting

Individual cell types were highly purified from PBMC by flow cytometric cell sorting on a MoFlo XDP cell sorter (Beckman Coulter). In this procedure, the cell sample is labelled with mAbs of interest and cells positive for these antibodies are acquired on the cell sorter. Cells are passed through a column of pressurised sheath fluid and as they are ejected from the nozzle on the cell sorter, they pass through the analysis point

consisting of laser beams which provide scattered light and fluorescence signals, which are then compared to pre-set sort criteria for the cells of interest. There is time delay between the analysis point and where the droplets break off at the end of the stream and it is referred to as the drop delay. Once the drop delay is calculated, an electric charge is applied to the stream at the precise moment the first drop forms, allowing individual drops to be independently charged. Once the charged droplets reach the end of the stream, they are passed through high-voltage charged deflection plates which cause the charged droplets to be deflected towards the oppositely charged plate and into collection tubes, while uncharged droplets are aspirated to waste.

PBMC were enriched by positive magnetic bead separation of γδ TCR^+ cells (Miltenyi Biotec) using the same methods as described previously. The γδ-enriched cells were stained with mAb specific for CD3 (10 µl per 1 x 10^6 cells) and Vδ2 (20 µl per 1 x 10^6 cells) in PBS for 15 min. The cells were washed in PBS and the pellet re-suspended in 0.5 ml PBS per 1 x 10^7 cells and sorted using a MoFlo XDP cell sorter (Beckman Coulter) which allowed retrieval of pure (> 97%) population of $CD3^+$ and $V\delta2^+$ cells (Fig. 2.2).

2. 3. 11 Generation of Vδ2 T cell lines

The magnetically enriched or sorted γδ T cells were plated in 24-well tissue culture plates at a cell density of 1 x 10^6 cells per ml and expanded by stimulating with (E)4-hydroxy-3-methyl-but-2enylpyrophosphate (HMB-PP, 10 nM) or zoledronate (5 µM) and culturing them with IL-2 supplemented (50 lU/ml) cRPMI medium. The medium was changed every 3-4 days by replacing with fresh IL-2-supplemented cRPMI to provide a constant supply of IL-2 to the cells which is required for T cell survival and expansion

(Smith, 1988). The cells were harvested after 14 of culture. Alternatively, Vδ2 T cells were expanded directly from PBMC using HMB-PP (10 nM) or zoledronate (5 µM) for 14 days.

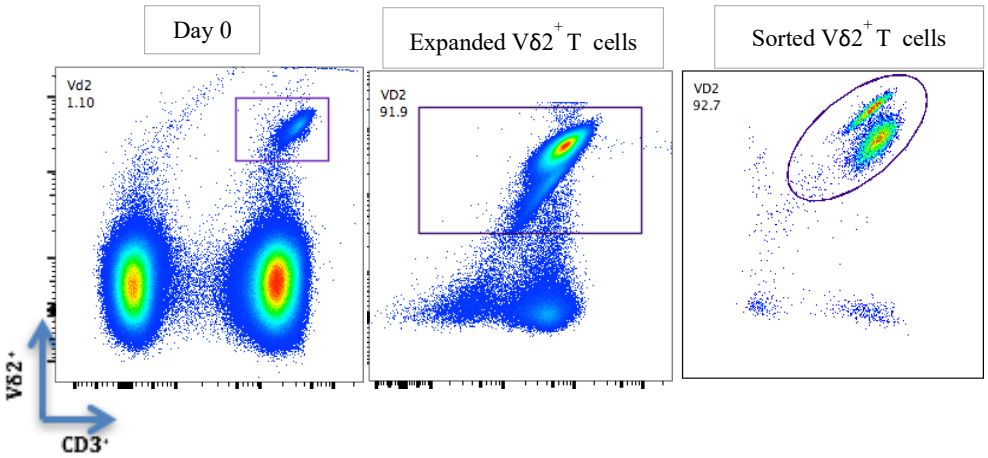

Figure 2. 2 The unsorted Vδ2$^+$ T cells vs expanded and sorted Vδ2$^+$ T cells

2. 4 Seahorse Metabolic Analysis

A Seahorse XFp Analyzer (Seahorse Bioscience) was used to measure the two major energy producing pathways of the cell – mitochondrial respiration and glycolysis - in a microplate, in real-time. It determines the real-time rates of oxidative phosphorylation and glycolysis in live cells. Oxidative phosphorylation was measured using the oxygen consumption rate (OCR) of the cells, while glycolysis was measured using the extracellular acidification rate (ECAR) caused by the live cells cultured under various conditions.

Each Seahorse cartridge was hydrated in a non-CO_2 37°C incubator with calibration buffer (200 ul). This was carried out for a minimum of 8 hours prior to use.

In order to stick the Vδ2 T cells to the bottom of the Seahorse plate to form an even monolayer, cell plates were coated with Cell-Tak (6 µg/ml). The Cell-Tak was diluted in sodium bicarbonate (0.1 M) with 0.15% (v/v) NaOH (1 M) and added to the bottom of each well (25 µl). It was left at room temperature for a minimum of 20 mins, removed from the plate and each well was washed twice with sterile ddH_2O (200µl).

Cells were washed twice in Glutamax Seahorse medium (containing 1 M glucose, adjusted to pH 7.4). Vδ2 T cells were added to each well ($0.35x10^6$ cells, 180 µl), while Seahorse medium (180 µl) was used in the blank wells. The cell plate was centrifuged at 200 g for 3 mins on break 1, and then placed in a non-CO_2 incubator for 30 mins prior to metabolic analysis.

The inhibitors (Table 2.6 and below) were pipetted into the cartridge. The cartridge was placed in the machine to allow for calibration, and then the cell plate was put in the machine for metabolic analysis.

For the determination of oxidative phosphorylation rates (that is cell respiration, measured as OCR), we used oligomycin as the ATP synthase inhibitor, Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP, is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential) as the electron transport chain accelerator, antimycin A as the electron transport chain complex III inhibitor and rotenone as the electron transport chain complex I inhibitor. Basal rates of OxPhos can be determined by the amount of oxygen being consumed over time.

While oligomycin inhibits ATP synthase, FCCP uncouples it which will dissipate the gradient of protons across the inner mitochondrial membrane and thus maximising OxPhos. Antimycin A and rotenone inhibit the electron transport chain thus any residual oxygen consumption can be calculated. This allows us to calculate the actual rates of OxPhos (Fig 2. 3).

For the determination of glycolytic rates (proton secretion related to glycolysis, measured as ECAR), glucose was used as the glycolysis substrate, oligomycin was used as the ATP synthase inhibitor and 2-deoxy-glucose (2-DG), a glucose analog, as the glycolysis inhibitor. Basal rates of glycolysis can be determined by the change in acidification in the media over time by release of protons during glycolysis. Oligomycin inhibits ATP synthase, thus blocking OxPhos and increasing glycolysis to maintain ATP production, allowing us to determine the maximum amount of glycolysis any given cell can undergo i.e. its glycolytic capacity. Lastly, 2DG, a direct glycolytic inhibitor blocks all glycolysis and thus any change in acidification of the media can be determined to be from a process other than glycolysis. This allows us to calculate the actual rates of glycolysis (Fig 2. 4).

Table 2. 6 Inhibitors used to analyse the metabolism of Vδ2 T cells.

	Compound	Injecting volume	Final concentration
A	Oligomycin	20 µl	2 µM
B	FCCP	22 µl	0.5 µM
C	Rotenone/ Antimycin A	20 µl	100 nM / 4µM
D	2-deoxyglucose	20 µl	30 mM

Oligomycin: inhibits ATP synthase, FCCP: ionophore that disrupts the proton gradient across the inner mitochondrial membrane, Rotenone/ Antimycin A: inhibits complex I and complex III respectively, 2-deoxyglucose: inhibits glycolysis.

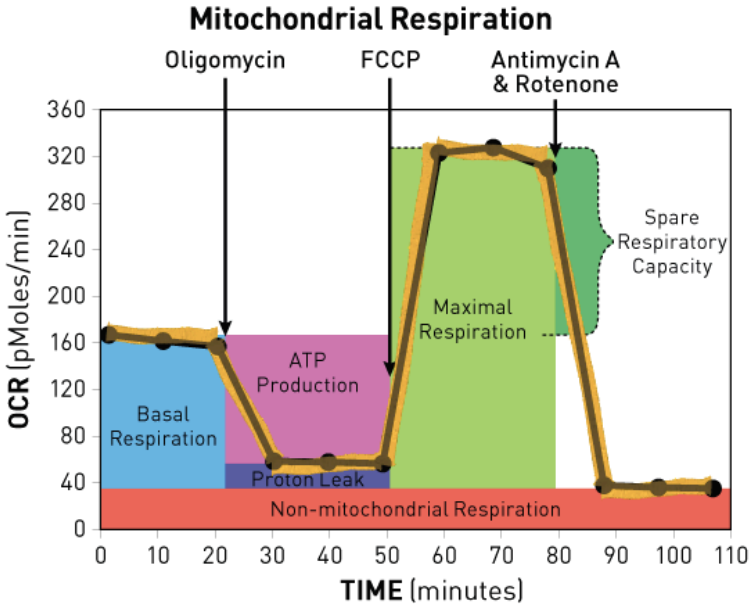

Figure 2. 3 The Seahorse XF Cell Mito Stress Test Profile

Oligomycin, FCCP, and rotenone/antimycin A are serially injected to measure ATP-linked respiration, maximal respiration, and non-mitochondrial respiration, respectively. Proton leak and spare respiratory capacity (SRC) are then calculated using basal respiration and these parameters. Basal rates of OxPhos can be determine by the amount of oxygen being consumed over time. While Oligomycin inhibits ATP synthase, FCCP uncouples it which will dissipate the gradient of protons across the inner mitochondrial membrane and thus maximising OxPhos. Antimycin A and Rotenone inhibit the Electron transport chain thus any residual oxygen consumption can be discarded. This allows us to calculate the actual rates of OxPhos. Figure adapted from Seahorse Bioscience.

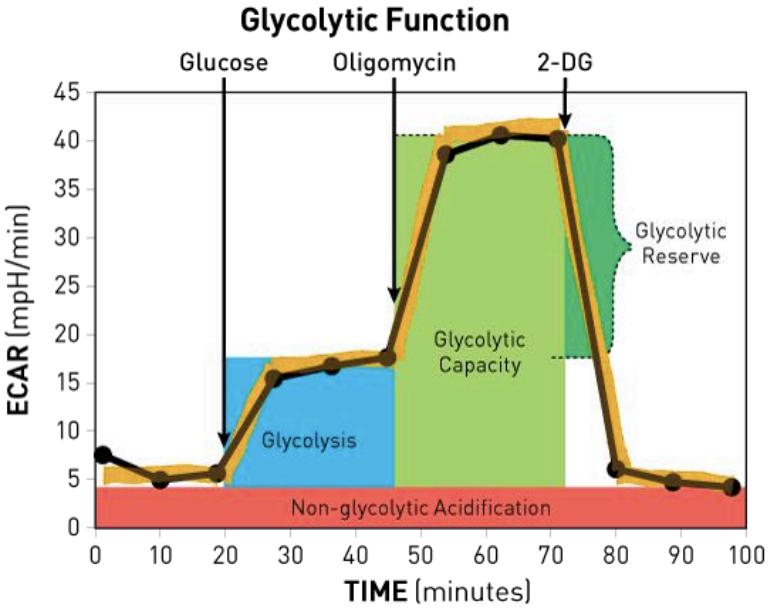

Figure 2. 4 The Glycolysis Stress Test Profile

Oligomycin is injected to block mitochondrial ATP production and measure maximal glycolysis. 2-DG is then injected to measure the full glycolytic capacity of the cells. Glycolytic reserve is then calculated using basal respiration and these parameters. Basal rates of glycolysis can be determined by the change in acidification in the media over time by release of protons during glycolysis. Oligomycin inhibits ATP synthase thus blocking OxPhos and increasing Glycolysis to maintain ATP production, this allows us to determine the maximum amount of glycolysis any given cell can undergo i.e. its glycolytic capacity. Finally, 2DG, a direct glycolytic inhibitor blocks all glycolysis and thus any change in acidification of the media can be determined to be from a process other than glycolysis. This allows us to calculate the actual rates of glycolysis. Figure adapted from Seahorse Bioscience.

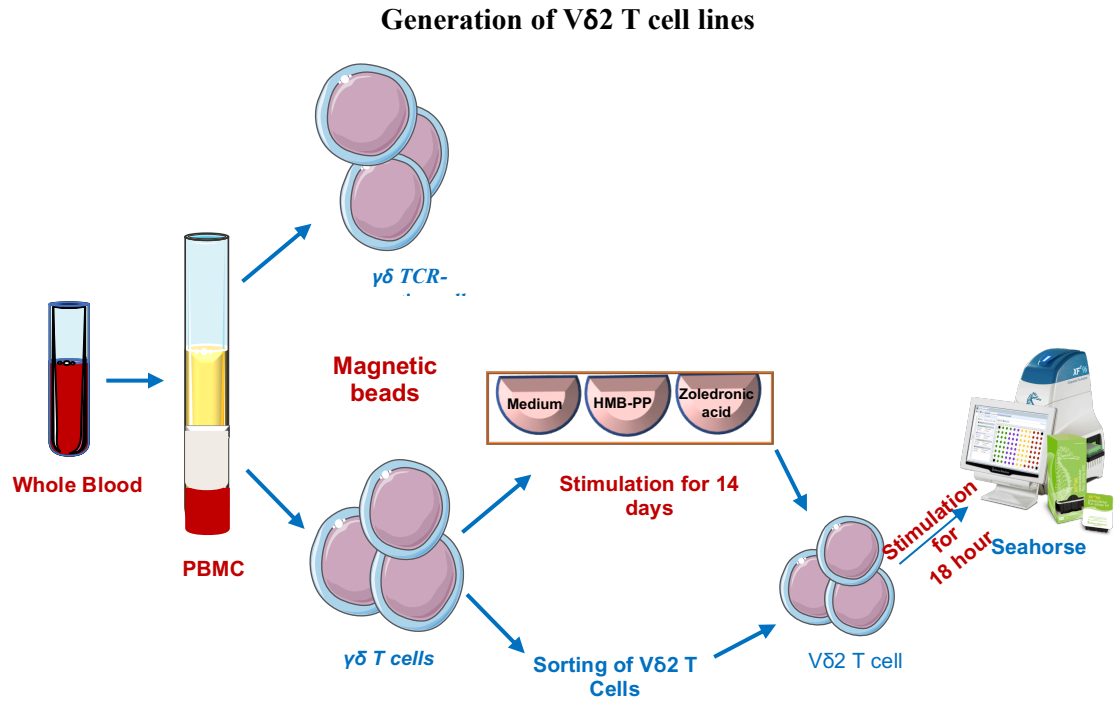

Figure 2. 5 Metabolic Analysis of Vδ2 T cells.

PBMC were isolated from blood packs. γδ T cells or Vδ2 T cells were isolated and purified using magnetic bead separation. γδ T cells were either stimulated for 14 days with HMB-PP (10 nM) or zoledronic acid (5 uM) or sorted for Vδ2 T cells. Vδ2 T cells were stimulated for 18 hours. Seahorse metabolic analysis was carried out.

2. 5 Statistical Analysis

For statistical analysis, GraphPad Prism 8 software was utilized. Data sets were compared using unpaired Mann-Whitney test when two data sets were being compared, the one-way ANOVA followed by a Kruskal–Wallis test or the two-way ANOVA when more than two data sets were being compared.

Chapter 3- Investigation of alteration in NK cells metabolism in patients with sepsis

3. 1 Introduction

Both pro-inflammatory and anti-inflammatory immune responses play roles in promoting and preventing the expansion of infectious agents and organ dysfunction during sepsis. As previously described, the immunological characteristic of sepsis includes two phases. The acute phase is initiated by the innate immune system, leading to a massive pro-inflammatory cytokine secretion termed a cytokine storm. This phase is followed by a compensatory anti-inflammatory phase, which can lead to profound immunosuppression (Biron et al., 2015, de Pablo et al., 2014, Hotchkiss et al., 2013, Toro et al., 2013).

Lymphocytes, such as NK cells and Vδ2 T cells play central roles in the early stages of immune activation and regulation, and therefore are likely to be important components of the dysregulated immune response that occur during sepsis. NK cells and Vδ2 T cells also have potential as therapeutic targets for sepsis, and immunotherapies using or targeting these cells are currently under investigation for other diseases. To our knowledge, the metabolic and functional profile of NK cells and Vδ2 T cells in patients with sepsis have not been fully determined and need to be investigated.

NK cells are cytotoxic lymphocytes that play a major role in host immunity against microbial infection and cancer. NK cells mediate antimicrobial responses as a result of their ability to identify pathogen-associated molecular patterns (PAMP) and cell-surface receptors that signify infection or tumour transformation. NK

cells can combat infection directly by producing cytotoxic mediators such as granzymes, perforin and pro-inflammatory cytokines such as IFN-γ, or indirectly through the activation of other innate and adaptive immune cells such as dendritic cells, monocytes, macrophages, and neutrophils (Colonna 2017, de Pablo et al., 2014, Walzer et al., 2005). $CD56^{bright}$ cells and $CD56^{dim}$ cells are two phenotypically and functionally different subsets of human NK cells based on cell-surface levels of CD56 receptor. The $CD56^{bright}$ subset produces more IFN-γ, whereas the $CD56^{dim}$ subset has more cytotoxic activity (Cooper et al., 2001; Fehniger et al., 2003).

Several studies have shown that NK cells are depleted in patients with sepsis (Hotchkiss, 2014, Giamarellos-Bourboulis et al., 2006, Puente et al., 1993, Bender et al., 1988). Furthermore, NK cells from sepsis patients have impaired ability to produce IFN-γ (Catherine Fitting et al., 2017, Forel et al., 2012). Indeed, NK cells have a significant role to play in sepsis pathology in order to prevent secondary infections (Chiche et al., 2011).

Given the importance of NK cells in fighting both bacterial and viral infections and the growing body of evidence that supports their prolonged importance during the immune response, it is possible that restoring their function in patients with sepsis, could be of therapeutic benefit. It has been shown that the metabolic changes that occur in NK cells are closely linked to function (Keating et al., 2016; Zaiatz-Bittencourt et al., 2018). Therefore, targeting metabolism might provide a novel therapeutic approach. However, nothing is known about the impact of overwhelming infection on NK cell metabolism. We hypothesised that NK cell metabolism would be dysregulated during the

immunosuppressive stage of sepsis. To investigate this, we retrospectively analysed the phenotypes, functions and metabolic pathways utilised by NK cells from patients admitted to the ICU unit of St James's Hospital in Dublin with sepsis.

The following sections in this chapter will discuss in detail the immunometabolic profile of the NK cells and their functions in patients with sepsis.

Hypothesis and Aims

We hypothesized that altered cellular metabolism in NK cells, in patients with sepsis, may affect their functions and contribute to the clinical phenotypes observed. We proposed the following objectives:

1) To compare the frequencies and phenotypes of circulating NK cells in healthy control subjects and sepsis patients.

2) To determine if nutrient receptor expression by freshly-isolated and *ex vivo*-stimulated circulating NK cells is altered in patients with sepsis.

3) To compare mitochondrial form and function in NK cells from healthy controls and sepsis patients.

4) To investigate if metabolism of NK cells is altered during sepsis, and to understand how this contributes to their function or dysfunction during disease.

3. 2 Results

3. 2. 1 NK cell frequencies in peripheral blood from patients with sepsis are similar to those in healthy controls

There is controversy in the literature regarding NK cell frequencies during sepsis (Guo et al., 2018). Therefore, in order to investigate if NK cells are altered in response to sepsis in our cohort of patients, we analysed the frequencies of circulating NK cells by flow cytometric analysis of isolated PBMC (Fig. 3. 1A). Using the $CD3^{-}$ $CD56^{+}$ lymphocytes phenotype to define NK cells, there was no difference in overall NK cell frequencies in patients with sepsis compared to controls (Fig. 3. 1B).

The frequencies $CD56^{bright}$ NK cells were significantly lower in patients with sepsis compared to healthy controls (Fig. 3.1C). Conversely, the frequencies of $CD56^{dim}$ NK cells were increased in patients (Fig. 3.1D).

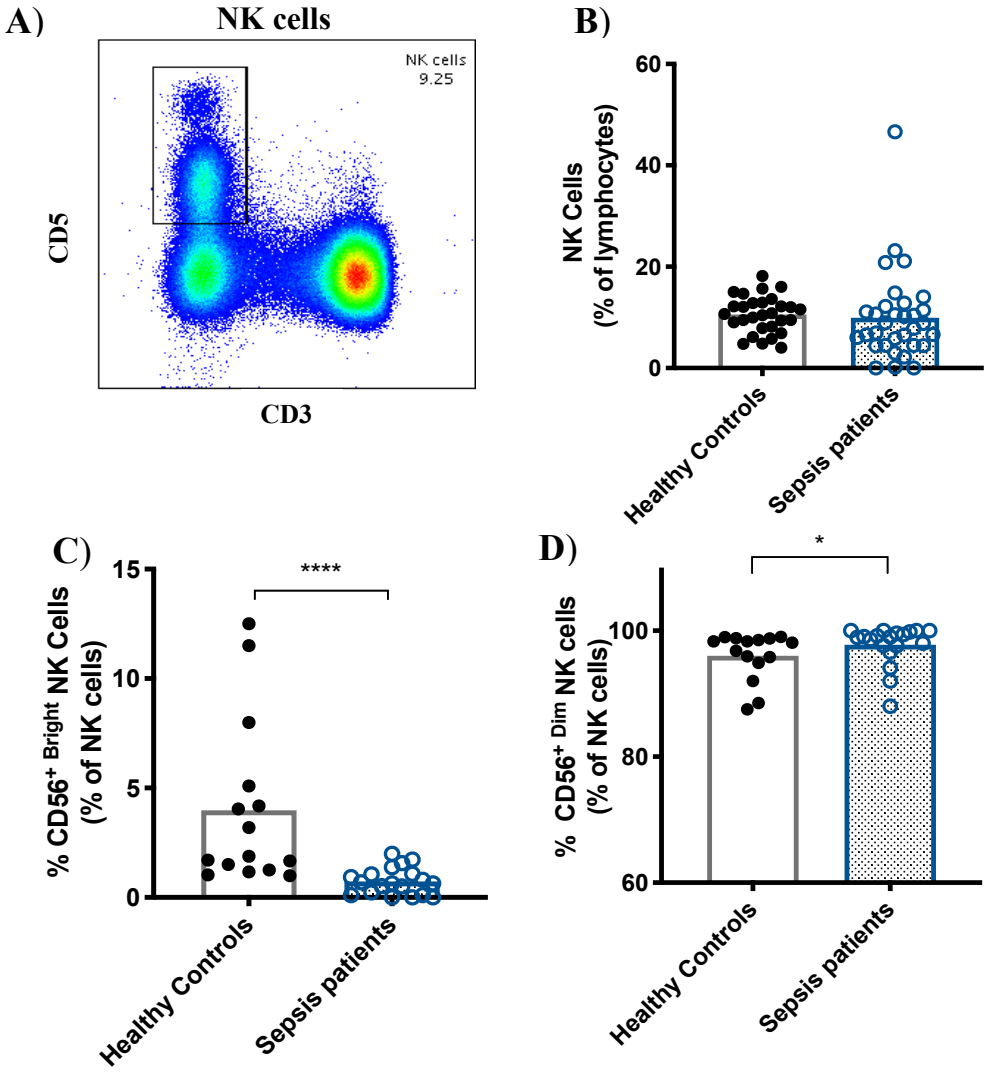

Figure 3. 1 The frequencies of circulating NK cells in patients with sepsis are similar to those in controls, but the proportions of CD56 ^{bright} and CD56 ^{dim} NK cells are altered.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3 and CD56 and the frequencies of NK cells (CD3$^-$ CD56$^+$) were determined by flow cytometry. (A) Flow cytometry dot plot showing CD3 and CD56 expression of gated lymphocytes. (B) Scatter plot showing the frequencies of NK cells, as percentages of total lymphocytes, in patients with sepsis (n = 32) and healthy control subjects (n = 29). (C and D) Frequencies of CD56^{bright}NK cells (C) and CD56^{dim} NK cells (D), as percentage of total NK cells, in 15 sepsis patients and 19 control subjects. Bars show means. Each dot represents 1 donor. Samples were compared using a non-parametric Mann-Whitney test. *P < 0.05, ****P <0.0001.

3. 2. 2 NK cells from sepsis patients display normal induction of CD69 expression in response to cytokine stimulation

In order to investigate if NK cells are activated, or primed for activation *in vivo* during sepsis, cell-surface expression of CD69 by NK cells was measured in freshly-isolated PBMC and after stimulation with IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours, by flow cytometry. CD69 expression on freshly isolated NK cells was slightly higher in patients compared to NK cells from controls (Fig. 3.2A). NK cells from both patients with sepsis and healthy subjects upregulated CD69 expression in response to stimulation with IL-12 and IL-15 (Fig. 3.2B-C). CD69 upregulation in response to cytokines occurred to the same level on NK cell from patients and as healthy controls (Fig. 3.2D).

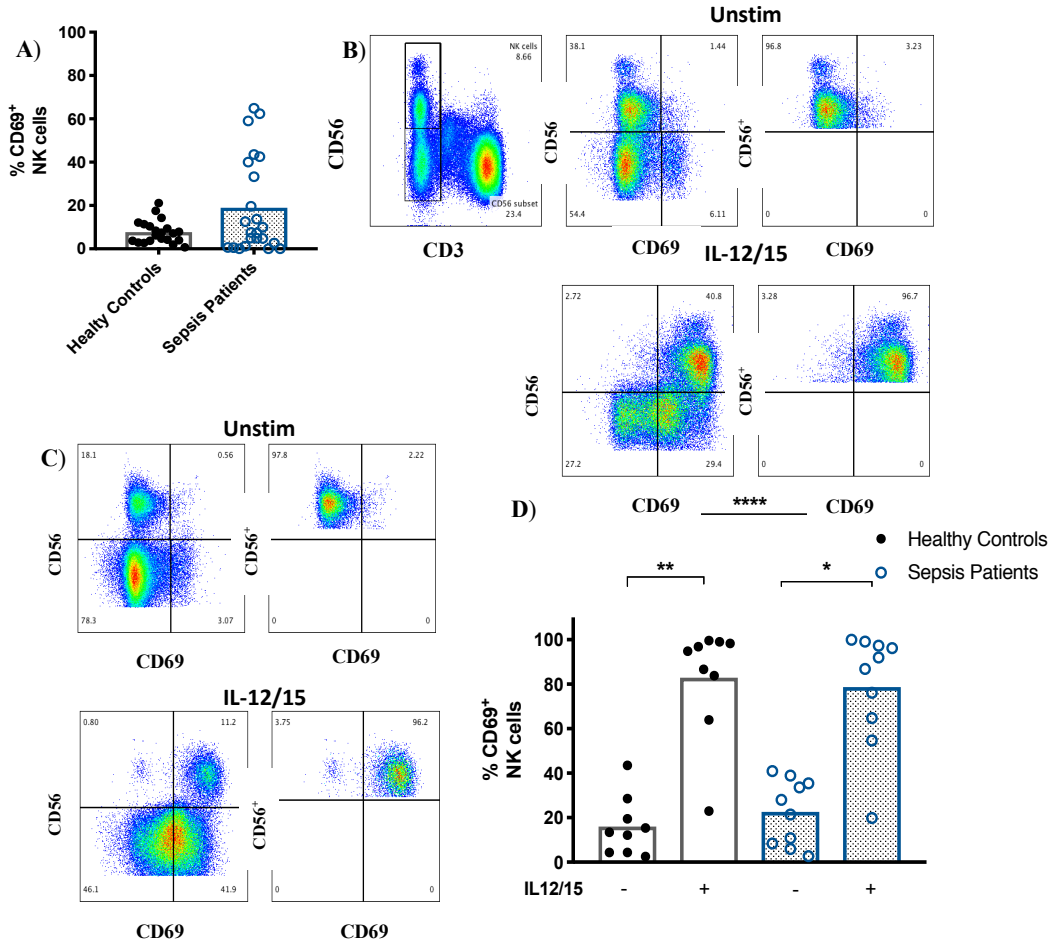

Figure 3. 2 NK cells from sepsis patients upregulate CD69 normally in response to cytokines.

*PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, CD56 and CD69 immediately after isolation or after stimulation with medium alone or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. The expression of CD69 by NK cells was then analysed by flow cytometry. (A) Frequencies of CD69-expressing NK cells from patients with sepsis (n = 23) and healthy control subjects (n = 22). (B) Representative flow cytometry dot plot showing CD69 upregulation on NK cells from a healthy control subject in response to stimulation. The left panel shows the expression of CD3 and CD56 by PBMC, while the centre and right panels show the expression of CD69 by gated CD3- cells and CD3-CD56+ NK cells, respectively. The top panels show CD69 expression by unstimulated CD3- cells and NK cells, whereas the lower panels show CD69 expression by IL-12 and IL-15-stimulated CD3- cells and NK cells. (C) Representative flow cytometry dot plot showing CD69 upregulation by NK cells from a patient with sepsis in response to stimulation. The left panel shows the expression of CD3 and CD56 by PBMC, while the centre and right panels show the expression of CD69 by gated CD3- cells and CD3-CD56+ NK cells, respectively. Upper and lower panels show resting and IL-12 and IL-15-stimiulated CD3- cells and NK cells, respectively. (D) Frequencies of CD69-expressing NK cells from patients with sepsis (n = 10) and healthy control subjects (n = 9) in response to treatment with medium or IL-12 + IL-15. Bars show means. Each dot represents 1 donor. Samples were compared using either a non-parametric Mann-Whitney test analysis (A) or a one-way ANOVA followed by a Kruskal–Wallis test (D). *P < 0.05, **P <0.001, ****P <0.0001.*

3. 2. 3 Effector functions NK cells from patents with sepsis

The above data show that NK cells from patients with sepsis upregulated CD69 normally upon cytokine stimulation, suggesting that they can be activated to a similar level. In order to investigate this further, expression the cytotoxicity markers associated with NK cells was assessed by intracellular flow cytometry. Expression of granzyme B (GnzB) by NK cells, was analyzed using freshly isolated PBMC and PBMC stimulated for 18 hours with cytokines (Fig. 3.3A). Freshly isolated NK cells from patients had similar GnzB expression compared to controls (Fig. 3.3B). Cytokine stimulation did not significantly upregulate GnzB expression in either sepsis patients or controls (Fig. 3.3 C and D).

We also investigated if NK cells from sepsis patients had an impaired ability to produce IFN-γ. NK cells from sepsis patients had reduced IFN-γ production upon IL-12/15 stimulation compared to controls (Fig. 3.4 A-C).

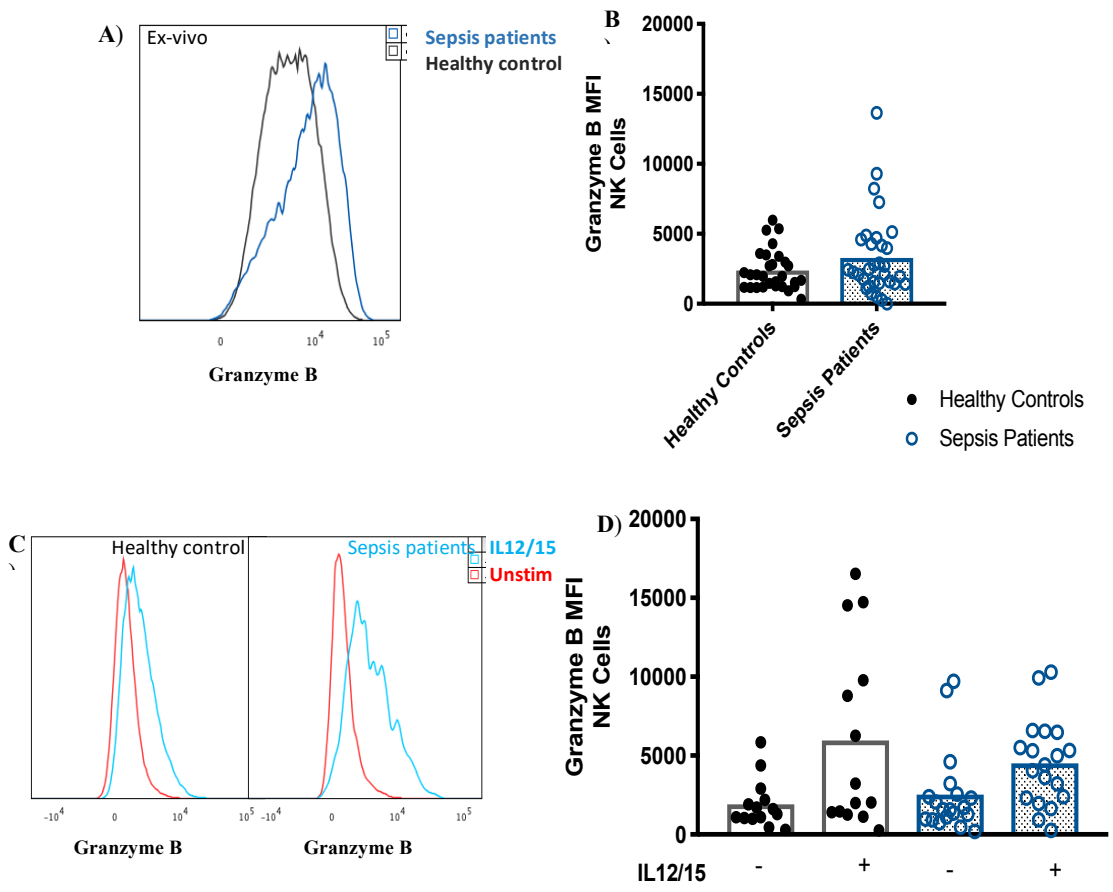

Figure 3. 3 Expression of GnzB by NK cells from sepsis patients and control subjects.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, CD56 and granzyme B immediately after isolation or after stimulation with medium alone or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. The expression of GnzB by NK cells was then analysed by intracellular flow cytometry. (A) Representative flow cytometry histogram showing the expression of GnzB by NK a patient with sepsis and a healthy control subject. (B) Mean fluorescence intensity (MFI) of GnzB staining of freshly isolated NK cells from patients with sepsis (n = 30) and healthy control subjects (n = 29). (C) Representative histograms comparing the expression of GnzB by NK cells from a healthy control subject in response to stimulation. (D) GnzB MFI of NK cells from patients with sepsis (n = 19) and healthy control NK cells (n = 14) in response to treatment with medium or IL-12 + IL-15. Bars show means. Each dot represents 1 donor. Samples were compared using either a non-parametric Mann-Whitney test analysis (B) or a one-way ANOVA followed by a Kruskal–Wallis test (D).

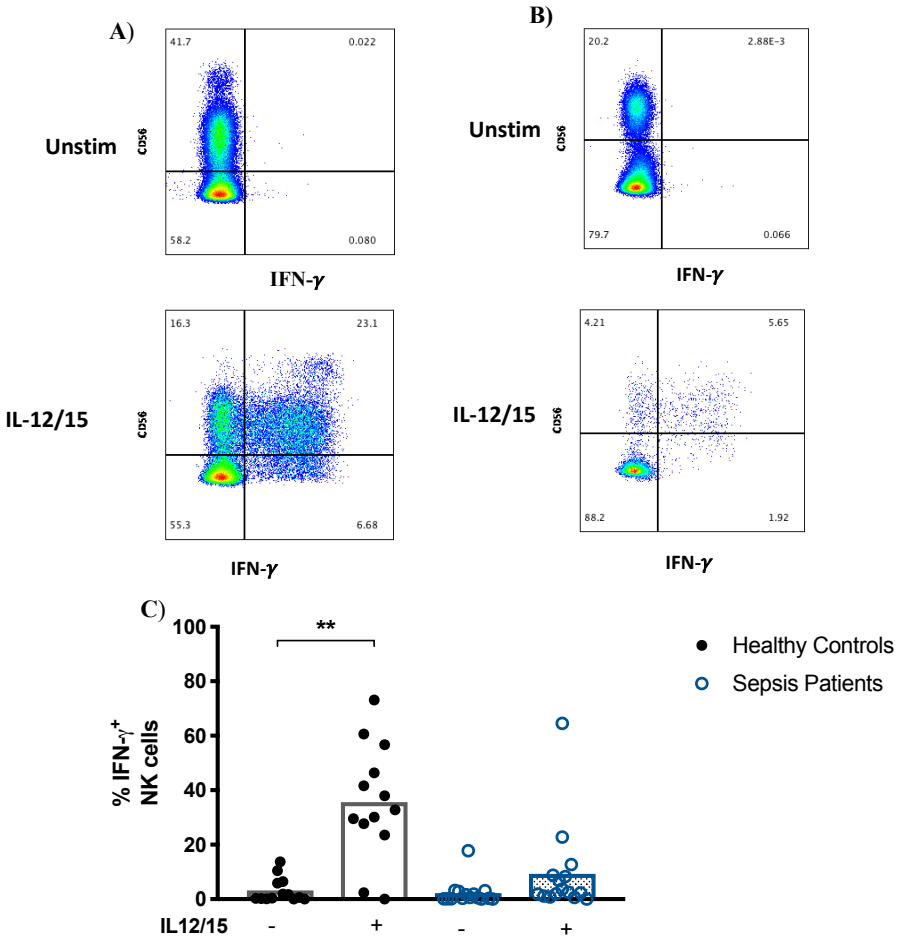

Figure 3. 4 NK cells from patients with sepsis have impaired upregulation of IFN-γ in response to cytokine stimulation.

*PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for cell-surface CD3, CD56 and intracellular IFN-γ after stimulation with medium alone or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. The production of IFN-γ by NK cells was then analysed by flow cytometry (A) Representative flow cytometry dot plots showing IFN-γ upregulation by NK cells from a healthy control subject in response to stimulation. The left and right panels show the expression of IFN-γ by gated CD3- cells and CD3-CD56+ NK cells, respectively. Upper and lower panels show unstimulated and IL-12/IL-15-stimulated cells, respectively. (B) Representative flow cytometry dot plot showing IFN-γ upregulation by NK cells from a patient with sepsis in response to stimulation. The left and right panels shows the expression of IFN-γ by gated CD3- cells and CD3-CD56+ NK cells, respectively, while the upper and lower panels show unstimulated and IL-12/IL-15-stimulated cells. (C) Frequencies of IFN-γ-expressing NK cells from patients with sepsis (n = 15) and healthy control subjects (n = 13) in response to treatment with medium or IL-12 + IL-15. Bars show means. Each dot represents 1 donor. Samples were compared using a one-way ANOVA followed by a Kruskal–Wallis test. **P <0.001.*

3. 2. 4 NK cells have impaired mTORC 1 activity in patients with sepsis

Mammalian target of rapamycin complex 1 (mTORC1) is an important metabolic regulator. It is a sensitive nutrient pathway that boosts anabolic cellular metabolism (lipids and nucleotides synthesis). mTORC1, activity in NK cells from healthy donors and sepsis patients was analysed by measuring the expression of phosphorylation of S6 ribosomal protein (pS6) by flow cytometry. pS6 levels were significantly lower in freshly isolated NK cells from patients with sepsis compared to NK cells from healthy donors (Fig. 3.5).

This data suggest that mTORC1 activity in NK cells is impaired in patients with sepsis.

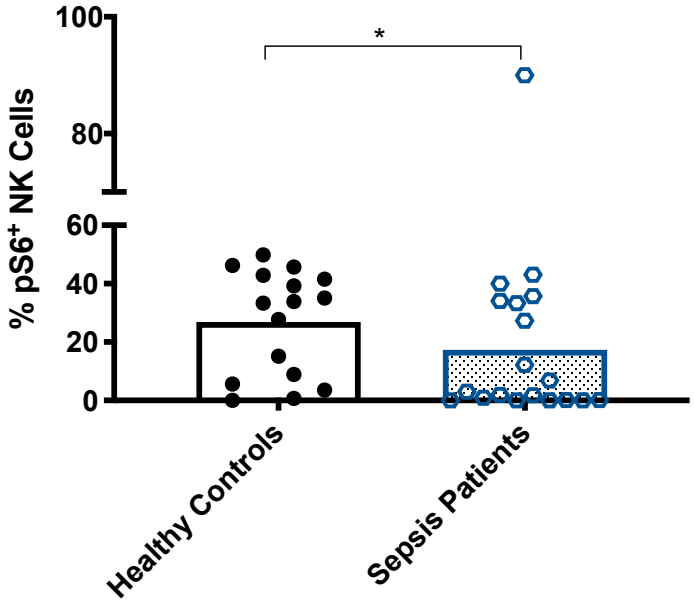

Figure 3. 5 NK cells from patients with sepsis have impaired mTORC1 activity.

*PBMC from patients with sepsis and healthy control subjects were stained using mAbs specific for CD3, CD56 and pS6 to determine mTORC1 activity of NK cells by flow cytometry. Frequencies of pS6-expressing NK cells from patients with sepsis (n = 19) and healthy control subjects (n = 16). Bars show means. Each dot represents 1 donor. Samples were compared using non-parametric Mann-Whitney test analysis. *P < 0.05.*

3. 2. 5 NK cell expression of nutrient receptors is similar in patients with sepsis and healthy controls

Nutrient uptake is an important first step in driving metabolic activation. To investigate if NK cells from patients with sepsis are primed to upregulate their metabolism, expression of nutrient receptors and uptake through the SLC7A5 nutrient receptor were measured.

Activity of the system L-amino acid transporter SLC7A5, which is crucial for c-Myc which is a key regulator of lymphocyte metabolism, was measured by measuring kynurenine uptake. Freshly-isolated NK cells from both healthy controls and sepsis patients exhibited a similar level of kynurenine uptake (Fig. 3.6).

CD71, the transferrin receptor for iron uptake, was expressed at a similar level on NK cells freshly isolated from patients with sepsis compared to controls (Fig. 3.7A). In addition, CD71 expression was similar on NK cells amongst patients with sepsis compared to controls after 18 hours of stimulation (Fig. 3.7).

The expression of CD98, a component of the L-amino acid transporter, was measured by flow cytometry. It was expressed similarly on all NK cells in PBMC freshly isolated from patients and controls (Fig. 3.8). Upon stimulation with IL-12/15, CD98 expression did not increase on NK cells from healthy donors or sepsis patients (Fig. 3.8).

Overall, these data show that NK cells from patients with sepsis upregulate their nutrient receptors normally compared to NK cells from healthy controls.

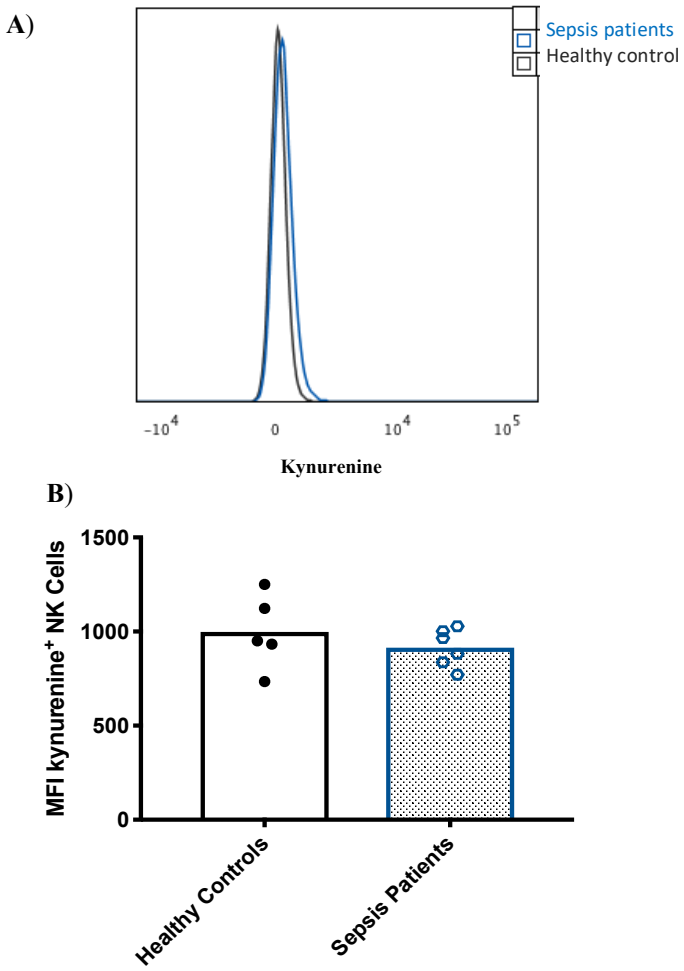

Figure 3. 6 NK cells from patients with sepsis showed similar nutrient up take through the SCL7A5 receptor.

Isolated PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, CD56 and incubated with kynurenine to determine the rate of kynurenine uptake of NK cells by flow cytometry. (A) Representative histogram showing the expression of kynurenine by NK cells from a patient with sepsis and a healthy control subject. (B) Mean fluorescence intensity (MFI) of kynurenine staining of freshly isolated NK cells from patients with sepsis (n = 6) and healthy control subjects (n = 5). Bars show means. Each dot represents 1 donor. Samples were compared using non-parametric Mann-Whitney test analysis.

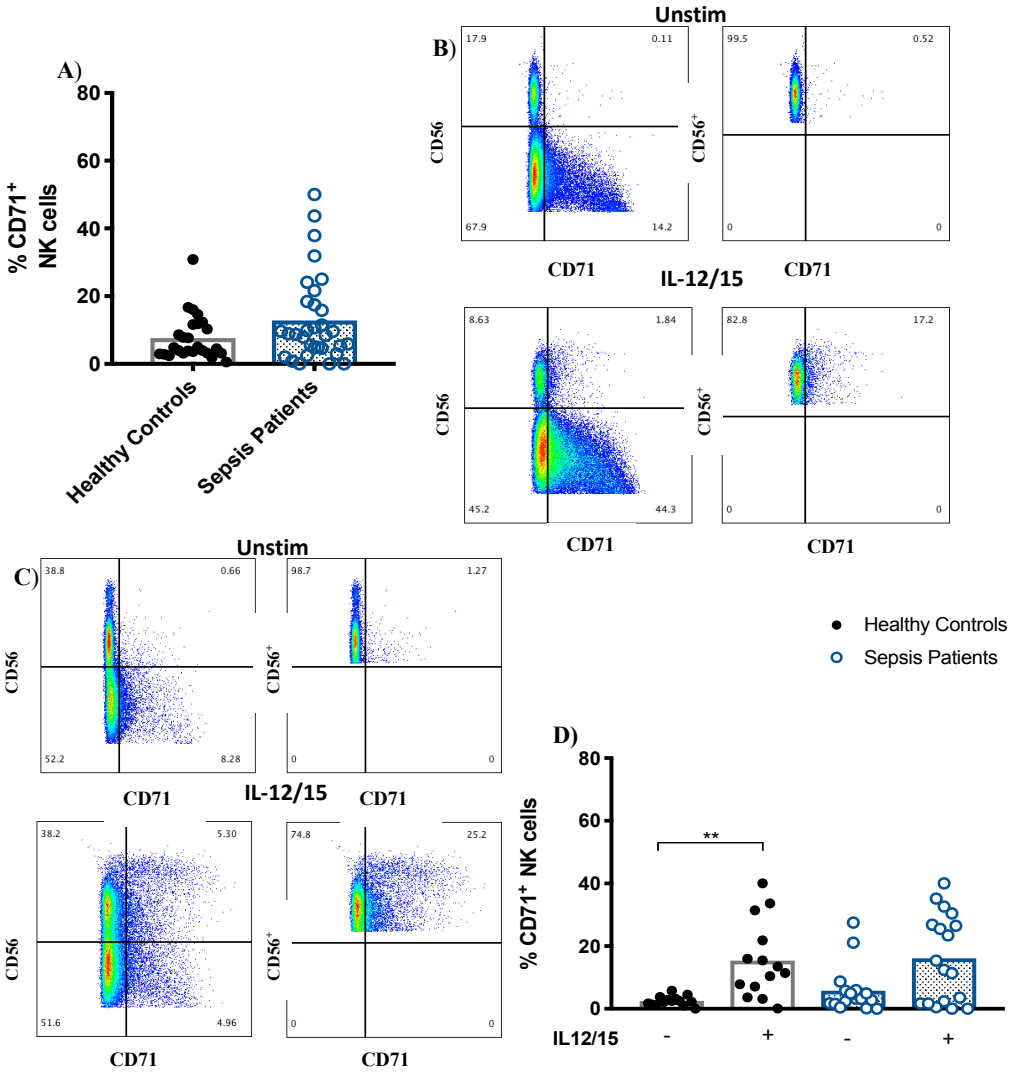

Figure 3. 7 NK cells from patients with sepsis displayed similar iron uptake receptor expression.

*PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, CD56 and CD71 immediately after isolation or after stimulation with medium alone or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. The expression of CD71 by NK cells was then analysed by flow cytometry. (A) Frequencies of CD71-expressing NK cells in freshly-isolated PBMC from patients with sepsis (n = 32) and healthy control subjects (n = 26). (B) Representative flow cytometry dot plots showing CD71 expression by NK cells from a healthy control subject in response to stimulation. The left and right panels show the expression of CD71 by gated CD3- cells and CD3-CD56+ NK cells, respectively. The upper and lower panels show unstimulated and IL-12/IL-15-stimulated cells, respectively. (C) Representative flow cytometry dot plot showing CD71 expression by NK cells from a patient with sepsis in response to stimulation. The left and right panels show the expression of CD71 by gated CD3- cells and CD3-CD56+ NK cells, respectively. The upper and lower panels show unstimulated and IL-12/IL-15-stimulated cells, respectively. (D) Frequencies of CD71-expressing NK cells from patients with sepsis (n = 18) and healthy control subjects (n = 14) in response to treatment with medium or IL-12 + IL-15. Bars show means. Each dot represents 1 donor. Samples were compared using either a non-parametric Mann-Whitney test analysis (A) or a one-way ANOVA followed by a Kruskal–Wallis test (D). **P <0.001.*

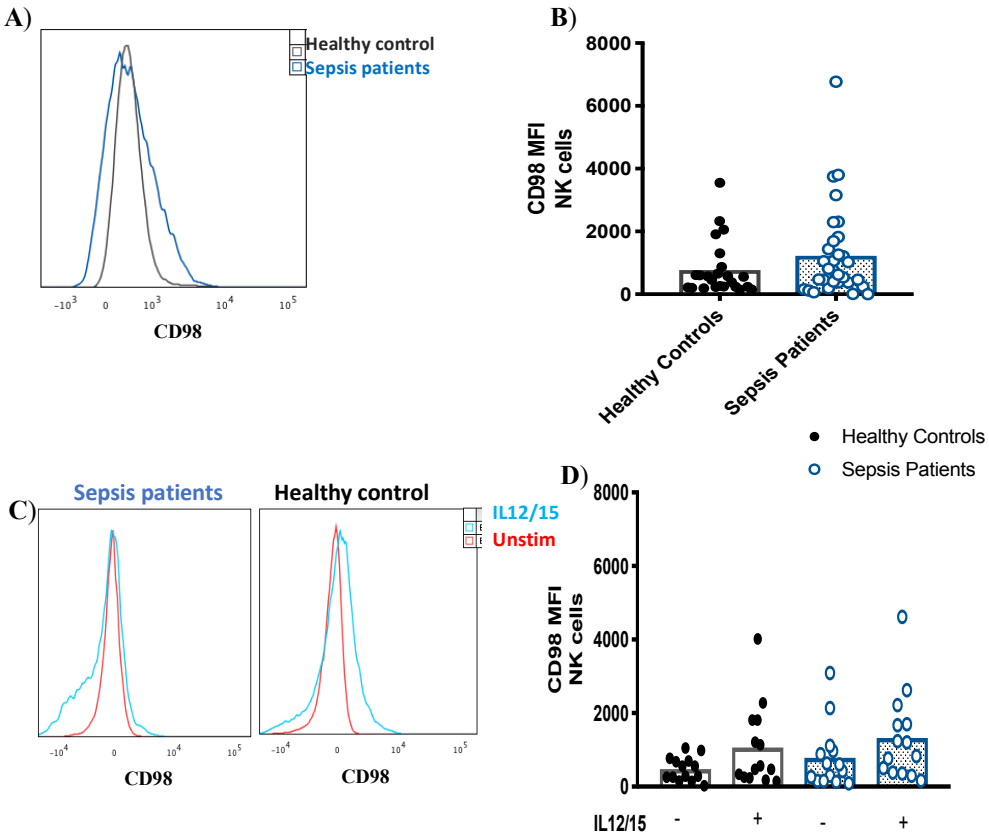

Figure 3. 8 NK cells from patients with sepsis express similar levels of amino acid uptake receptors.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, CD56 and CD98 immediately after isolation or after stimulation with medium alone or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. The expression of CD98 by NK cells was then analysed by flow cytometry. (A) Representative flow cytometry histogram comparing CD98 expression by NK cells from a patient with sepsis and a healthy control subject. (B) Mean fluorescence intensity (MFI) of CD98 staining of freshly isolated NK cells from patients with sepsis (n = 32) and healthy control subjects (n = 24). (C) Representative histogram comparing the expression of CD98 by NK cells from a patient with sepsis and a healthy control subject in response to stimulation. (D) CD98 MFI of NK cells from patients with sepsis (n = 14) and healthy control (n = 14) in response to treatment with medium or IL-12 + IL-15. Bars show means. Each dot represents 1 donor. Samples were compared using either a non-parametric Mann-Whitney test analysis (B) or a one-way ANOVA followed by a Kruskal–Wallis test (D).

3. 2. 6 The mitochondrial structure and function of NK cells from sepsis patients are similar to those of NK cells from healthy controls

In cellular metabolism, the mitochondria have an essential role to play. Therefore, their structure and functions were analysed in freshly isolated NK cells from healthy control subjects and sepsis patients in order to examine possible effects of sepsis on NK cells metabolism.

3. 2. 6. 1 NK cells from sepsis patients have normal mitochondrial mass

As mitochondrial mass can indicate a cell's mitochondrial health and give insight into the pathways of cell mitophagy and mitochondrial biogenesis, the mitochondrial mass of NK cells from healthy controls and patients with sepsis was measured directly ex-vivo and after 18 hours stimulation with IL-12/15 by staining the cells with Mitotracker Green FM. Mitochondrial mass of freshly isolated NK cells from both healthy controls and sepsis patients was similar (Fig. 3. 9 A-B). After stimulation of PBMC with IL-12 and IL-15 in vitro, the mitochondrial mass in NK cells from both sepsis patients and control subjects did not increase (Fig. 3. 9C).

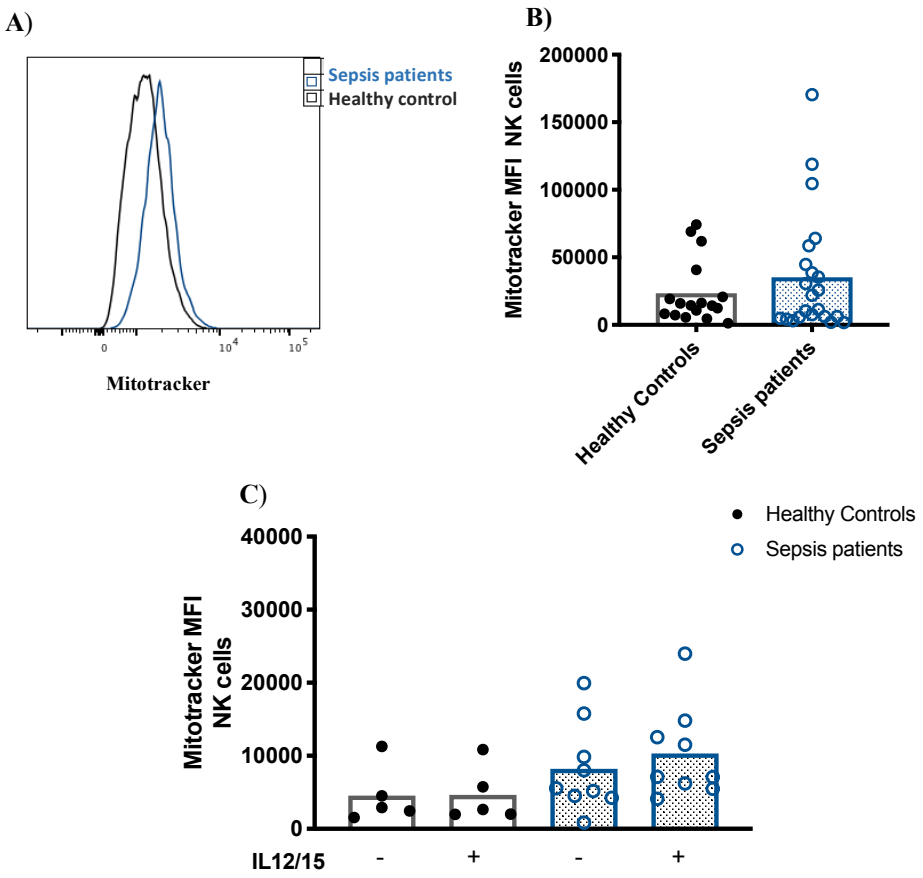

Figure 3. 9 NK cells from patients with sepsis display similar mitochondrial mass.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, CD56 and Mitotracker Green FM (100 nM) immediately after isolation or after stimulation with medium alone or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. Cells were analyzed by flow cytometry. (A) Representative histogram showing the expression of Mitotracker Green FM by NK cells freshly isolated from a patient with sepsis and a healthy control subject. (B) Mean fluorescence intensity (MFI) of mitochondrial mass (Mitotracker) staining of freshly isolated NK cells from patients with sepsis (n = 22) and healthy control subjects (n = 15). (C) Mitochondrial mass MFI of NK cells from patients with sepsis (n = 9) and healthy controls (n = 5) in response to treatment with medium or IL-12 + IL-15. Bars show means. Each dot represents 1 donor. Samples were compared using either a non-parametric Mann-Whitney test analysis (B) or a one-way ANOVA followed by a Kruskal–Wallis test (C).

3. 2. 6. 2 NK cells from sepsis patients have normal levels of ATP synthase

ATP synthase is an enzyme that produces cellular ATP by utilizing the electrochemical gradient of the mitochondrial membrane. ATP5B, is the key catalytic subunit which was measured directly ex-vivo and after 18 hours stimulation with IL-12/15 using flow cytometry. ATP5B expression in fresh isolated NK cells from healthy controls and patients were similar (Fig. 3. 10A). Stimulation of PBMC with IL-12 and IL-15 in vitro did not result in any changes in ATP synthase levels in NK cells from healthy donors and patients with sepsis (Fig. 3. 10B-C).

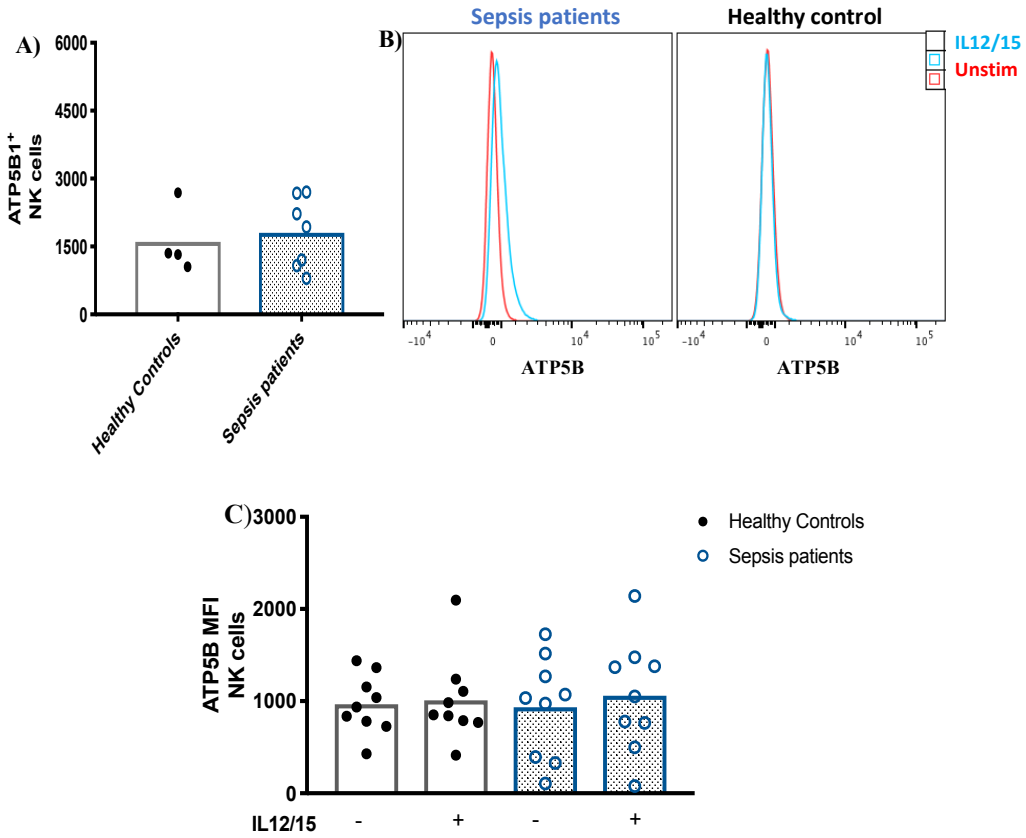

Figure 3. 10 NK cells from patients with sepsis express normal levels of ATP synthase.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, CD56 and ATP5B immediately after isolation or after stimulation with medium alone or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. Cells were analyzed by flow cytometry. (A) Mean fluorescence intensity (MFI) of ATP5B staining of freshly isolated NK cells from patients with sepsis (n = 7) and healthy control subjects (n = 5). (B) Representative flow cytometry histogram showing the expression of ATP5B by unstimulated and IL-12/IL-15-stimulated NK cells from a patient with sepsis and a healthy control subject. (C) ATP5B MFI of NK cells from patients with sepsis (n = 9) and healthy controls (n = 9) in response to treatment with medium or IL-12 + IL-15. Bars show means. Each dot represents 1 donor. Samples were compared using either a non-parametric Mann-Whitney test analysis (A) or a one-way ANOVA followed by a Kruskal–Wallis test (C).

3. 2. 6. 3 NK cells from sepsis patients have similar levels of mROS to those of NK cells from healthy controls

Mitochondrial dysfunction is commonly linked with high levels mROS. mROS can react with protein, lipids, and DNA in the mitochondria, and can influence signalling pathways. MitoSOX was used as a measurement of mitochondrial ROS. Mitochondrial superoxide levels were measured directly ex-vivo by MitoSOX staining using flow cytometry. Freshly isolated NK cells from patients and controls had similar levels of mROS (Fig. 3. 11A-B).

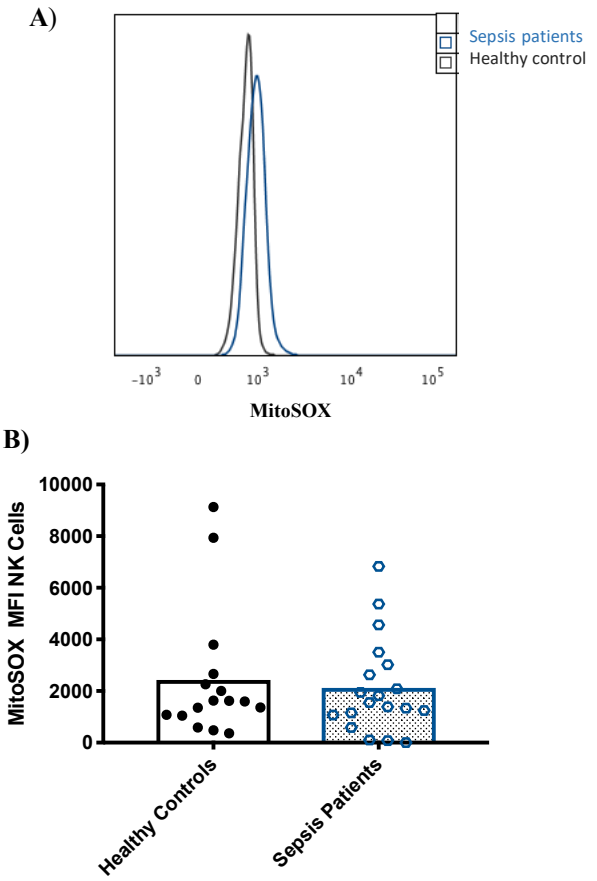

Figure 3. 11 NK cells from patients with sepsis have normal levels of mROS.

Isolated PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, CD56 and MitoSOX Red dye (1.5 µm) for 15 min at 37°C. Cells were analysed by flow cytometry. (A) Representative histogram showing the expression of MitoSOX by freshly isolated NK cells from a patient with sepsis and a healthy control subject. (B) Mean fluorescence intensity (MFI) of MitoSOX staining of freshly isolated NK cells from patients with sepsis (n = 19) and healthy control subjects (n = 15). Bars show means. Each dot represents 1 donor. Samples were compared using non-parametric Mann-Whitney test analysis.

3. 2. 6. 4 NK cells from sepsis patients produce similar levels of cellular ROS to those of NK cells from healthy controls

High levels of cROS in the cytosol may affect and damage the cells and their signalling pathways. The levels of cROS in freshly isolated NK cells from healthy controls and sepsis patients were investigated using flow cytometry assay CellROX.

Similar to what was observed for mROS, NK cells in freshly-isolated PBMC from patients with sepsis had similar levels of cROS compared to NK cells from healthy control subjects (Fig. 3. 12A-B). This suggests that no evidence of mitochondrial dysfunction in NK cells during sepsis.

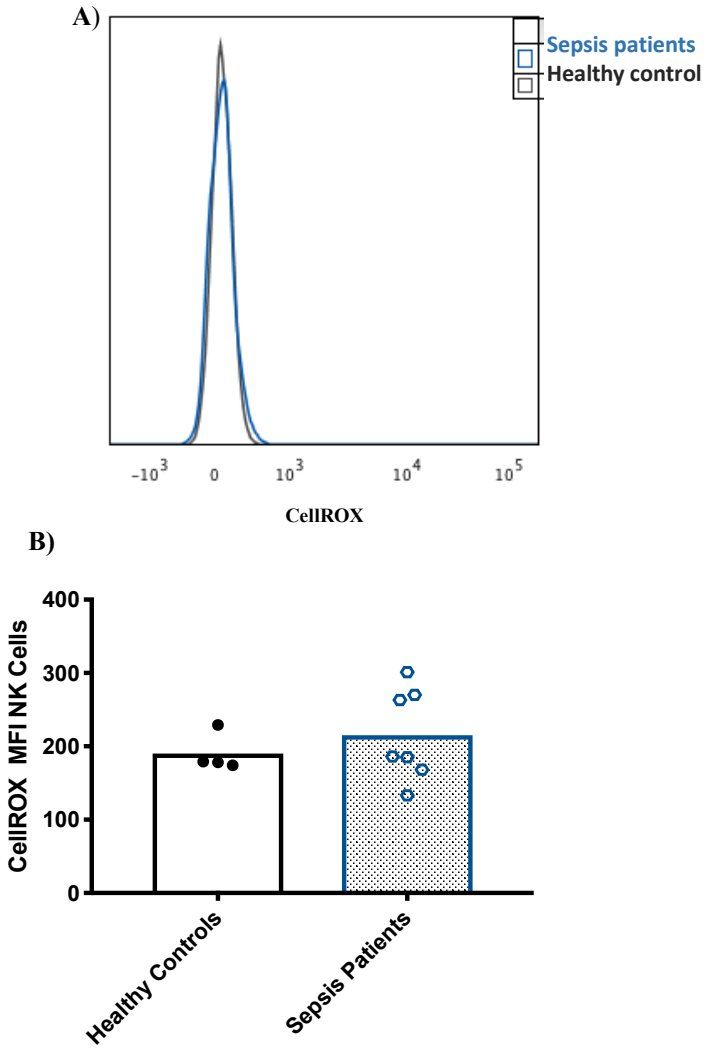

Figure 3. 12 NK cells from patients with sepsis express normal levels of CellROX.

Isolated PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, CD56 and CellROX (1µm) for 30 min at 37°C. Cells were analysed by flow cytometry. (A) Representative histogram showing the expression of CellROX by freshly isolated NK cells from a patient with sepsis and a healthy control subject. (B) Mean fluorescence intensity (MFI) of CellROX staining of freshly isolated NK cells from patients with sepsis (n = 7) and healthy control subjects (n = 4). Bars show means. Each dot represents 1 donor. Samples were compared using non-parametric Mann-Whitney test analysis.

3. 2. 6. 5 Relationship of severity scores of SOFA and APACHE II and immunological functions in NK cells from sepsis

To examine the relationship between severity and cytotoxic function of NK cells. First, we analysed the correlation between mTORC1 activity and the production IFN-γ and we showed that there is no linear relationship between the the levels of pS6 in NK cells from patients with sepsis and the production of IFN-γ (r = 0.3478, P < 0.202; Fig. 3.13 A). The levels of severity scores of both SOFA and APACH II also showed a statistically negative correlation with IFN-γ production(SOFA r = -0.1325, P <0.2637, APACH II r = -0.1325 P < 0.649; Fig. 3.13B) and granzyme B activity (SOFA: r = -0.1802, P < 0.5, APACHE II: r = -0.3067, P < 0.1450; Fig. 3.13C).

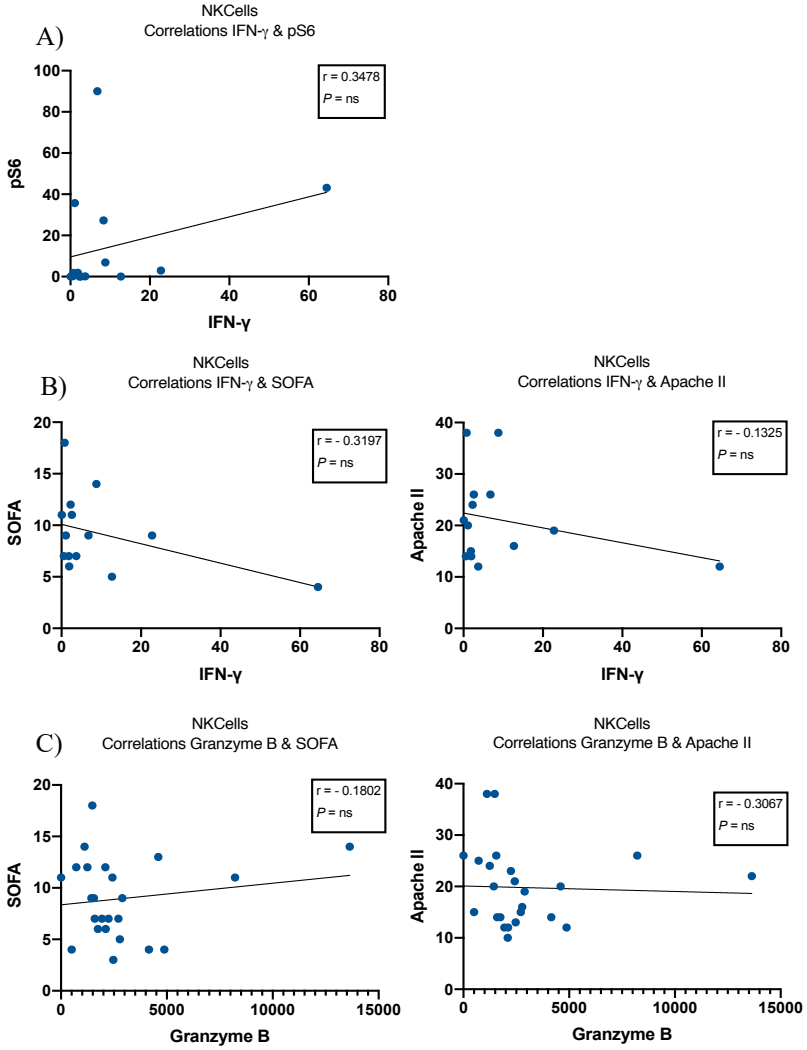

Figure 3. 13 Correlation of severity scores of SOFA and APACHE II and immunological functions in NK cells from sepsis.

(A) Correlation of mTORC1 activity (pS6) with IFN-γ production (r = 0.3478, P < 0.202). (B) correlation of IFN-γ production with the levels of severity scores of both SOFA and APACH II (SOFA: r = -0.1325, P <0.2637, APACH II: r = -0.1325 P < 0.649).(C) The correlation between granzyme B cellular activity with the levels of severity scores of both SOFA and APACH II (SOFA: r = -0.1802, P < 0.5, APACHE II: r = -0.3067, P < 0.1450). The correlation between variables was analyzed by nonparametric spearman correlation test.

3. 3 Discussion

Sepsis is the result of an abnormal immune response to infection or trauma. The initial strong pro-inflammatory response fails to control the infection and sepsis can develop. In time, sepsis can cripple the immune system resulting in a profound immunosuppression. Studying what drives the early pro-inflammatory response is important but is often difficult as patients are not identified clinically at this point. Therefore, studying the later stages of sepsis may provide a more realistic approach to identifying the pathogenic mechanisms and restoring immune function in this vulnerable cohort of patients.

Although NK cells are best known as mediators of immune responses against viruses and tumours, they also have well-documented roles in immune defense against bacteria, fungi and parasites (Mody et al., 2019). Once NK cells recognize microbial infection and become activated, they carry out their immune effector functions primarily by 2 mechanisms - cytotoxicity and the production of cytokines (Zhang & Huang, 2017).

NK cells become activated during bacterial infections by directly recognizing bacteria or their products or by recognising bacteria-infected cells (Horowitz et al., 2012). Thus, NK cells may be important in preventing sepsis, making them potential therapeutic targets for bacterial infections (Guo et al., 2018.; Horowitz et al., 2012). Direct antimicrobial activity can occur when NK cells contact pathogens via NKp30, an activating NK cell receptor. This interaction triggers the production of cytotoxic molecules, mainly

granulysin and perforin to clear bacterial infection such as *Cryptococci, Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Ernst et al., 2000; Lu et al., 2014; Ma et al., 2002, 2004; Markham et al., 1985; Walch et al., 2005). NK cells produce IFN-γ as a result of recognition of bacterial pathogen-associated molecular patterns by TLR2 (Chalifour et al., 2016). IFN-γ is also produced by NK cells that recognize bacterial superantigens such as Staphylococcal enterotoxin B (SEB) and Streptococcal pyrogenic exotoxin A (SPEA) (D'Orazio et al., 1995; Sacks et al., 1991; Souza-Fonseca-Guimaraes et al., 2012). Granzyme B produced by NK cells cleaves proteins, which function in vital biosynthetic and metabolic pathways that are critical for bacterial survival under diverse environmental conditions, leading to apoptosis (Dotiwala et al., 2017).

The role of NK cells in bacterial sepsis is still unclear. Some studies have shown that the numbers of circulating NK cells were markedly decreased in patients with sepsis, and their low numbers were associated with high mortality rate (Raúl de Pablo et al., 2012; Hiraki et al., 2007; Ono et al., 2018). The frequencies of circulating NK cells are thought to be a beneficial biomarker to determine the survival of patients with sepsis (Andaluz-Ojeda et al., 2011; Giamarellos-Bourboulis et al., 2006). Our data show that the percentages of circulating NK cells in peripheral blood from our cohort of patients with sepsis are similar to those in healthy control subjects. Human NK cells have two subsets. $CD56^{bright}$ NK cells that produce more cytokine, mainly IFN-γ. $CD56^{dim}$ NK cells are considered to be more cytotoxic than $CD56^{bright}$ (Michel et al., 2016). In this study, the frequencies of $CD56^{bright}$ NK cells were significantly lower in sepsis patients than in control subjects, with a resulting increase in $CD56^{dim}$ NK cells. A previous clinical study

also reported that $CD56^{bright}$ NK cell frequencies are lower in patients in early stage sepsis (Toro et al., 2013). Decreases or increases in circulating NK cells numbers could be a result of the migration of NK cells to infected tissues or due to death by apoptosis, which may depend on the stage of sepsis or septic shock and the type of pathogens. Physiological aging is an evolutionarily conserved process which is correlated to an inadequate or disabled function of immune cells, including NK cells. The reduced function of NK cells is identified as NK cell immunosenescence. Age-associated NK cell immunosenescence contributes to the higher frequency of viral infection and cancer induction. Also, NK cell-mediated exclusion of senescent cells decreases on aging and leads to the accumulation of aged cells in tissue or organs which damages tissue homeostasis and their function. NK cell-mediated elimination of senescent cells is a direct exclusion (migration, recognition, binding, and elimination of their targets) manner which is achieved by the NK^{dim} cell by the granule exocytosis pathway (Sagiv et al., 2013). Previous study investigated the effect of aging on the cytokine production of NK bright cells and reported that the production of cytokines (IFN-γ, MIP-1α, IL-8, etc.) is significantly lower in older NK^{bright} cells than in younger NK^{bright} cells (Chan et al., 2007).

One of fastest evolving areas of medicine is immunometabolism. Immunometabolism describes the intracellular metabolic pathways of energy generation and utilisation. Each of the interlocking pathways represent a unique manner for a cell to generate and consume energy, that impacts on its ability to hold out its functions. Stimulation of an immune cell changes its functions, thus this can be related to a complementary amendment in metabolism, called metabolic reprogramming (O'Neill et al., 2016). At

rest, NK cells use glucose as source of energy via glycolysis linked to oxidative phosphorylation resulting high levels of energy. Once NK cells become activated, they increase the rate of aerobic glycolytic metabolism increasing the biosynthetic precursors for cytokine and granule production (Donnelly et al., 2014; Keating et al., 2016; Marçais et al., 2014). Inhibition of OxPhos strongly inhibited IFN-γ production (Keating et al., 2016). Also, inhibition of either glycolysis or OxPhos has the potential to inhibit ATP production (Gardiner, 2019; Keating et al., 2016; Wang et al., 2015). The mechanism behind metabolic changes linking metabolism and immunity during the immunosuppressive stage of sepsis are still being investigated. Hence, we hypothesized that dysregulated metabolism would underpin NK cell dysfunction in the immunosuppressive stage of sepsis.

To determine if NK cells from sepsis patients have altered metabolism, we analysed the expression of metabolic and functional markers by NK cells within freshly-isolated PBMC and after stimulation with IL-12 and IL-15, cytokines known to activate NK cells (Fehniger et al., 1999; Keating et al., 2016). We found that the early activation marker CD69 was expressed at slightly higher levels on NK cells in PBMC freshly isolated from patients compared to controls, a finding that is in agreement with a previous study in acute sepsis in patients (Boomer et al., 2012). This suggests that NK cells may be activated in vivo as a result of infection or sepsis and that these cells may be playing a role in the pathology. However, this increase was not statistically significant.

After stimulation for 18 hours with IL-12 and IL-15, we found that NK cells from patients had a similar expression of CD69 in comparison with those from controls. This suggests that NK cells from sepsis patients respond normally to stimulation in vitro.

Granzyme B is expressed by all NK cells. Granzymes are serine proteases that are implicated in cytotoxicity. Our analysis of NK cells freshly isolated from patients with sepsis revealed that the expression of granzyme B was similar in patients compared to controls. In contrast, another clinical study showed an increase in both granzyme B and granzyme A in NK cells of patients with sepsis and septic shock. Multi-organ dysfunction and high mortality rates were observed in these patients (Zeerleder et al., 2005).

Previous studies have investigated and reported that the levels of granzymes A and B in plasma of patients with severe sepsis was raised (Napoli et al., 2012; Zeerleder et al., 2005). Also, increased production of granzyme A and B was reported in whole blood upon stimulation with Gram-positive and Gram-negative bacteria (Lauw et al., 2000). García-Laorden *et al.* (2017) reported an increase in NK cells expressing granzyme A and B in blood from a murine model of sepsis in response to *E.coli* stimulation (García-Laorden et al., 2017). The current study is the first, to our knowledge, showing granzyme B expression by NK cells from sepsis patients in response to cytokine stimulation. We found that NK cells from sepsis patients and healthy control were similar in their ability to upregulate GnzB after IL-12/15 stimulation. Other studies on blood samples from healthy controls and septic patients indicated that NK cells exhibited similar degranulation and cytotoxicity (Alter et al., 2004; Forel et al., 2012).

These results suggest that the NK cell cytotoxic response is not altered in patients with bacterial infection and sepsis.

Next, we analysed the production of IFN-γ by NK cells after stimulation with IL-12 and IL-15 using flow cytometry. We found that NK cells from patients with sepsis have impaired upregulation of IFN-γ in response to cytokine stimulation. Similarly, Boomer *et al.* (2012) reported impaired secretion of IFN-γ by NK cells from acute sepsis patients following stimulation *in vitro* (Boomer et al., 2012). Both $CD56^{bright}$ and $CD56^{dim}$cells subset from sepsis patients exhibited a decline in IFN-γ secretion in response to stimulation (Fernando Souza-Fonseca-Guimaraes et al., 2012). These data suggest a possible link between NK cell effector function and sepsis pathology where the impaired antimicrobial activity of NK cells may contribute to the immunosuppression that is characteristic of sepsis and is correlated with mortality or development of secondary infection.

mTORC1 and c-Myc have been highlighted as crucial regulators of NK cell metabolism that is essential for NK cell effector functions (Keating et al., 2016; Loftus et al., 2018; Marçais et al., 2014). Both have important roles to play in the regulation of glycolysis and OxPhos, which directly impacts on NK cell functions. Upon cytokine stimulation, NK cells undergo metabolic reprogramming, increasing mTORC1 activity and enhance glycolytic flux. However, mTORC1 inhibition by rapamycin can leads to inhibition of glycolysis, cell size and CD71 and CD98 expression (Donnelly et al., 2014; Keating et al., 2016). We noted that NK cells from patients had lower levels of mTORC1 activity by measuring the phosphorylation of pS6 directly ex-vivo. Similarly, a previous study has

reported a significant reduction in glycolysis, OxPhos, ATP levels and mTORC1 activity in T cells from septic patients (Venet et al., 2017).

We showed that NK cells freshly isolated from patients with sepsis had a similar expression of CD98, CD71 and kynurenine uptake compared to controls. The expression of CD98 and CD71 was also similar on NK cells from patients with sepsis after *in vitro* stimulation. Our data suggest that resting and activated NK cells from patients with sepsis and from control subjects have similar nutrient requirements.

Mitochondria are key organelles responsible for energy generation. Keating and co-workers reported that NK cells function is profoundly dependent on mitochondrial OxPhos (Keating et al., 2016). We investigated wither the mitochondria of NK cells from patients with sepsis have changed their metabolism in response to sepsis. Upon examination of these vital organelles, we found that NK cells from sepsis patients had similar mitochondrial mass and normal levels of mROS, cROS and ATP synthase compared to healthy control subjects. These data suggest that the mitochondria of NK cells from patients with sepsis are carrying out their function and metabolism normally and no evidence of mitochondrial dysfunction in NK cells during sepsis. Variations in cellular metabolic pathways may have critical roles in immunoparalysis in sepsis, including a shift from OxPhos to glycolysis and may be mediated by epigenetic changes induced by inflammation (Van der Poll et al., 2017). Manipulation of cellular metabolism may lead to the discovery of novel immunotherapies for sepsis by targeting specific cell types.

In summary, this chapter has investigated and described the immunometabolic profile of the NK cells and their functions in patients with sepsis. Our results suggest that patients with sepsis exhibit a similar level of cytotoxicity and nutrient uptake by NK cells, suggesting that these cells from sepsis patients display similar functional and metabolic profiles to those from healthy controls. The only significant differences found in the present study were a decrease in the proportions of NK cells with $CD56^{bright}$ phenotypes and increase in the proportions with $CD56^{dim}$ phenotypes, and impaired mTORC1 activation and IFN-γ production in response to cytokine stimulation (Figure 3.14). The cause of infection, the environment of the host, and metabolic status are all intricately related to each other. Future studies on the metabolic status of NK cells over time as a patient progresses from the pro-inflammatory to the immunosuppressive stages of sepsis, may lead to identification of metabolic changes that underlie the changes in mTORC1 activation and IFN-γ production.

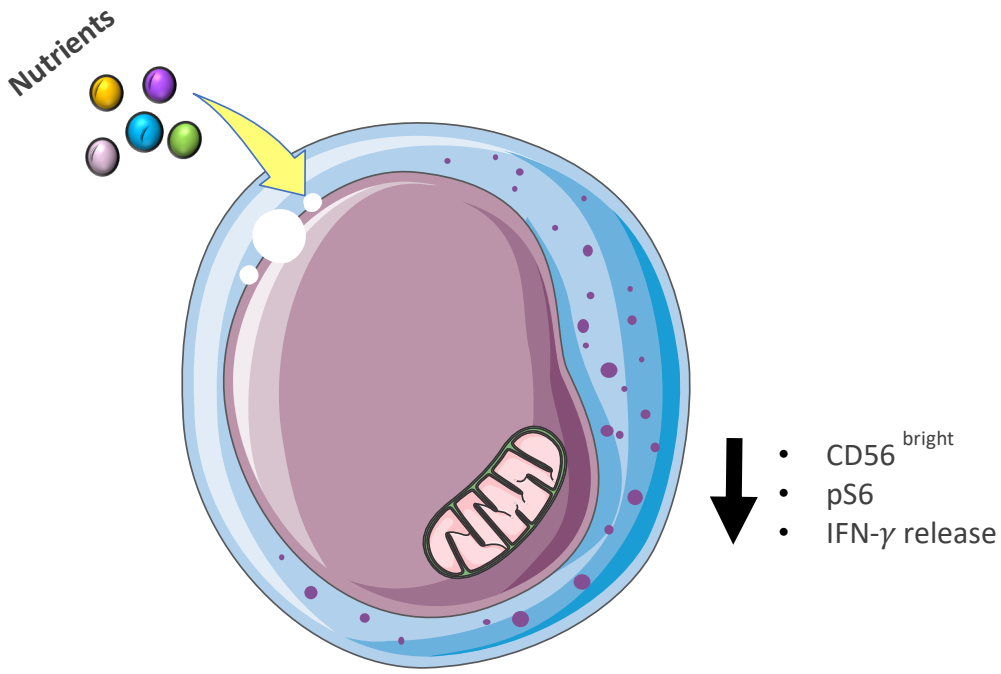

Figure 3. 14 Summary NK cells from patients with sepsis.

Patients with sepsis have significantly fewer $CD56^{bright}$, NK cells and higher frequencies of $CD56^{dim}$ NK cells. Their NK cells exhibit reduced mTORC1 activation but a normal level of cytotoxicity. Patients' NK cells displayed normal levels of nutrient receptors and normal nutrient uptake, suggesting a normal functional and metabolic profile. The production of IFN-γ by NK cells was impaired upon stimulation with IL-12 and IL-15.

Chapter 4 - Metabolic profile of Vδ2 T cells in patients with sepsis

4. 1 Introduction

Sepsis is one of the leading causes of death worldwide, with a mortality rate of up to 30%. The pathogenesis of sepsis can be described as a race toward death between a dysregulated systemic immune response and a microbial infection or trauma that leads to organ failure and death. The antimicrobial response is biphasic with an overwhelming inflammatory response preceding a period of profound immune suppression (Hotchkiss, 2014). Cells of both innate and adaptive immune systems may become impaired in their function as a result of immunosuppression in sepsis patients (de Pablo et al., 2014, Hotchkiss et al., 2013).

Innate lymphocytes are central to immunity against infection and may be altered in patients with sepsis. Innate lymphocytes include MHC-unrestricted T cells whose antigen receptors display specificity for conserved non-peptide antigens. They also include a number of non-T cells, such as NK cells and innate lymphoid cells. They are characterized by memory phenotypes in the absence of deliberate immunization (de Pablo et al., 2014, Bendelac et al., 2001) and the ability to respond rapidly, to kill infected and tumour cells and to release cytokines that activate, polarise and regulate adaptive immune responses (Monserrat et al., 2012; Rodriguez-Zapata et al., 1996).

γδ T cells express T cell receptor (TCR) γ and δ chains along with the CD3 molecule (Carding & Egan, 2002). The TCRs of γδ T cells recognise a variety of non-protein antigens, including glycolipids and pyrophosphates, and stress-inducible molecules expressed by virus-infected and tumour cells. The activation of γδ T cells can also occur

non-specifically via PAMPs, by superantigens (Hedges et al., 2005), and by ligation of a number of receptors that are typically found on NK cells, such as natural killer groups 2 receptors (NKG2C and NKG2D). Cytokine production and cytotoxic activity are key effector functions of γδ T cells (Lawand et al., 2017; Todaro et al., 2009; Wrobel et al., 2007).

In humans, γδ T cells have three main subsets, based on their Vδ chain expression Vδ1 T, Vδ2 T and Vδ3 T cells (Hayday, 2000). Vδ2 T cells are the most abundant subset of γδ T cells, representing 1-5% in healthy human peripheral blood (Caccamo et al., 2006; Vantourout & Hayday, 2013).

The TCR of Vδ2 T cells can recognize the expression of phosphoantigen intermediates in the methyl-erythritol phosphate biochemical (non-mevalonate) pathway, used by some bacteria, fungi and parasites. The most potent phosphoantigen identified is the microbial metabolite (E)-4-hydroxy-3-methyl-but-2-enyl (HMB-PP) which activates and expands Vδ2 T cells (Champagne, 2011; Gober et al., 2003). Once activated, Vδ2 T cells can kill infected and tumour cells and release pro-inflammatory cytokines such as IFN-γ, TNF-α, GM-CSF, IL-4, IL-10, and IL-17. Furthermore, they play a vital role in linking innate and adaptive immunity by promoting the differentiation of different subsets of antigen presenting cells (APCs) (Tyler et al., 2015). In septic patients, reductions in the frequencies of circulating γδ T cells but increased activation, including increased CD69 expression and increased pro-inflammatory and anti-inflammatory cytokine production (IFN-γ, IL-17, IL-10 and TGF- β) in vivo has been reported (Liao et al., 2017). However, in other studies, the expression of IFN-γ was impaired in γδ T cells. In a large study of sepsis

patients and controls, Andreu-Ballester *et al.* (2013 & 2018) found that the numbers of all subsets of lymphocytes are reduced in sepsis patients but the largest reduction was seen with γδ T cells, whose numbers inversely correlated with severity and mortality (Andreu-Ballester et al., 2013, 2018). It is likely that the numerical and functional changes to Vδ2 T cells result from the altered energy requirements that these cells have as a result of infection and inflammation. Manipulation of cellular metabolism may lead to the discovery of novel immunotherapies for sepsis by targeting specific cell types. In this chapter, the immunometabolic profile of the Vδ2 T cells and their functions in patients with sepsis and healthy control subjects, is investigated.

Hypothesis and Aims

We hypothesized that altered cellular metabolism in Vδ2 T cells, in patients with sepsis, may affect their functions and contribute to the clinical phenotypes observed. We proposed the following objectives:

1) To compare the frequencies and phenotypes of circulating Vδ2 T cells in healthy control subjects and sepsis patients.

2) To investigate if nutrient receptor expression by freshly-isolated and *ex vivo*-stimulated circulating Vδ2 T cells is altered in patients with sepsis.

3) To compare mitochondrial form and function in Vδ2 T cells from healthy controls and sepsis patients.

4) To investigate if metabolism of Vδ2 T cells is altered during sepsis, and to understand how this contributes to their function or dysfunction during disease.

4. 2 Results

4. 2. 1 Circulating Vδ2 T cells frequencies in peripheral blood from patients with sepsis are significantly lower than those in healthy controls

In order to investigate if Vδ2 T cells are altered in response to sepsis in our cohort of patients, we analysed the frequencies of circulating Vδ2 T cells by flow cytometric analysis of isolated PBMC (Fig. 4.1A). Using the $CD3^+$ Vδ2 TCR^+ lymphocyte phenotype to define Vδ2 T cells, there was a significant decrease in Vδ2 T cell frequencies in patients with sepsis compared to controls (Fig. 4.1B).

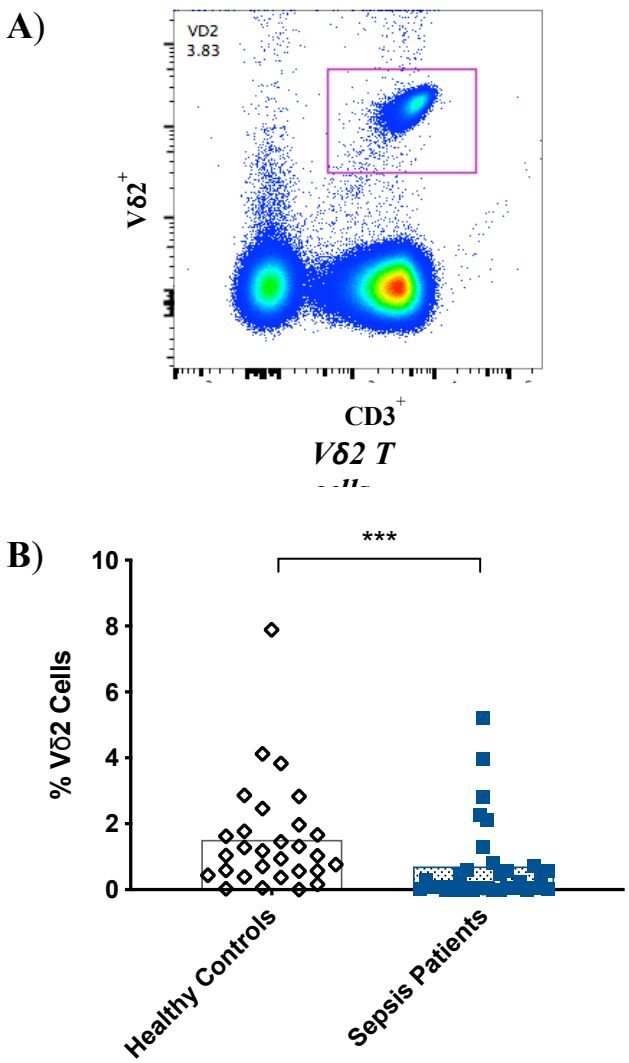

Figure 4. 1 The frequencies of circulating Vδ2 T cells in patients with sepsis are significantly lower than those in control subjects.

*PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3 and the Vδ2 TCR and the frequencies of Vδ2 T cells ($CD3^-$ Vδ2 TCR^+) were determined by flow cytometry. (A) Flow cytometry dot plot showing CD3 and the Vδ2 TCR expression by gated lymphocytes. (B) Scatter plot showing the frequencies of Vδ2 T cells, as percentages of total lymphocytes, in patients with sepsis (n = 32) and healthy control subjects (n = 29). Bars show means. Each dot represents 1 donor. Samples were compared using a non-parametric Mann-Whitney test. ***P <0.0001*

4. 2. 2 Vδ2 T cells from sepsis patients display lower frequencies of expression of the activation marker CD69 compared to control subjects but can upregulate CD69 upon stimulation *ex vivo*

In order to investigate the activation of Vδ2 T cells in vivo during sepsis, cell-surface expression of CD69 by Vδ2 T was measured in freshly-isolated PBMC and in PBMC after stimulation for 18 hours with IL-12 (30 ng/mL) and IL-15 (100 ng/ml) or HMB-PP (10 nM), using flow cytometry (Fig. 4.2A-B). CD69 expression on freshly isolated Vδ2 T cells was low as expected; however, it was significantly lower on patient compared to control cells (Fig. 4.2C). Vδ2 T cells from both patients with sepsis and healthy subjects upregulated CD69 expression in response to stimulation with IL-12 and IL-15 or HMB-PP. CD69 upregulation in response to cytokines occurred to the same level on Vδ2 T cells from patients and as healthy controls (Fig. 4.2D-E).

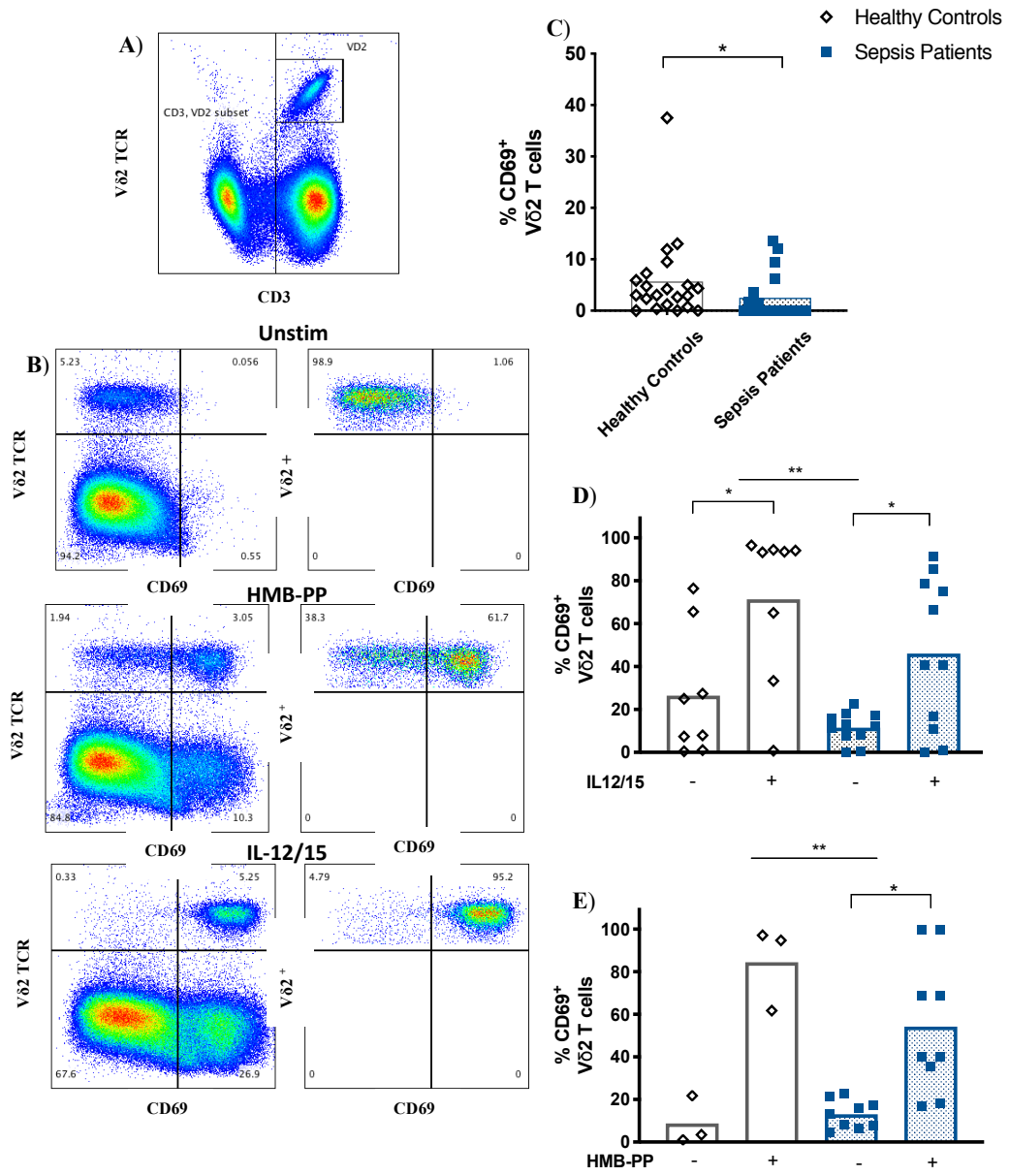

Figure 4. 2 Vδ2 T cells from sepsis patients display lower CD69 expression compared to Vδ2 T cells from healthy donors but upregulate CD69 normally in response to activation.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, Vδ2 TCR and CD69 immediately after isolation or after stimulation with medium alone or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) or HMB-PP (10 nM) for 18 hours at 37°C. The expression of CD69 by Vδ2 T cells was then analysed by flow cytometry. (A) Representative flow cytometry dot plot showing CD3 and Vδ2 TCR expression by PBMC. (B) Representative flow cytometry dot plots showing CD69 upregulation by gated Vδ2 T cells from a healthy control subject in response to stimulation. The left panels shows the expression of CD69 and *Vδ2 TCR* by total PBMC, while the right panels show the expression of CD69 by gated *Vδ2 T cells*. *The top panels show CD69*

*expression by unstimulated PBMC and Vδ2 T cells, whereas the centre and lower panels show CD69 expression by HMB-PP-stimulated and by IL-12 and IL-15-stimulated PBMC and Vδ2 T cells, respectively. (C) Frequencies of CD69-expressing Vδ2 T cells from patients with sepsis (n = 19) and healthy control subjects (n = 21). (D) Frequencies of CD69-expressing Vδ2 T cells from patients with sepsis (n = 11) and healthy control subjects (n = 8) in response to treatment with medium or IL-12 + IL-15. (E) Frequencies of CD69-expressing Vδ2 T cells from patients with sepsis (n = 9) and healthy control subjects (n = 3) in response to treatment with medium or HMB-PP. Bars show means. Each dot represents 1 donor. Samples were compared using either a non-parametric Mann-Whitney test analysis (C) or a one-way ANOVA followed by a Kruskal–Wallis test (D, E). *P < 0.05, **P <0.001.*

4. 2. 3 Effector functions of Vδ2 T cells from sepsis patients and healthy donors

The above data show that Vδ2 T cells from patients with sepsis upregulated CD69 normally upon stimulation, suggesting that they can be activated to a similar level. In order to investigate this further, expression of markers of cytotoxicity by Vδ2 T cells were assessed by flow cytometry (Fig. 4.3A). Expression of granzyme B (GnzB) by Vδ2 T cells, was analyzed using freshly isolated PBMC and PBMC stimulated for 18 hours with medium alone, HMB-PP or IL-12 and IL-15. Freshly isolated Vδ2 T cells from patients had similar GnzB expression compared to controls (Fig. 4.3B). Neither, cytokine nor HMB-PP stimulation upregulated GnzB expression in either sepsis patients or controls (Fig. 4.3C-D).

We also investigated if Vδ2 T cells from sepsis patients had an impaired ability to produce IFN-γ. The production of IFN-γ associated with Vδ2 T cells in response stimulation was assessed by flow cytometry. Vδ2 T cells from sepsis patients had reduced IFN-γ production upon IL-12/15 or HMB-PP stimulation compared to controls (Fig. 4.4).

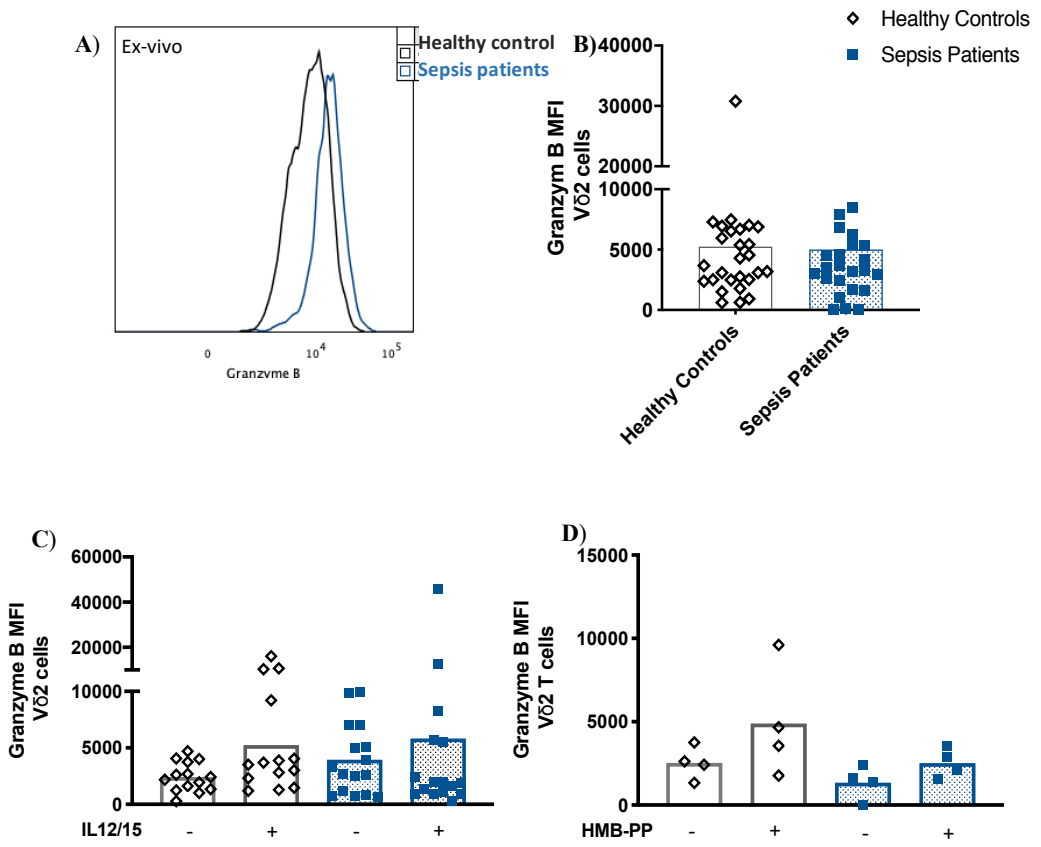

Figure 4. 3 Expression of GnzB by Vδ2 T cells from sepsis patients and control subjects.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for cell surface CD3, Vδ2 TCR and intracellular granzyme B immediately after isolation or after stimulation with medium alone, HMB-PP (10 nM) or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. The expression of GnzB by Vδ2 T cells was then analysed by flow cytometry. (A) Representative flow cytometry histogram showing the expression of GnzB by Vδ2 T cells from a patient with sepsis and a healthy control subject. (B) Mean fluorescence intensity (MFI) of GnzB staining of freshly isolated Vδ2 T cells from patients with sepsis (n = 27) and healthy control subjects (n = 28). (C) MFI of GnzB expression by Vδ2 T cells from patients with sepsis (n = 16) and healthy control subjects T (n = 14) in response to treatment with medium or IL-12 + IL-15. (D) GnzB MFI of Vδ2 T cells from patients with sepsis (n = 4) and healthy control Vδ2 T (n = 4) in response to treatment with medium or HMB-PP. Bars show means. Each dot represents 1 donor. Samples were compared using either a non-parametric Mann-Whitney test analysis (B) or a one-way ANOVA followed by a Kruskal–Wallis test (C, D).

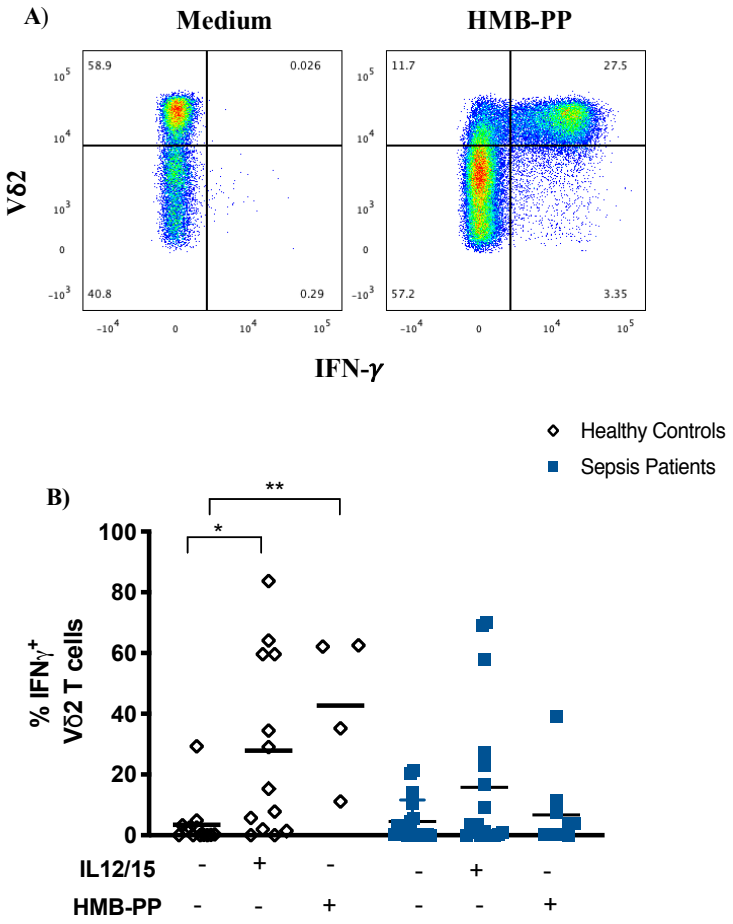

Figure 4. 4 Vδ2 T cells from patients with sepsis have impaired production of IFN-γ in response to cytokine and HMB-PP stimulation.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for cell-surface CD3, Vδ2 TCR and intracellular IFN-γ immediately after isolation or after stimulation with medium alone, HMB-PP (10 nM) or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. (A) Representative flow cytometry dot plots showing IFN-γ production by Vδ2 T cells from a healthy control subject in response to stimulation. The left panel shows unstimulated cells, while the centre and right panels show HMB-PP-stimulated cells, respectively. *(B)The production of IFN-γ by Vδ2 T cells was then analysed by intracellular flow cytometry Frequencies of IFN-γ -expressing Vδ2 T cells from patients with sepsis (n = 18-10) and healthy control subjects (n = 13, 4) in response to treatment with medium or IL-12 + IL-15 and HMB-PP. Bars show means. Each dot represents 1 donor. Samples were compared using a one-way ANOVA followed by a Kruskal–Wallis test (B).* *P < 0.05, **P <0.001.

4. 2. 4 Vδ2 T cells have a normal mTORC 1 activity in response to sepsis

Mammalian target of rapamycin complex 1 (mTORC1) is an important metabolic regulator. It is a nutrient-sensitive pathway that boosts anabolic cellular metabolism (lipid and nucleotide synthesis), which occurs during cell activation. mTORC1, activity in Vδ2 T cells from healthy donors and patients with sepsis was analysed by measuring phosphorylation of S6 ribosomal protein (pS6) by flow cytometry. Freshly isolated Vδ2 T cells from patients with sepsis had similar levels of pS6 compared to Vδ2 T cells from healthy donors (Fig. 4.5).

This data suggest that mTORC1 activity in Vδ2 T cells is normal in patients with sepsis.

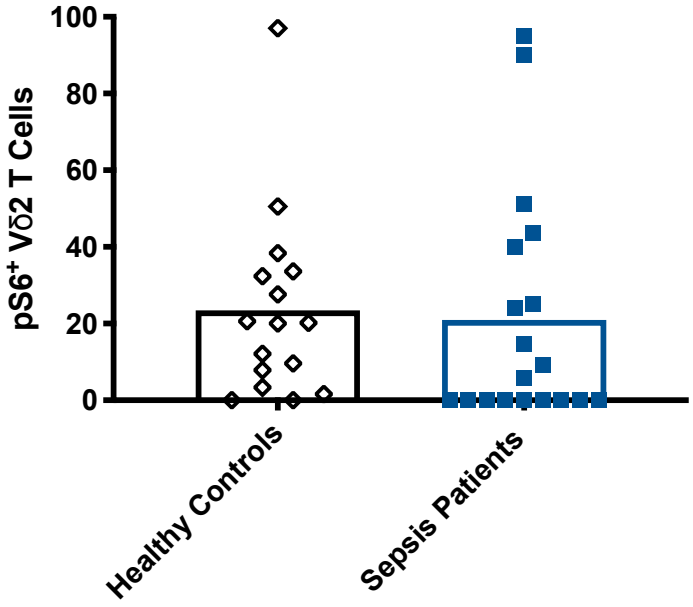

Figure 4. 5 Vδ2 T cells from patients with sepsis have a normal mTORC1 activity.

*PBMC from patients with sepsis and healthy control subjects were stained using mAbs specific for CD3, Vδ2 TCR and phosphorylated ribosomal protein pS6 to determine mTORC1 activity of Vδ2 T cells by flow cytometry. Graph shows frequencies of pS6-expressing Vδ2 T cells from patients with sepsis (n = 19) and healthy control subjects (n = 16). Bars show means. Each dot represents 1 donor. Samples were compared using a non-parametric Mann-Whitney test analysis. *P < 0.05.*

4. 2. 5 Altered nutrient receptor expression by Vδ2 T cells from patients with sepsis

Nutrient uptake is an important first step in driving energy metabolism required for cell activation. To investigate if Vδ2 T cells from patients with sepsis are primed to upregulate their metabolism, expression of nutrient receptors were measured.

The expression of CD71, the transferrin receptor for iron uptake, by freshly-isolated and stimulated Vδ2 T cells was assessed by flow cytometry (Fig. 4.6A). CD71, was expressed at similar levels on Vδ2 T cells freshly isolated from patients with sepsis and controls (Fig. 4.6B). Stimulation of the PBMC with IL-12 and IL-15 resulted in an upregulation of CD71 expression by Vδ2 T cells from both sepsis patients and control subjects, with no differences observed the two groups (Fig. 4.6C). Upon stimulation with HMB-PP, a non-significant increase in CD71 expression by Vδ2 T cells from both healthy donors and sepsis patients was observed (Fig. 4.6D). Again, there was no difference in the expression of CD71 by Vδ2 T cells from healthy donors and sepsis patients.

The expression of CD98, a component of the L-amino acid transporter, by Vδ2 T cells from sepsis patients and healthy donors was also measured by flow cytometry (Fig. 4.7A). CD98 was expressed at significantly higher levels on Vδ2 T cells in PBMC freshly isolated from sepsis patients compared to controls (Fig. 4.7B). Upon stimulation with IL-12/15 or HMB-PP, CD98 expression by Vδ2 T cells from healthy donors or sepsis patients did not increase significantly and no differences were observed in the expression of this

marker on Vδ2 T cells from the 2 subject groups (Fig. 4. 7B-D).

Overall, these data show that Vδ2 T cells from patients with sepsis upregulated their nutrient receptors normally compared to Vδ2 T cells from healthy controls.

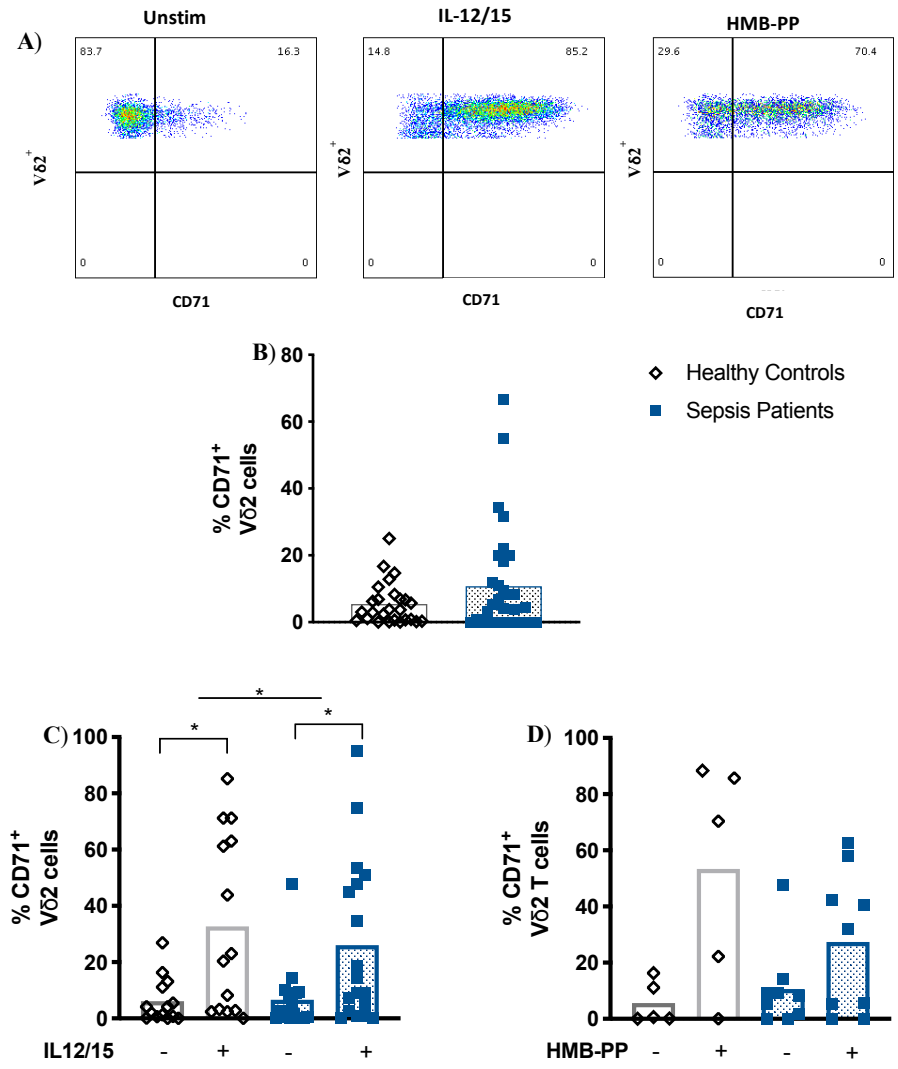

Figure 4. 6 Vδ2 T cells from patients with sepsis and healthy donors display similar CD71 expression and upregulation in response to activation.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, Vδ2 TCR and CD71 immediately after isolation or after stimulation with medium alone, HMB-PP (10 nM) or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. (A) Representative flow cytometry dot plots showing CD71 expression by Vδ2 T cells from a healthy control subject in response to stimulation. (B) Frequencies of CD71-expressing Vδ2 T cells in freshly-isolated PBMC from patients with sepsis (n = 32) and healthy control subjects (n = 26). (C) Frequencies of CD71-expressing Vδ2 T cells from patients with sepsis (n = 18) and healthy control subjects (n = 14) in response to treatment with medium or IL-12 + IL-15. (D) Frequencies of CD71-expressing Vδ2 T cells from patients with sepsis (n = 9) and healthy control subjects (n = 5) in response to treatment with medium or IL-12 + IL-15. Bars show means. Each dot

represents 1 donor. Samples were compared using either a non-parametric Mann-Whitney test analysis (B) or a one-way ANOVA followed by a Kruskal–Wallis test (C, D). *P < 0.05.

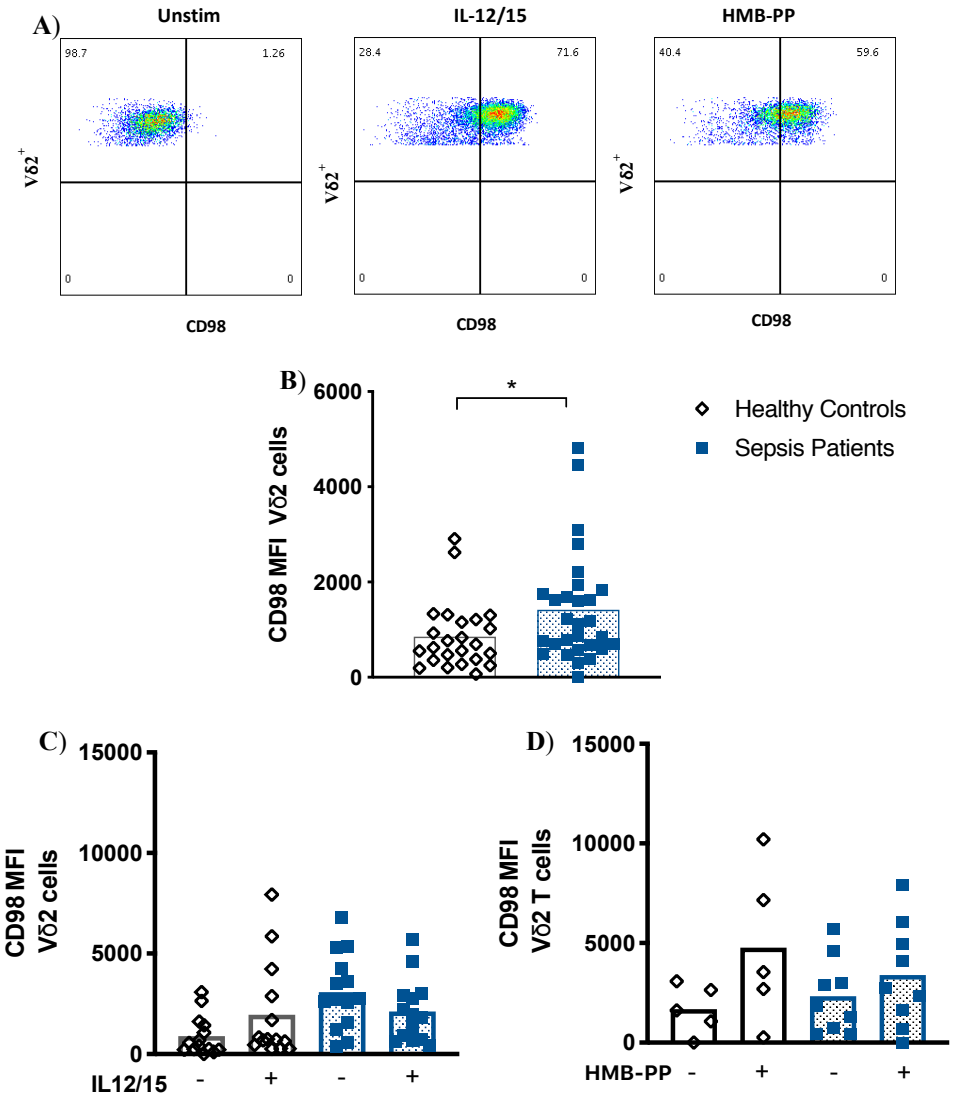

Figure 4. 7 Vδ2 T cells from patients with sepsis express higher levels of CD98 compared to Vδ2 T cells from healthy donors.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, Vδ2 TCR and CD98 immediately after isolation or after stimulation with medium alone, HMB-PP (10 nM) or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. The expression of CD98 by Vδ2 T cells was then analysed by flow cytometry. (A) Representative flow cytometry dot plots showing CD98 expression by Vδ2 T cells from a healthy control subject after stimulation with medium alone (left panel), HMB-PP (centre) and IL-12 + IL-15 (right). (B) Mean fluorescence intensity (MFI) of CD98 staining of freshly isolated Vδ2 T cells from patients with sepsis (n =29) and healthy control subjects (n = 24). (C) MFI of CD98 expression by Vδ2 T cells from patients with sepsis (n = 14) and healthy control (n = 14) in response to treatment with medium or IL-12 + IL-15. (D) MFI of CD98 expression by Vδ2 T cells from patients with sepsis (n = 9) and healthy control (n = 5) in response to treatment with medium or HMB-PP. Bars show means. Each dot

represents 1 donor. *Samples were compared using either a non-parametric Mann-Whitney test analysis (B) or a one-way ANOVA followed by a Kruskal–Wallis test (C, D).* *P < 0.05.

4. 2. 6 The mitochondrial structure and function of Vδ2 T cells from sepsis patients are similar to those in healthy controls

In cellular metabolism, the mitochondria have an essential role to play in energy (ATP) production and provision of the biological elements for protein, lipids and nucleic acid synthesis (Gardiner, 2019). Mitochondrial structure is controlled by the metabolic requirements of the cell and mitochondria can be converted from fissed (ovoid) structures, found in effector T cells which rely on glycolysis as an energy source, to fused (elongated tubular networks) mitochondria found in memory cells, which rely on OxPhos (Buck et al., 2016; Pagliuso et al., 2018). Therefore, mitochondrial structure and functions were analysed in freshly isolated Vδ2 T cells from healthy control subjects and sepsis patients in order to examine possible effects of sepsis on Vδ2 T cells metabolism.

4. 2. 6. 1 Vδ2 T cells from sepsis patients have normal mitochondrial mass

As mitochondrial mass can indicate a cell's mitochondrial health, and give insight into the pathways of cell mitophagy and mitochondrial biogenesis, the mitochondrial mass of Vδ2 T cells from healthy controls and patients with sepsis was measured directly ex-vivo and after 18 hours stimulation with IL-12/15 or HMB-PP by staining the cells with Mitotracker Green FM. Mitochondrial mass of freshly isolated Vδ2 T cells from both healthy controls and sepsis patients was similar (Fig.4.8 A-B). After stimulation of PBMC with IL-12 and IL-15 or HMB-PP in vitro, the mitochondrial mass in Vδ2 T cells from both sepsis patients and control subjects did not change (Fig. 4.8C-D).

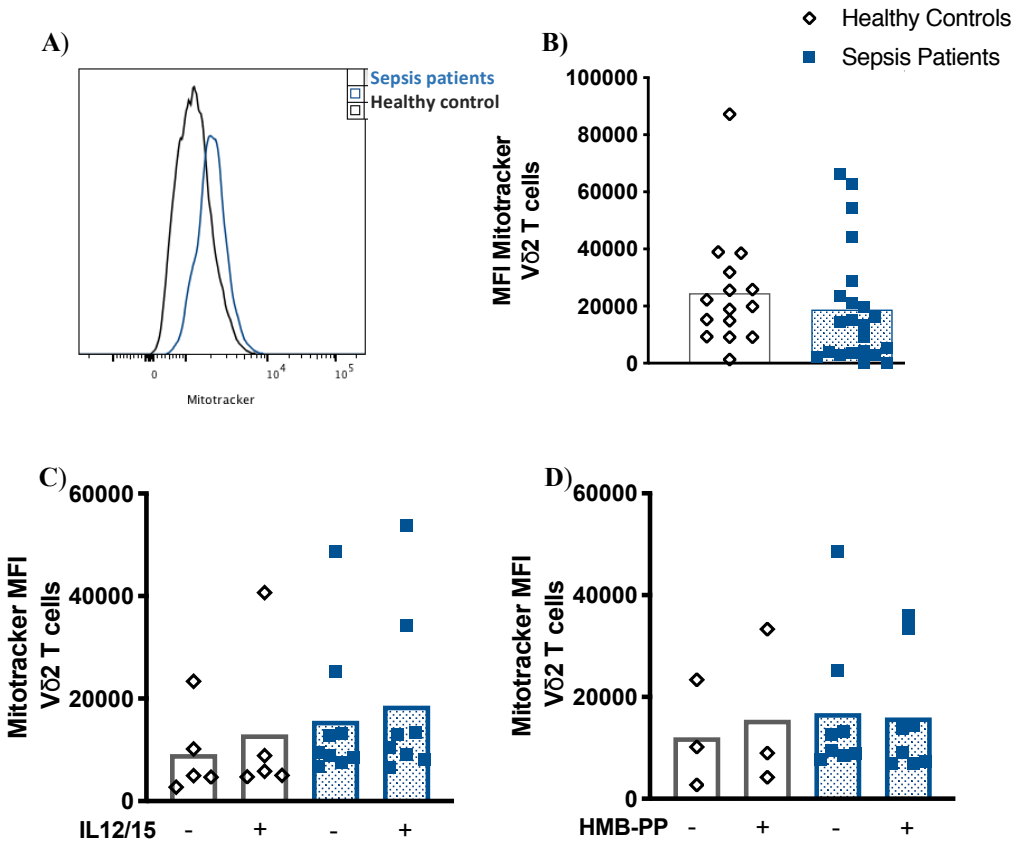

Figure 4. 8 Vδ2 T cells from patients with sepsis and healthy donors display similar mitochondrial mass.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, Vδ2 TCR and Mitotracker Green FM (100 nM) immediately after isolation or after stimulation with medium alone, HMB-PP (10 nM) or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. Cells were analyzed by flow cytometry. (A) Representative histogram showing the expression of Mitotracker Green FM by Vδ2 T cells freshly isolated from a patient with sepsis and a healthy control subject. (B) Mean fluorescence intensity (MFI) of mitochondrial mass (Mitotracker) staining of freshly isolated Vδ2 T cells from patients with sepsis (n = 22) and healthy control subjects (n = 17). (C) Mitochondrial mass MFI of Vδ2 T cells from patients with sepsis (n = 9) and healthy controls (n = 5) in response to treatment with medium or IL-12 + IL-15. (D) Mitochondrial mass MFI of Vδ2 T cells from patients with sepsis (n = 8) and healthy controls (n = 3) in response to treatment with medium or HMB-PP. Bars show means. Each dot represents 1 donor. Samples were compared using either a non-parametric Mann-Whitney test analysis (B) or a one-way ANOVA followed by a Kruskal–Wallis test (C, D).

4. 2. 6. 2 Vδ2 T cells from sepsis patients have normal levels of ATP synthase

ATP synthase is an enzyme that produces cellular ATP by utilizing the electrochemical gradient of the mitochondrial membrane. ATP5B, the key catalytic subunit of ATP synthase was measured directly ex vivo and after 18 hours stimulation with IL-12/15 using flow cytometry. ATP5B expression in freshly isolated Vδ2 T cells from healthy donors and patients with sepsis were similar (Fig. 4.9A). Stimulation of PBMC with IL-12 and IL-15 or HMB-PP in vitro did not result in any changes in ATP synthase levels in Vδ2 T cells from healthy donors and patients with sepsis (Fig. 4.9B -C).

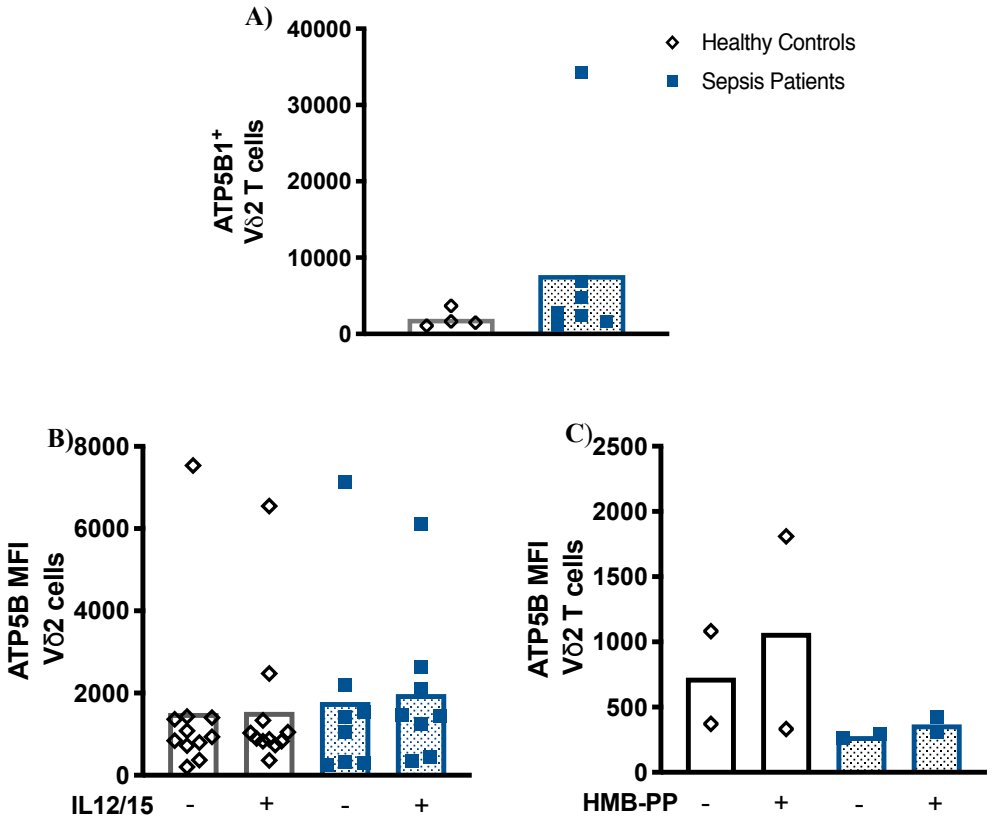

Figure 4. 9 Vδ2 T cells from patients with sepsis express normal levels of ATP synthase.

PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, Vδ2 TCR and ATP5B immediately after isolation or after stimulation with medium alone, HMB-PP (10 nM) or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) for 18 hours at 37°C. Cells were analyzed by flow cytometry. (A) Mean fluorescence intensity (MFI) of ATP5B staining of freshly isolated Vδ2 T cells from patients with sepsis (n = 7) and healthy control subjects (n = 4). (B) ATP5B MFI of Vδ2 T cells from patients with sepsis (n = 8) and healthy controls (n = 11) in response to treatment with medium or IL-12 + IL-15. (C) ATP5B MFI of Vδ2 T cells from patients with sepsis (n = 2) and healthy controls (n = 2) in response to treatment with medium or HMB-PP. Bars show means. Each dot represents 1 donor. Samples were compared using either a non-parametric Mann-Whitney test analysis (A) or a one-way ANOVA followed by a Kruskal–Wallis test (B, C).

4. 2. 6. 3 Vδ2 T cells from sepsis patients have similar mROS levels to those in healthy controls

Mitochondrial dysfunction is commonly linked with high levels mitochondrial ROS (mROS). mROS can react with protein, lipids, and DNA in the mitochondria, and can influence signalling pathways. Mitochondrial superoxide levels were measured directly ex-vivo by MitoSOX staining using flow cytometry (Fig. 4.10A). Freshly isolated Vδ2 T cells from patients and controls had similar levels of mROS (Fig. 4.10B).

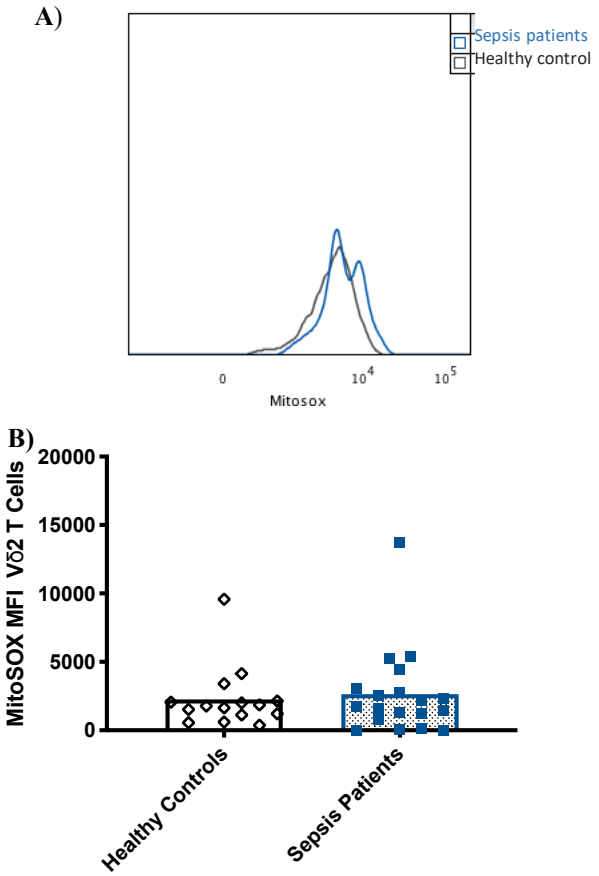

Figure 4. 10 Vδ2 T cells from patients with sepsis and healthy controls have similar levels of mROS.

Isolated PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, Vδ2 TCR and MitoSOX Red dye (1.5 µM) for 15 min at 37°C. Cells were analysed by flow cytometry. (A) Representative histogram showing the expression of MitoSOX by freshly isolated Vδ2 T cells from a patient with sepsis and a healthy control subject. (B) Mean fluorescence intensity (MFI) of MitoSOX staining of freshly isolated Vδ2 T cells from patients with sepsis (n = 19) and healthy control subjects (n = 16). Bars show means. Each dot represents 1 donor. Samples were compared using *non-parametric Mann-Whitney test analysis*.

4. 2. 6. 4 Vδ2 T cells from sepsis patients and healthy controls produce similar levels of cellular ROS

High levels of ROS in the cytosol (cROS) may affect and damage the cells and their signalling pathways. The levels of cROS in freshly isolated Vδ2 T cells from healthy controls and sepsis patients were investigated by staining PBMC with CellROX and analysis by flow cytometry (Fig. 4.11A).

Similar to what was observed for mROS, Vδ2 T cells in freshly-isolated PBMC from patients with sepsis had similar levels of cROS compared to Vδ2 T cells from healthy control subjects (Fig. 4.11B). This shows no evidence of mitochondrial dysfunction in Vδ2 T cells from patients with sepsis.

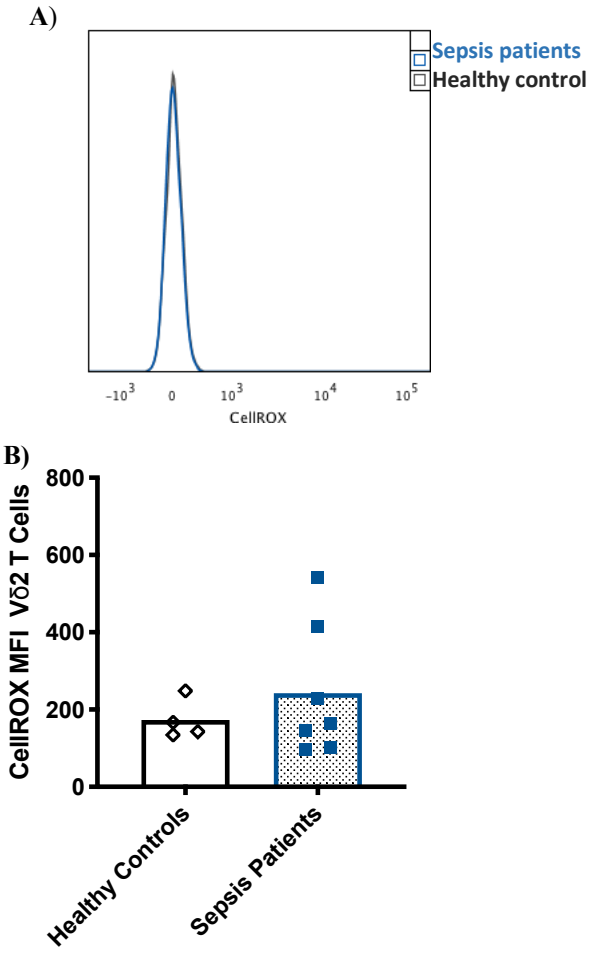

Figure 4. 11 Vδ2 T cells from patients with sepsis express normal levels of CellROX.

Isolated PBMC from patients with sepsis and healthy control subjects were stained with mAbs specific for CD3, Vδ2 TCR and CellROX (1 µm) for 30 min at 37°C. Cells were analysed by flow cytometry. (A) Representative histogram showing the expression of CellROX by freshly isolated Vδ2 T cells from a patient with sepsis and a healthy control subject. (B) Mean fluorescence intensity (MFI) of CellROX staining Vδ2 T cells within freshly isolated PBMC from patients with sepsis (n = 7) and healthy control subjects (n = 4). Bars show means. Each dot represents 1 donor. Samples were compared using *non-parametric Mann-Whitney test*.

4. 2. 6. 5 Relationship of severity scores of SOFA and APACHE II and immunological functions in Vδ2 T cells from sepsis

To examine the relationship between severity and cytotoxic function of Vδ2 T cells. First, we analysed the correlation between mTORC1 activity and the production IFN-γ and we showed that there is no linear relationship between the the levels of pS6 in Vδ2 T cells from patients with sepsis and the production of IFN-γ (r = 0.1304, P < 0.6149; Fig. 4.12A). The levels of severity scores of both SOFA and APACH II also showed a statistically negative correlation with IFN-γ production (SOFA r = 0.1076, P <0.6890, APACH II r = 0.1329, P < 0.6201; Fig. 4.12B) and granzyme B activity (SOFA: r = - - 0.1802, P < 0.3994, APACHE II: r = -0.3067, P < 0.1450; Fig. 4.12C).

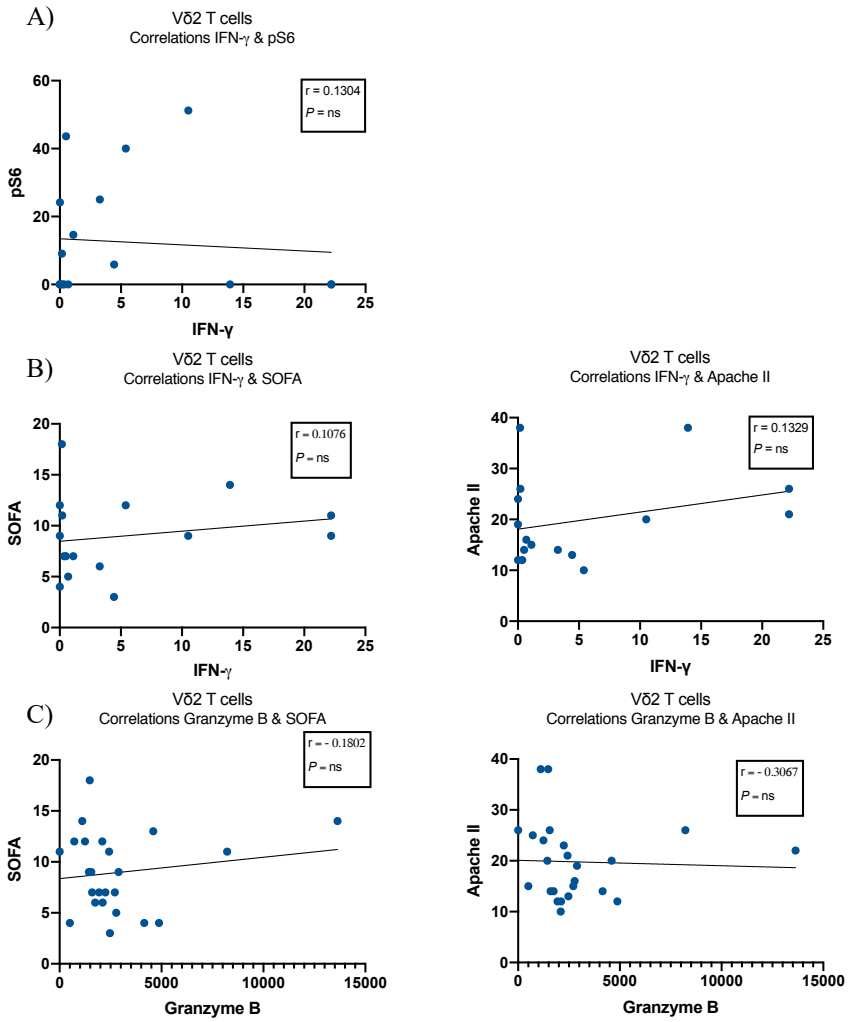

Figure 4. 12 Correlation of severity scores of SOFA and APACHE II and immunological functions in Vδ2 T cells cell from sepsis.

(A) Correlation of mTORC1 activity (pS6) with IFN-γ production r = *0.1304,* P < *0.6149). (B) correlation of IFN-γ production with the levels of severity scores of both SOFA and APACH II (SOFA* r = *0.1076,* P <*0.6890, APACH II* r = *0.1329,* P < *0.6201).(C) The correlation between granzyme B cellular activity with the levels of severity scores of both SOFA and APACH II (SOFA:* r = - -*0.1802,* P < *0.3994, APACHE II:* r = -*0.3067,* P < *0.1450). The correlation between variables was analyzed by nonparametric spearman correlation test.*

4. 3 Discussion

Many studies have reported reductions in γδ T cells in sepsis patients which are associated with high mortality (Andreu-Ballester et al., 2013; Heffernan et al., 2014; Venet et al., 2005). This is of importance because γδ T cells are considered to be a first line of protection against microbial infection in mucosa and inducers of αβ T cells activation and development. Galley *et al.* (2015) reported a reduction in proliferative responses by γδ T cells in *in vitro* culture for 7 days in response to IL-2 and zoledronate stimulation in septic patients in compared to healthy controls, indicating a defect in γδ T cells responses in septic patients (Galley et al., 2015). However, a previous study found a lower circulating number of γδ T cells, but higher expression of CD69, in patients with sepsis compared to healthy controls, implying an early activation of γδ T cells in patients with sepsis (Galley et al., 2015; Matsushima et al., 2004). These results suggest that γδ T cells play a significant role in the pathology of sepsis.

As Vδ2 T cells are the most abundant subset of γδ T cells in human peripheral blood and they play important early roles in immunity against bacteria, we investigated if the frequencies of circulating Vδ2 T cells are altered in patients with sepsis. Davey *et al.* (2018) did an extensive study to find out what niches these cells occupy and what functions they manifest. In both the adults and newly born, there was a similar repertoire of T cell diversity without any specific clonal focussing across development. They also show that $V\delta2^+$ subsets with and without $V\gamma9^+$ are different from each other. The subset without Vγ9 did not respond to the phosphoantigens, unlike the other subset of cells. $V\gamma9^-V\delta2^+$ also did not express levels of $V\delta2^+$ as high as the ones bearing Vγ9, and

also had elevated levels of CD27. They responded to stimulation of CD3 and CD28, but not to IL12/IL18. The $V\gamma9^{+}V\delta2^{+}$, however, responded to both (Davey et al., 2017). Thus, $V\gamma9^{-}V\delta2^{+}$ set of cells is more similar to Vδ1+ subset, in terms of phenotype and receptor expression. We found that the frequencies of circulating Vδ2 T cells are lower in patients with sepsis compared with healthy donors. This finding is in agreement with a previous study by Toro *et al.* (2013) who reported a significant decrease in the frequencies of Vδ2 T cells and increase in Vδ1 T cells in patients with sepsis (Toro et al., 2013). Similar findings have been reported in studies of patients with HIV, which also have expansions of Vδ1 T cells and depletions of Vδ2 T cells. This finding was even more pronounced in patients with HIV who were co-infected with opportunistic infection (Maher et al., 2015; Hui Wang et al., 2007). The increase of Vδ1 T cells could be related to recirculation of those cells from mucosal tissues (Poles et al., 2003), while the reduction of Vδ2 T cells should be related to apoptosis susceptibility via sustained activation (Dieli et al., 2003; Poggi et al., 2004).

Vδ2 T cells recognise the pyrophosphate metabolite HMB-PP produced in the non-mevalonate pathway of isoprenoid synthesis (Morita et al., 2007), found in many pathogenic bacteria. Upon activation they kill bacteria, release cytokines, and promote antigen presentation to T cells of the adaptive immune system. They also activate and regulate other anti-bacterial cells of the immune system, such as neutrophils and macrophages (Hintz et al., 2001; Morita et al., 2007). Our data show that the expression of CD69, an early activation marker, was significantly lower on Vδ2 T cells from patients with sepsis, suggesting an impaired ability to defend against bacterial infection. In

contrast, previous studies indicated that patients with sepsis have a higher basal level of CD69 on γδ T cells in compared to healthy controls (Liao et al., 2017; Matsushima et al., 2004). However, CD69 expression on Vδ2 T cells in response to stimulation has not yet been evaluated and whether or not changes in CD69 expression after stimulation are associated with patient prognosis remains unknown. Therefore, we measured and analysed CD69 expression in response to IL-12/15 and HMB-PP stimulation. Our result showed that Vδ2 T cells from healthy controls and patients increased the expression of CD69 upon stimulation. However, Liao *et al.* (2017) demonstrated an impaired of CD69 expression on γδ T cells from sepsis patients, suggesting that these cells from patients are refractory to activation, possibly due to chronic stimulation (Liao et al., 2017). Vδ2 T cells are considered to be part of the first line to defence against microbial infection and their depletion in sepsis patients, may underlie the failure to clear infections. It has been suggested that the reductions of circulating γδ T cells, in particular Vδ2 T cells, in critically ill sepsis patients could be related to increased apoptosis due to chronic activation and should be used as an indicator of immunosuppression (Chung et al., 2006; Correia et al., 2016; Poggi et al., 2004; Venet et al., 2005).

The present study showed that there was no difference in the expression of GnzB on Vδ2 T cells freshly isolated from patients with sepsis and controls. We also found that stimulation of Vδ2 T cells from sepsis patients and control subjects with IL-12 and IL-15 or HMB-PP stimulation failed to upregulate GnzB expression. Therefore, the cytotoxic activities of Vδ2 T cells do not seem to be different in patients with sepsis and control subjects.

Compared to Vδ2 T cells from healthy donors, we demonstrated that the production of IFN-γ by stimulated Vδ2 T from patients was impaired. Our result was consistent with previous studies that reported a decrease of IFN-γ gene expression in PBMC of severe sepsis patients (Grealy et al., 2013; White, Mahon, et al., 2011; White, Martin-Loeches, et al., 2011). Liao *et al.* (2017) also reported that upon stimulation, IFN-γ expression by γδ T cells was significantly lower in septic patients compered to healthy controls (Liao et al., 2017). This finding in contrasts with that of another study that found that γδ T cells from patients with sepsis produced similar amounts of IFN-γ to controls (Galley et al., 2015). Future studies are required to confirm if defective IFN-γ by stimulated γδ T cells can contribute to immunosuppression during sepsis.

Lymphocytes undergo metabolic changes to cope with changing environments, such as changes in glucose and oxygen availability (Loftus & Finlay, 2016; O'Neill et al., 2016; Pearce et al., 2013). Metabolic changes are also required to shape the immune effector function and differentiation of a cell (O'Neill et al., 2016; Pearce et al., 2013). Different cell types with different functions utilise different metabolic pathways to satisfy their energy requirements and to provide essential biomolecules (Kedia-Mehta & Finlay, 2019). We investigated if the metabolic regulator 1 (mTORC1) and nutrient receptors are altered in patients with sepsis. Metabolic analysis showed that freshly isolated Vδ2 T cells from sepsis patients had similar levels of mTORC1 activity and expression of CD71, but higher levels of CD98 significantly in comparison to heathy controls. Interestingly, after stimulating PBMC for 18 hours, with IL-12 and IL-15, Vδ2 T cells from

healthy controls upregulated CD71, but not CD98, to a greater degree than Vδ2 T cells from sepsis patients. In contrast, HMB-PP failed to significantly upregulate CD71 and CD98 expression by Vδ2 T cells from both healthy controls and patients with sepsis. These data suggest that Vδ2 T cells from patients with sepsis may have higher activity of the amino acid transporter LAT1 (heterodimer of CD98 and Slc7a5) which import of amino acids, particularly leucine, via LAT1 turns on mTORC1.

Mitochondria are the engine of aerobic metabolism and the place where the cells generate most of their ATP under resting conditions. We investigated if mitochondria from patients with sepsis are altered in patients with sepsis. Upon examination of these vital organelles, we found that Vδ2 T cells from sepsis patients had similar mitochondrial mass, and levels of mitochondrial ROS to those of healthy donors, suggesting a lack of mitochondrial stress. Cellular ROS and ATP synthase staining was also comparable in Vδ2 T cells from sepsis patients compared to healthy control subjects suggesting that these cells have normally functioning mitochondria. IL-12/15 and HMB-PP stimulation did not increase the mitochondrial mass and ATP syntheses levels in Vδ2 T cells. Therefore, it seems that the mitochondria of Vδ2 T from patients with sepsis are carrying out their functions and metabolizing normally. Interestingly, Venet *et al*. (2017) reported a large reduction in both basal level and stimulated OxPhos, glycolysis, ATP levels and mTORC1 activity in T lymphocytes from septic patients, while subsequent stimulation failed to upregulate these parameters, implying the T cells from sepsis patients are metabolically dysfunctional (Venet et al., 2017). Treatment with IL-7 of dysfunctional T cells upregulated both glucose metabolism and mTORC activity in sepsis

patients (Venet et al., 2017). This highlights the important role of immunometabolism in understanding and preventing sepsis pathology and immune defects which can lead to developing novel immunotherapy.

In conclusion, our data suggest that patients with sepsis display a similar level of iron uptake, similar mitochondrial mass, and levels of mitochondrial ROS, cellular ROS and ATP synthase, suggesting that these cells from sepsis patients have similar mitochondrial metabolic profiles to those from healthy controls. No mitochondrial dysfunction was observed in Vδ2 T cells from patients with sepsis. However, we found that a significant decrease in Vδ2 T cell frequencies, higher level of L-amino acid uptake, impaired IFN-γ production and failure to upregulated CD98 in response to HMB-PP and cytokine stimulation. These properties may predispose patients with bacterial infections to developing sepsis or to mortality from sepsis (Figure 4.12).

Overall, many factors need to be taken into consideration in order to get a better understanding of Vδ2 T cells function and metabolic profile in patients with sepsis. These may include the numbers of patients and criteria such as age, gender and type of infection, for instance, Gram-Positive and Gram-negative bacteria or fungi, and the sample types, such as blood, tissue or bronchoalveolar lavage. The influence of other innate and adaptive immune cells, and the stage and duration of the disease are also likely to influence the metabolic activities of Vδ2 T cells.

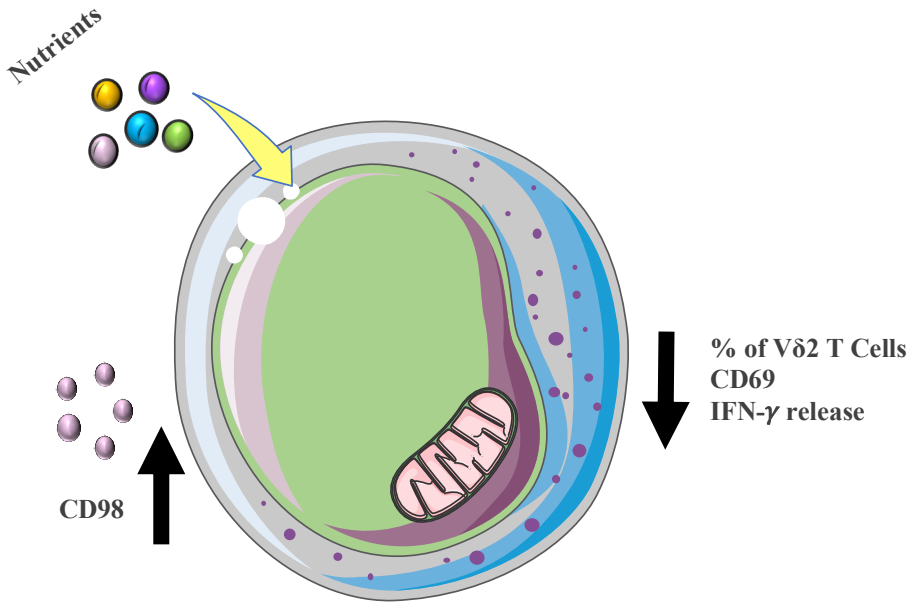

Figure 4. 13 Summary Vδ2 T cells from patients with sepsis.

Freshly isolated Vδ2 T cells are found at lower frequencies in patients with sepsis compared to healthy donors. They display significantly lower expression of the activation marker CD69 and the cytotoxic molecule granzyme B and higher levels of CD98, L-amino acid uptake, and a normal level of CD71 iron uptake displaying a normal mitochondrial metabolic profile. The production of IFN-γ by Vδ2 T cells was impaired upon stimulation with HMB-PP or IL-12 and IL-15.

Chapter 5- Investigation of the metabolic requirements of Vδ2 T cells during proliferation and effector function

5. 1 Introduction

Human γδ T cells represent a minor population T cells in peripheral blood and a larger population in epithelial tissues. They play a critical role in recognizing pathogens and tumours and rapidly respond by killing bacteria and infected and transformed host cells. They also promote the differentiation and activation of other immune cells. Humans have three dominant subsets of γδ T cells - Vδ1, Vγ9Vδ2 and Vδ3 T cells. The TCRs of these cells can recognize a variety of metabolites and components of pathogens and stress-inducible ligands on host cells in an major histocompatibility complex (MHC)-unrestricted manner (Hayday, 2000).

Vγ9Vδ2 T cells (Vδ2 T cells) are the principle γδ T cell subset in blood accounting for 1-5% of T cells in healthy individuals. However, they expand dramatically in microbial infections and reach upward of > 50% of all T cells in sites of infection (Chien et al., 2014; Hara et al., 1992; Morita et al., 2007; Vantourout & Hayday, 2013).

Vδ2 T cells do not recognize peptides presented by MHC molecules. Instead, the Vδ2 T cell receptor (TCR) recognizes a number of non-peptide pyrophosphate molecules termed phosphoantigens (pAgs) which are produced as intermediates in the isoprenoid biosynthetic pathway. The mevalonate pathway and the non-mevalonate pathway are two distinct pathways of isoprenoid synthesis. The non-mevalonate pathway of isoprenoid synthesis is used by bacteria, mycobacteria, protozoa and parasites (Chen & Letvin, 2003; Morita et al., 2007). It results in the production of (E)-4-hydroxy-3-

methylbut-2-enly pyrophosphate (HMB-PP). HMB-PP is a unique ligand that can activate Vδ2 T cells and has a specific part to play in recognition of foreign pathogens by Vδ2 T cells. Isopentenyl pyrophosphate (IPP) is a metabolite of the mevalonate pathways used by most eukaryotes which can serve as self-ligand in human. However, IPP levels in healthy cells are not sufficient to initiate Vδ2 T cells activation. Cell stress which could be caused by infection or tumour transformation can result in increased IPP levels sufficient to activate Vδ2 T cells (Eberl et al., 2003; Gober et al., 2003; Idrees et al., 2013; Morita et al., 2007; Riganti et al., 2012).

Additionally, aminobisphosphonates drugs, used to treat osteoporosis, can activate Vδ2 T cells by inhibiting the enzyme that is required for IPP breakdown, resulting in IPP accumulation. As a result, aminobisphosphonates such as zoledronate and pamidronate can trigger Vδ2 T cell proliferation *in vivo* and *in vitro* in anti-tumour immunotherapies (Dieli et al., 2007; Fisher et al., 2014; Hoeres et al., 2018; Nussbaumer & Koslowski, 2019). It was shown that butrophilin 3A1 complexed with CD27 binds to pAg within cells, leading to Vδ2 T cells activation (Harly et al., 2012; Sandstrom et al., 2014).

Cytokines such as IL-12, IL-18 and IL-15 are also capable of activating Vδ2 T cells in the absence of specific antigen. Cytokine-activated Vδ2 T cells are capable of proliferation (Domae et al., 2017), production of IFN-γ (García et al., 1998) and perforin/granzyme-mediated cytotoxicity against tumour cell lines (Domae et al., 2017) and granulysin-mediated killing of bacteria (Tanaka et al., 1994).

Activated Vδ2 T cells can also promote the survival, differentiation and activation of number of cell types into APC, including monocytes , neutrophils, dendritic cells and B cells (Davey et al., 2014;Chien et al., 2014; Hedges et al., 2005; Kabelitz & He, 2012; Morita et al., 2007; Petrasca et al., 2013).

Since Vδ2 T cells appear to be involved in the pathology of sepsis, we have initiated more detailed studies on the metabolic regulation of their effector functions using lines of Vδ2 T cells expanded from peripheral blood of healthy donors, which yielded sufficient numbers of purified Vδ2 T cells to use Seahorse technology. Since Vδ2 T cells can be activated by many different stimuli, it was first necessary to compare the effects of different modes of stimulation on proliferation and effector functions and features of these cells. This would then allow metabolic analysis to be done to identify pathways important for these functions. To achieve this, we tested the ability of HMB-PP, combinations of cytokines, anti-CD3 and anti-CD28 mAbs, and the aminobisphonate drug, zoledronate to induce proliferation and activation of Vδ2 T cells *in vitro*.

Hypothesis and Aims

We hypothesized that altered cellular metabolism in Vδ2 T cells upon treatment with HMB-PP, IL-12/15 or zoledronic acid, may differentially affect their functions. We proposed the following objectives:

1) To compare the effects of stimulating PBMC from healthy human donors with HMB-PP, zoledronate, anti-CD3 and CD28 mAbs, and IL-12 + IL-15 on the proliferation and expansion of Vδ2 T cells *in vitro*.

2) To compare the effects of stimulating expanded human Vδ2 T cell lines with HMB-PP, zoledronate and IL-12 + IL-15 on their cytolytic activities and cytokine secretion profiles.

3) To characterize the metabolic profile of resting and activated Vδ2 T cells, using Vδ2 T cell lines or freshly isolated Vδ2 T cells from healthy donors using Seahorse technology.

5. 2 Results

5. 2. 1 HMB-PP and zoledronate induce Vδ2 T cell expansion from PBMC

In order to investigate if Vδ2 T cells expand in response to different modes of activation, PBMC were isolated from 4 healthy donors and an aliquot of the cells was analysed by flow cytometry to determine the frequencies of Vδ2 T cells at time 0, using the $CD3^+$ Vδ2 TCR^+ lymphocyte phenotype to define Vδ2 T cells (Fig. 5. 1A). Cells were plated in 24-well tissue culture plates at a density of 1 x 10^6 cells per ml and expanded by giving them a single stimulation with either HMB-PP (10 nM), zoledronate (5 µM), or IL-12 (30 ng/ml) with IL-15 (100 ng/ml), and culturing them with IL-2 supplemented (50 lU/ml) cRPMI medium. The medium was changed every 3-4 days by replacing with fresh IL-2-supplemented cRPMI.

The results indicate that HMB-PP and zoledronate induced expansions of Vδ2 T cells with zoledronate inducing superior proliferation (Fig. 5.2A-B), indicating that both stimulations are ideal to generate Vδ2 T cell lines from PBMC, with purity up to 90% on day 14. However, stimulation with IL-12 with IL-15 did not induce Vδ2 T c proliferation.

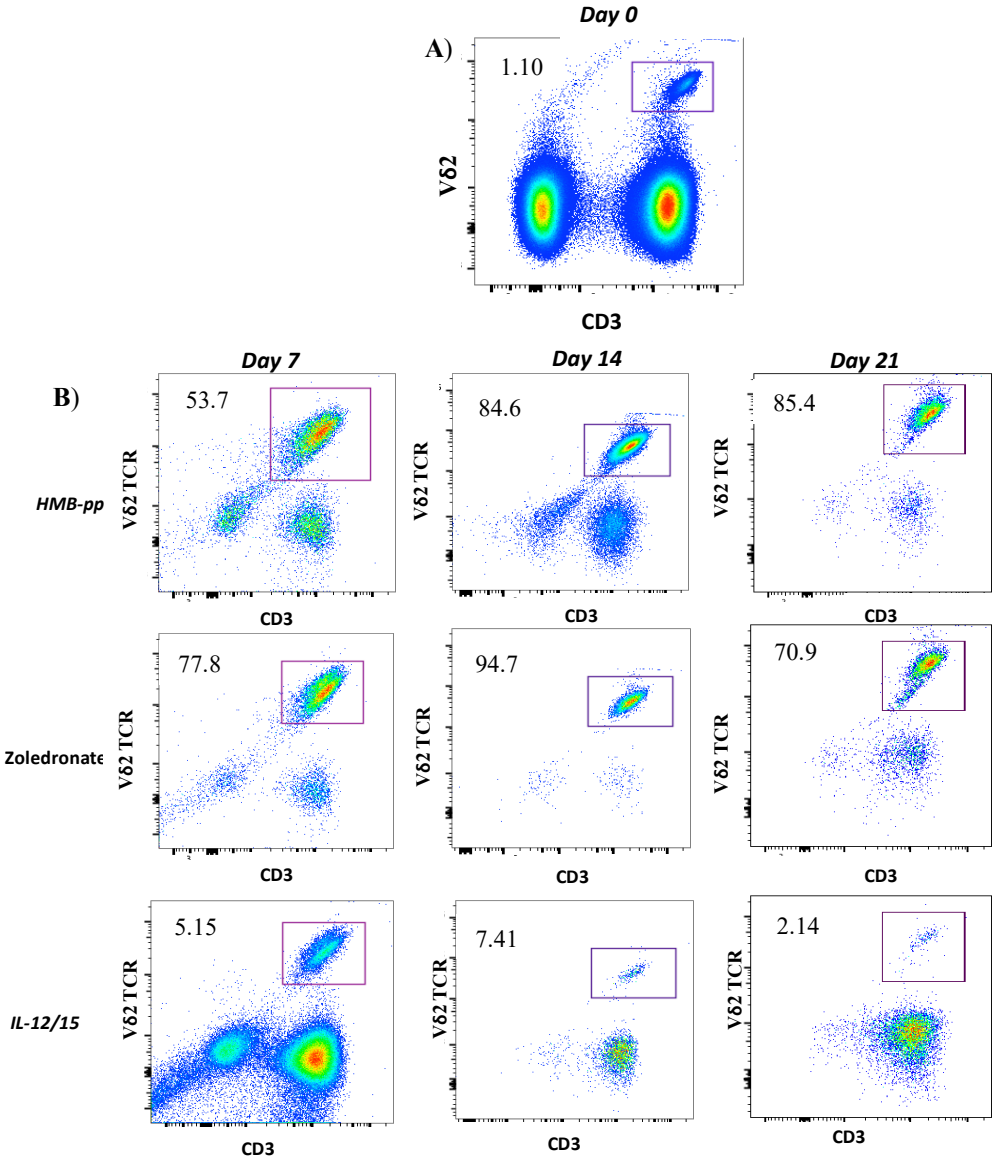

Figure 5. 1 HMB-PP and zoledronate can be used to selectively expand Vδ2 T cells from PBMC.

PBMC from healthy control subjects were stained with mAbs specific for CD3 and the Vδ2 TCR and the frequencies of Vδ2 T cells (CD3⁺ Vδ2 TCR ⁺) were determined by flow cytometry immediately after isolation or after stimulation with medium alone or HMB-PP (10 nM), or zoledronate (5 µM) or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) (denoted IL-12/15) for different time point (day 7, 14 and 21) at 37°C. (A) Flow cytometry dot plot showing CD3 and Vδ2 TCR expression by gated lymphocytes on day 0. (B) Flow cytometry dot plots showing CD3 and the Vδ2 TCR expression by gated lymphocytes after 7, 14 and 21 days post stimulation (n = 4).

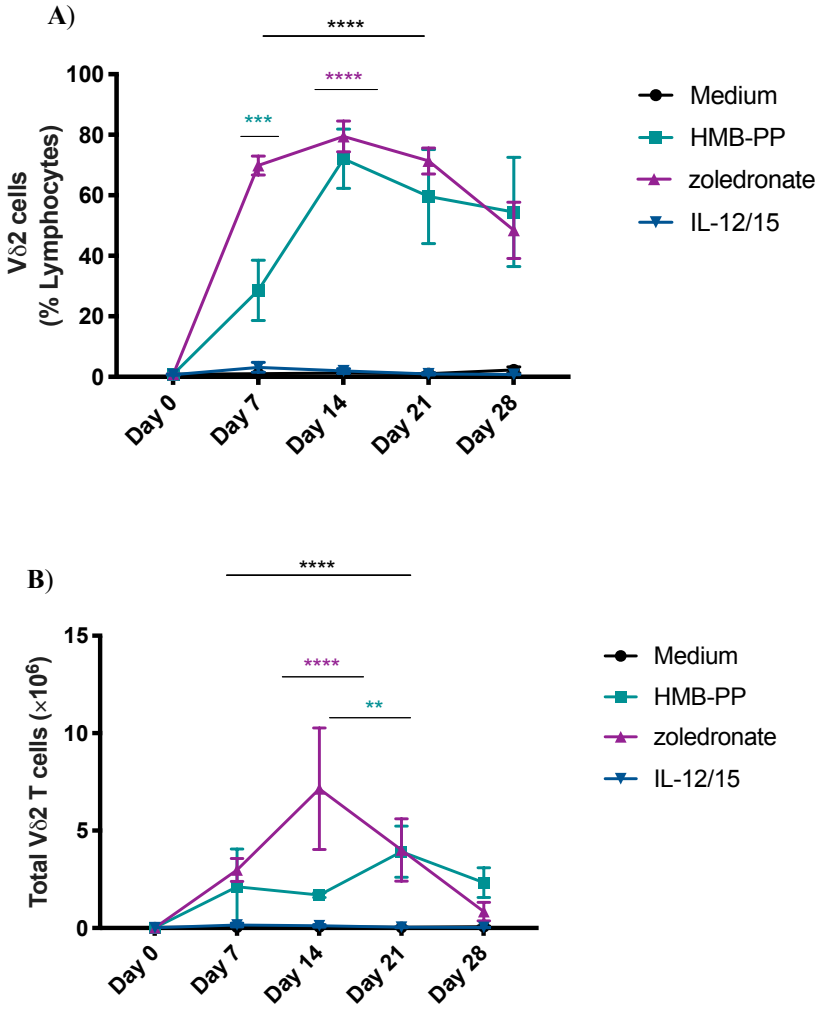

Figure 5. 2 HMB-PP and zoledronate can induce expansion Vδ2 T cell from PBMC.

*PBMC from healthy control subjects were stained with mAbs specific for CD3 and the Vδ2 TCR and the frequencies of Vδ2 T cells (CD3$^-$ Vδ2 TCR$^+$) were determined by flow cytometry immediately after isolation or after stimulation with medium alone or HMB-PP (10nM), zoledronate (5 µM), IL-12 (30 ng/mL) and IL-15 (100 ng/ml) (denoted IL-12/15), or anti-CD3 mAb (1 µg/ml) and anti-CD28 mAb (1 µg/ml) (denoted anti-CD3/CD28) for 7, 14, 21 and 28 days at 37°C. (A) Graph showing mean (± SEM) frequencies of Vδ2 T cells as percentages of lymphocytes in the cultures at each time point. (n = 4). (B) Graph showing mean (± SEM) absolute numbers of Vδ2 T cells in the cultures at each time point (n = 4). Bars show means. Samples were compared using a two-way ANOVA. ***P < 0.0002, ***P <0.0001.*

5. 2. 2 Altered nutrient receptor expression by expanded Vδ2 T cells from healthy donors

We next investigated if expanded Vδ2 T cells from healthy donors upregulated their nutrient receptors in response to treatment with medium, HMB-PP, zoledronate or IL-12 + IL-15. The expression of CD71 and CD98 by Vδ2 T cells in response to stimulation was assessed by flow cytometry. Vδ2 T cells upregulated both nutrient receptors to similar levels in response to different stimulation conditions (Fig. 5.3A-B & 5.4A-B). However, activation with HMB-PP resulted in the highest levels of both CD71 and CD98, seven days after activation. Activation-induced CD71 and CD98 expression by Vδ2 T cells was long lived, being detectable after 28 days, but the levels of both markers, as detected from the intensity of staining, dropped dramatically by day 14. Based on the results in figures 5.1, 5.2 and 5.3, zoledronate in the presence of IL-2 is the most potent expander of Vδ2 T cells from PBMC but HMB-PP most potently induces their expression of nutrient receptors.

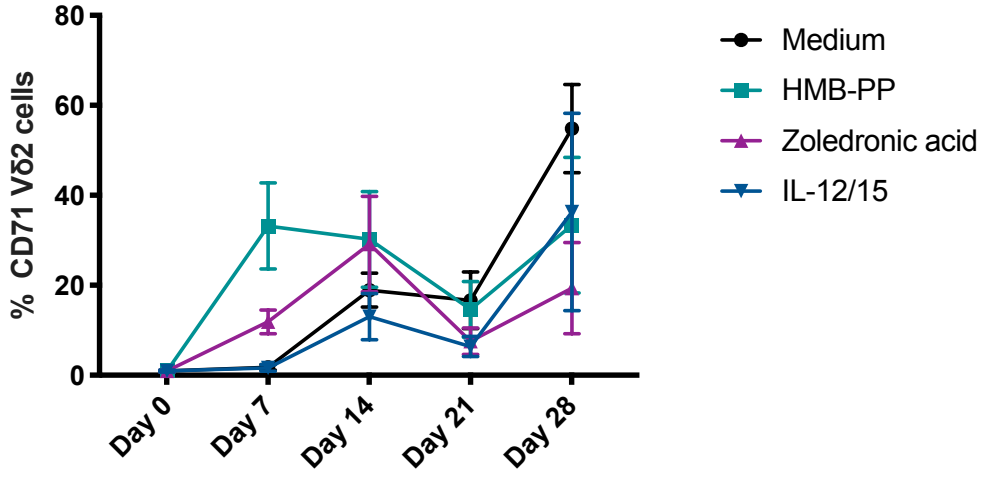

Figure 5. 3 Kinetics of CD71 expression by activated Vδ2 T cells.

PBMC from healthy control subjects were stained with mAbs specific for CD3 and the Vδ2 TCR and the frequencies of Vδ2 T cells (CD3⁺ Vδ2 TCR ⁺) were determined by flow cytometry immediately after isolation or after stimulation with medium alone or HMB-PP (10nM), zoledronate (5 µM), or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) (denoted IL-12/15) for 7, 14, 21 and 28 days at 37°C. Frequencies of CD71-expressing Vδ2 T cells for healthy donors (n = 4) in response to each treatment over time. Bars show means. Samples were compared using a two-way ANOVA.

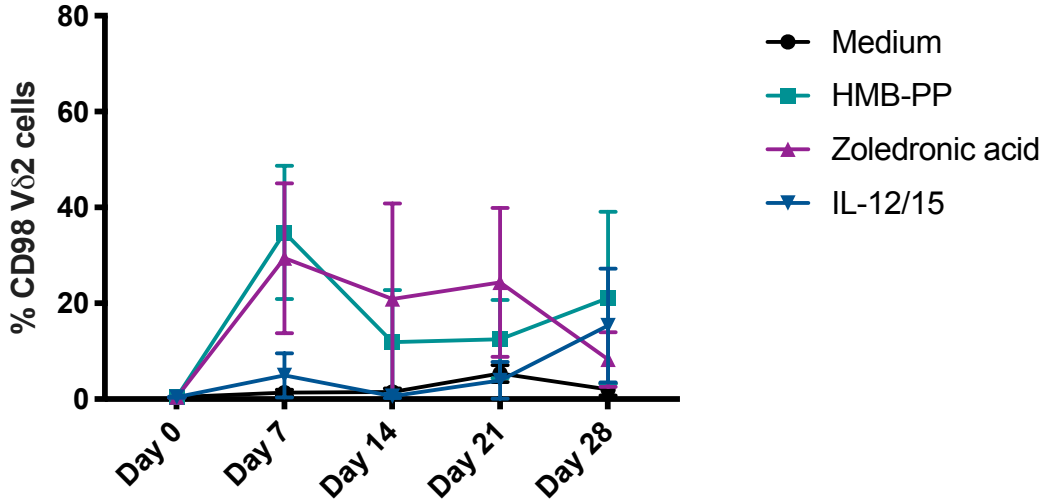

Figure 5. 4 Kinetics of CD98 expression by activated Vδ2 T cells.

PBMC from healthy control subjects were stained with mAbs specific for CD3 and the Vδ2 TCR and the frequencies of Vδ2 T cells (CD3^{+} Vδ2 TCR $^{+}$) were determined by flow cytometry immediately after isolation or after stimulation with medium alone or HMB-PP (10nM), zoledronate (5 µM), or IL-12 (30 ng/mL) and IL-15 (100 ng/ml) (denoted IL-12/15) for 7, 14, 21 and 28 days at 37°C. Frequencies of CD98-expressing Vδ2 T cells for healthy donors (n = 4) in response to each treatment over time. Bars show means. Samples were compared using a two-way ANOVA.

5. 2. 3 Activation of expanded Vδ2 T cells did not result in increased GrzB expression

In order to investigate the functional profile of Vδ2 T cells after stimulation using different modes of activation, Vδ2 T cell lines generated by stimulating PBMC using zoledronic acid were re-stimulated for 4 hours with HMB-PP (10 nM), zoledronate (5 μM) or PMA/ionomycin for 4 hours. The intracellular expression of granzyme B (GnzB) was analyzed by flow cytometry. Figure 5.5 shows that none of the stimuli significantly upregulated GnzB expression by Vδ2 T cells.

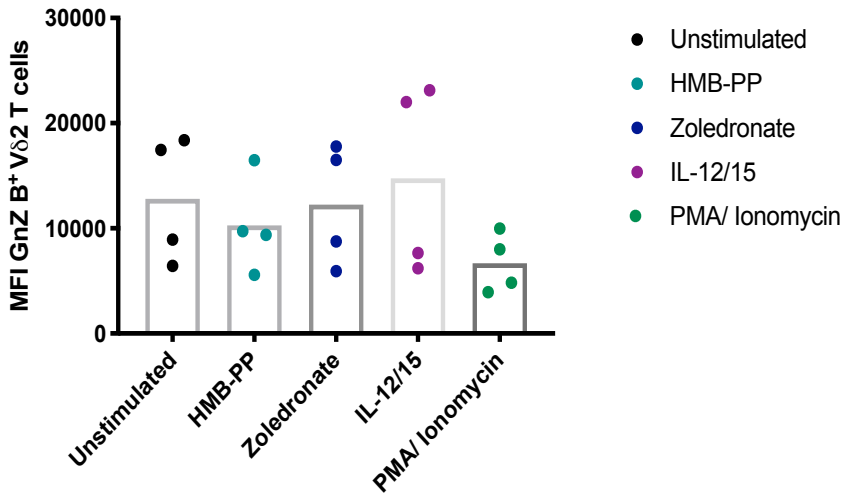

Figure 5. 5 Expression of GnzB by expanded and re-stimulated Vδ2 T cells.

Zoledronate-expanded Vδ2 T cell lines were stained with mAbs specific for cell-surface CD3, Vδ2 TCR and intracellular granzyme B immediately after isolation or after stimulation with medium alone, HMB-PP (10 nM), IL-12 (30 ng/mL) and IL-15 (100 ng/ml), zoledronate (5 µM) or PMA/Ionomycin for 4 hours at 37°C. The expression of GnzB by Vδ2 T cells was then analysed by flow cytometry. Graph shows MFI of GnzB expression by Vδ2 T cells (n = 4) in response to the treatments. Bars show means. Each dot represents 1 donor. Samples were compared using a one-way ANOVA followed by a Kruskal–Wallis test.

5. 2. 4 Re-stimulation of expanded Vδ2 T cells induces IFN-γ but not IL-4 or IL-17 production

In order to define the cytokine profile of Vδ2 T cells, Vδ2 T cells expanded from PBMC using zoledronic acid, were re-stimulated with HMB-PP (10 nM) or zoledronate (5 µM) or PMA/Ionomycin for 4 hours and examined for expression IFN-γ, IL-17 and IL-4 by flow cytometry. We found that when zoledronate-expanded Vδ2 T cells were stimulated with HMB-PP or PMA/ionomycin, there was a significant increase in IFN-γ production (Fig. 5.6A-B), but not IL-17 (Fig. 5.4C) or IL-4 (Fig. 5.6D) compared to other treatments. These results confirm previous findings (Dunne et al., 2010) that Vδ2 T cells predominantly produce Th1 cytokines upon activation.

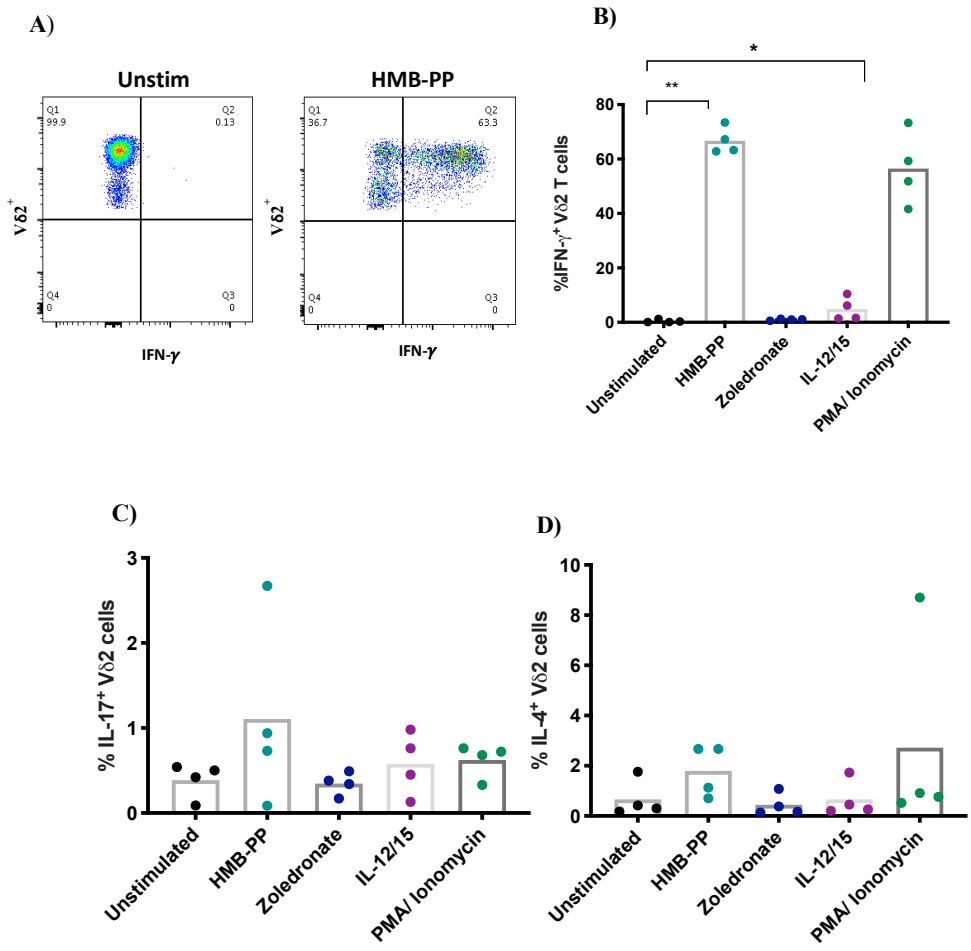

Figure 5. 6 Expanded Vδ2 T cells produce IFN-γ in response to HMB-PP and PMA/Ionomycin stimulation.

Vδ2 T cells expanded from PBMC using zoledronic acid were stained with mAbs specific for cell-surface CD3, Vδ2 TCR and intracellular IFN-γ immediately after isolation or after re-stimulation with medium alone, HMB-PP (10 nM), IL-12 (30 ng/mL) and IL-15 (100 ng/ml), zoledronate (5 µM), or PMA/Ionomycin for 4 hours at 37°C. The production of IFN-γ by Vδ2 T cells was then analysed by flow cytometry. (A) Representative flow cytometry dot plots showing IFN-γ production by Vδ2 T cells in response to HMB-PP *stimulation. (B) Frequencies of IFN-γ-expressing Vδ2 T cells from Vδ2 T cell lines (n = 4) in response to each treatment. (C) Frequencies of IL-17-expressing Vδ2 T cells from Vδ2 T cell lines (n = 4) in response to stimulation. (D) Frequencies of IL-4 -expressing Vδ2 T cells from Vδ2 T cell lines (n = 4) in response to stimulation. Bars show means. Each dot represents 1 donor*. Samples were compared using a one-way ANOVA followed by a Kruskal–Wallis test *analysis*. **P < 0.05, **P <0.001.*

5. 2. 5 Expanded and sorted Vδ2 T cells did not upregulate rates of glycolysis and oxidative phosphorylation in response to HMB-PP stimulation

The Seahorse XFp Analyser was used to compare relative rates of glycolysis and oxidative phosphorylation by unstimulated and HMB-PP-stimulated Vδ2 T cells. Biochemical inhibitors were added to cells at various times to interrogate the type of metabolism occurring (see section 2. 4, Fig 2. 4. 1& 2. 4. 2). To date, no studies have been done on Vδ2 T cells using Seashore technology. First needed to explore and optimize this method. In initial experiments, we attempted to use zoledronate-expanded Vδ2 T cells, which were purified by flow cytometric cell sorting. Figure 5.7 (A and B) shows that, surprisingly, the rates of both glycolysis and oxidative phosphorylation were lower in HMB-PP-re-stimulated Vδ2 T cells than in unstimulated Vδ2 T cells. This suggests that these cells may be dying as a result of intense stimulation.

As shown in Figure 5.7, expanded and sorted Vδ2 T cells did not upregulate rates of glycolysis or oxidative phosphorylation in response to stimulation. We therefore next investigated rates of glycolysis and OxPhos in sorted Vδ2 T cells freshly-isolated from PBMC from 3 donors, after stimulation with HMB-PP. Figure 5.8 (A and B) show that the rates of glycolysis and oxidative phosphorylation did not increase in response to activation with HMB-PP. This failure to upregulate rates of glycolysis and OxPhos may reflect damage to the cells as a result of flow cytometric cell sorting. In support of this possibility, very low levels of Vδ2 T cells were recovered.

In order to overcome this issue, Vδ2 T cells were obtained from 500 mL of fresh blood by positive magnetic bead separation of PBMC. PBMC were stained with a PE-labelled anti-CD3 mAb and subsequently, anti-PE Microbeads were used to isolate Vδ2 T cells (see section 2.3.9). Using this method, up to 6 X 10^6 Vδ2 T cells were obtained from each donor. Seahorse analysis of varying numbers of cells (0.8 X 10^6 and 0.5 X 10^6, data not shown) indicated that 0.5 X 10^6 cells per well was optimal for the comparison of ECAR and OCR by Seahorse analysis.

Purified Vδ2 T cells were stimulated overnight with HMB-PP or zoledronate, before subjecting them to metabolic analysis. HMB-PP and zoledronate treatment both increased the rate of glycolysis, as measured by ECAR, in Vδ2 T cells (Fig 5.9A for representative ECAR traces). The levels of basal glycolysis (glycolysis of the cells at rest) were significantly higher when Vδ2 T cells was stimulated with HMB-PP than with zoledronic acid (Figure 5.9B). In addition, higher maximum glycolytic rates (the total capacity of the cells to carry out glycolysis) was observed in HMB-PP stimulated Vδ2 T cells (Fig 5.9C). Since the addition of 2DG shuts down ECAR almost completely, these observations confirm that Vδ2 T cells use glycolysis as a means of generating energy. Furthermore, while HMB-PP provided a stronger signal, the impact of zoledronate was relatively modest.

Next, we investigated if Vδ2 T cells from healthy donors are capable OxPhos. Purified fresh Vδ2 T cells from healthy donors were stimulated with HMB-PP or zoledronate overnight, before metabolic analysis. HMB-PP and zoledronate stimulations both

increased the rates of oxygen consumption and levels of OxPhos (Fig 5.10A for representative OCR trace). We found that Vδ2 T cells used OxPhos basally, and when HMB-PP and Zoledronate both increased OCR in Vδ2 T cells but the effect of Zoledronate was much more modest. In terms of respiratory capacity, HMB-PP but not Zoledronate increased respiratory capacity (Fig 5.10A-C). A small increase in ATP-linked respiration was observed when the Vδ2 T cells were stimulated with HMB-PP (Fig 5.10D). While most results were consistent, the impact of antimycin/rotenone was variable. In some experiments, OCR was reduced completely (consistent with all oxygen consumed through OxPhos) while in others (as illustrated in the representative seahorse trace), there was more variability. Increased experiments are required to investigate this further to see if a consistent pattern of data is obtained.

In summary, HMB-PP increased metabolic activity of Vδ2 T cells in terms of both glycolysis and OxPhos. In contrast, Zoledronate was relatively poor in this regard and only induced a modest increase in OxPhos. These data demonstrate that primary Vδ2 T cells from humans engage both glyolysis and OxPhos upon stimulation.

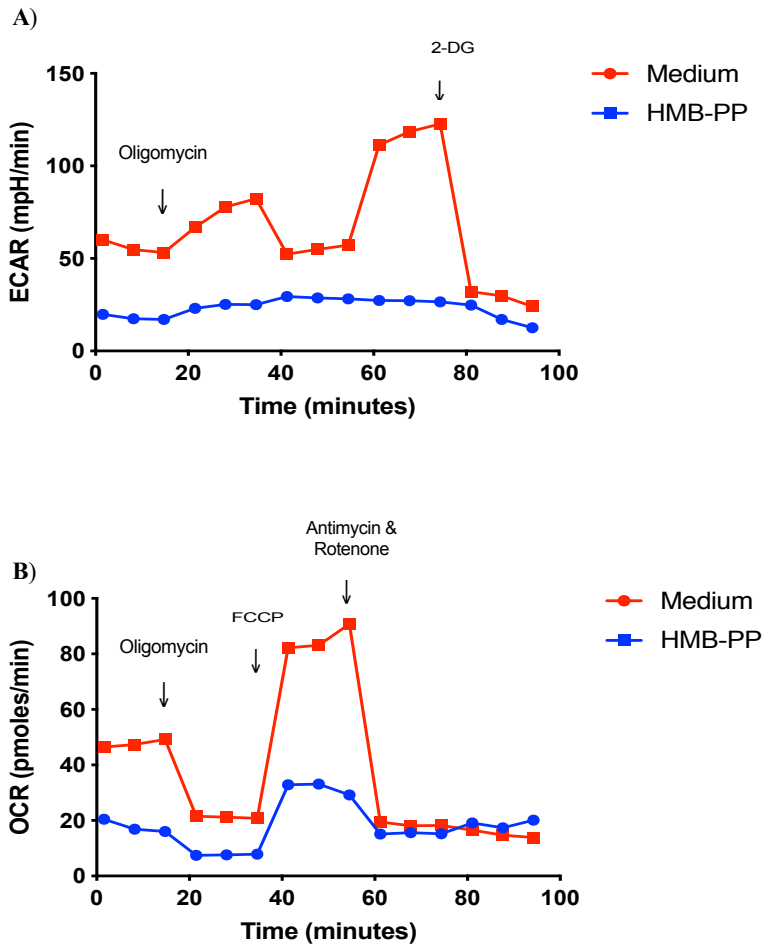

Figure 5. 7 Attempts to measure changes in glycolysis and oxidative phosphorylation by expanded and sorted Vδ2 T cells activated with HMB-PP.

Vδ2 T cells were isolated from cultures of zoledronate-expanded Vδ2 T cell lines by flow cytometric cell sorting and stimulated with medium alone or HMB-PP (10 nM) for 18 hours at 37°C. The extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) were then measured using a Seahorse XFp Analyser to examine changes in rates of glycolysis and oxidative phosphorylation, respectively, in response to sequential addition of the inhibitor of ATP synthase, oligomycin, the oxidative phosphorylation uncoupler FCCP, the electron transport chain inhibitors antimycin and rotenone and the glycolysis inhibitor 2-deoxy-glucose (2-DG). (A) Representative ECAR trace of unstimulated and HMB-PP stimulated expanded Vδ2 T cells from a healthy donor. (B) Representative OCR trace of unstimulated and HMB-PP stimulated expanded Vδ2 T cells from a healthy donor. Results are representative of experiments using preparations of Vδ2 T cells from 4 healthy donors.

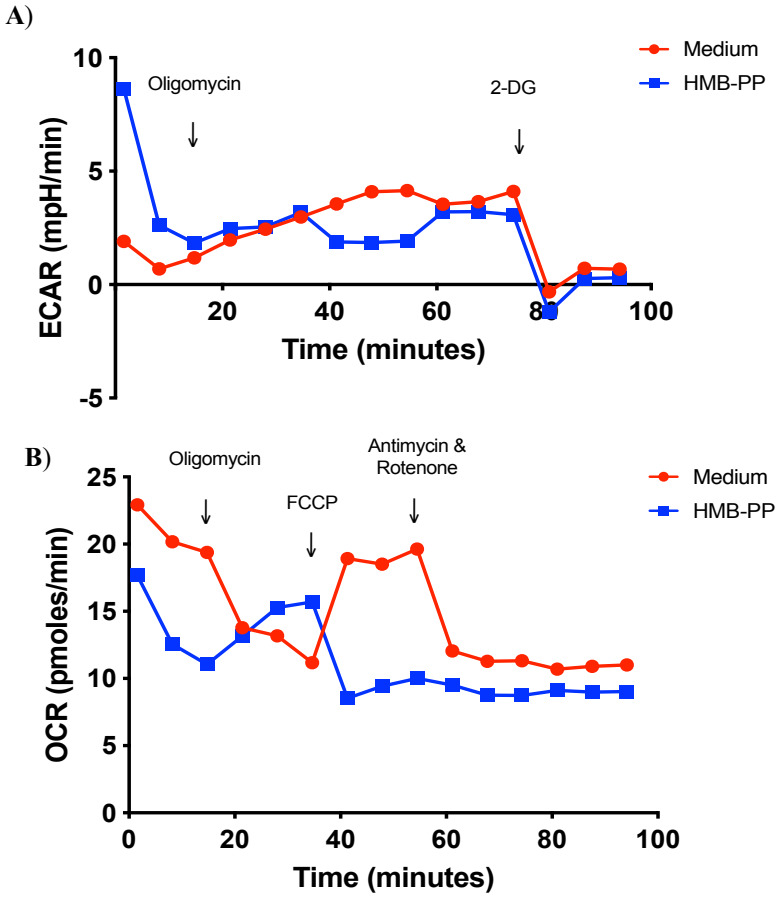

Figure 5. 8 Attempts to measure changes in glycolysis and oxidative phosphorylation by fresh, unexpanded Vδ2 T cells activated with HMB-PP.

Vδ2 T cells were isolated from freshly-isolated PBMC by flow cytometric cell sorting and stimulated with HMB-PP (10 nM) for 18 hours at 37°C. The extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) were then measured using a Seahorse XFp Analyser to examine changes in rates of glycolysis and oxidative phosphorylation, respectively, in response to sequential addition of the inhibitor of ATP synthase, oligomycin, the oxidative phosphorylation uncoupler FCCP, the electron transport chain inhibitors antimycin and rotenone and the glycolysis inhibitor 2-deoxy-glucose (2-DG). (A) Representative ECAR trace of unstimulated and HMB-PP stimulated fresh sorted Vδ2 T cells from a healthy donor. (B) Representative OCR trace of unstimulated and HMB-PP stimulated fresh sorted Vδ2 T cells from a healthy donor analysed on the same day. Results are representative of experiments using preparations of Vδ2 T cells from 3 healthy donors.

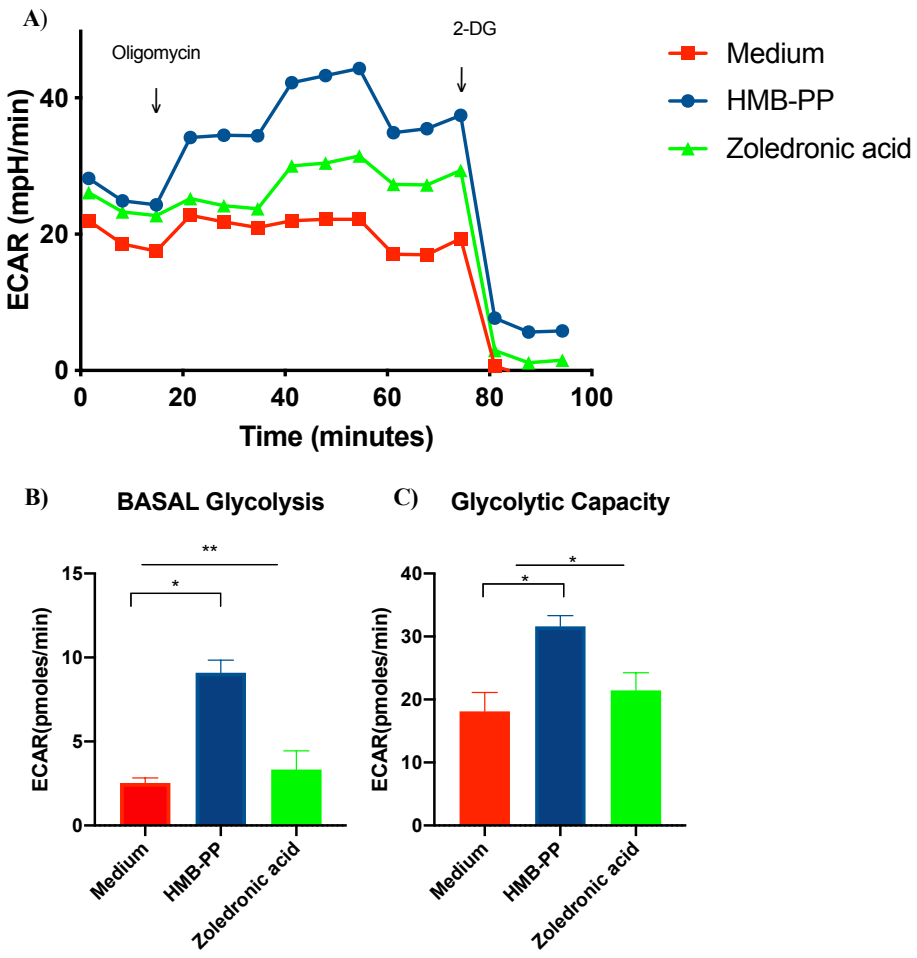

Figure 5. 9 Freshly-isolated Vδ2 T cells from healthy donors upregulate glycolysis in response to stimulation with HMB-PP or zoledronate.

*Freshly isolated and magnetic bead-sorted Vδ2 T cells were stimulated with HMB-PP (10 nM) or zoledronate (5 µM) for 18 hours at 37°C and metabolic analysis was performed using the Seahorse XFp Analyser after sequential addition of oligomycin, FCCP, antimycin and rotenone and 2-DG. (A) Representative ECAR trace of unstimulated, HMB-PP and zoledronate stimulated Vδ2 T cells from a healthy donor analysed on the same day. (B) Comparison of basal glycolytic rates in unstimulated, HMB-PP and zoledronate stimulated Vδ2 T cells from healthy donors. (C) Comparison of the glycolytic capacity in unstimulated, HMB-PP and zoledronate stimulated Vδ2 T cells from healthy donors (n = 4). Samples were compared using a one-way ANOVA followed by a Kruskal–Wallis test analysis. *P < 0.05, **P <0.001.*

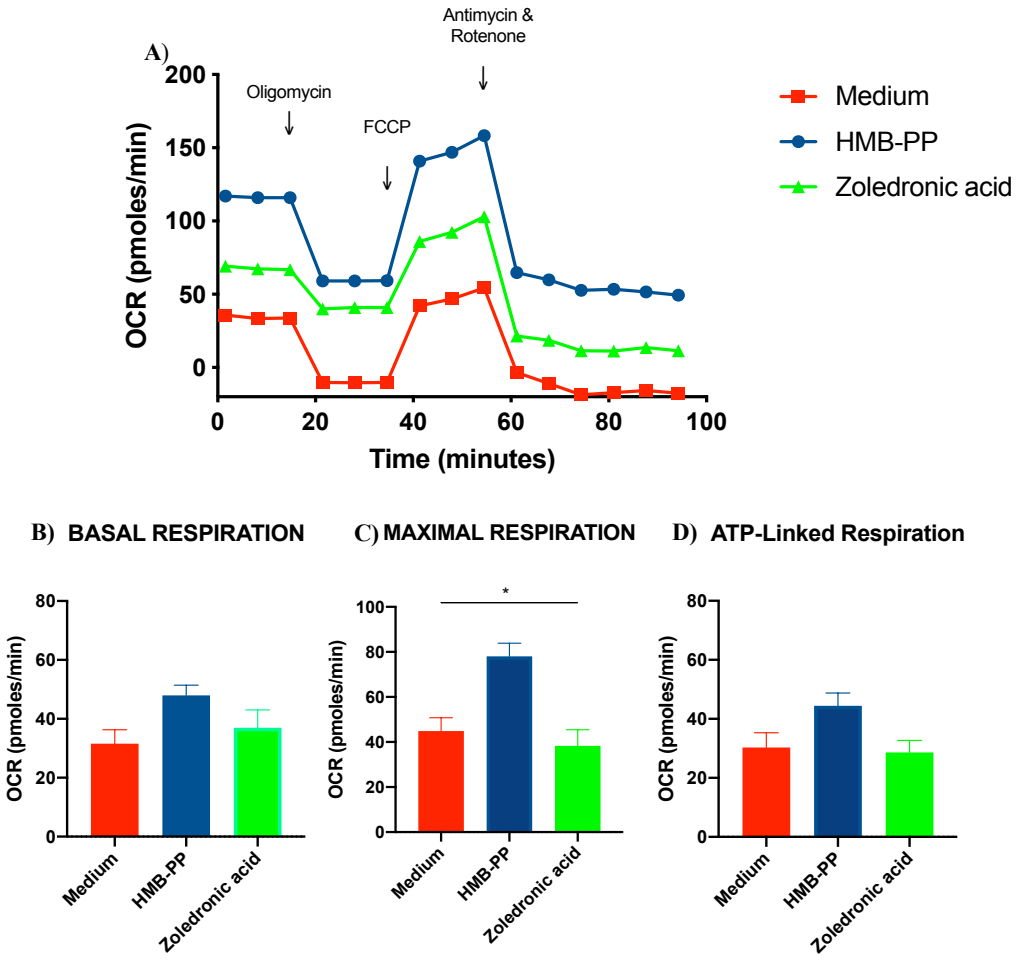

Figure 5. 10 Vδ2 T cells from healthy donors exhibit increased mitochondrial respiration in response to stimulation.

*Freshly isolated Vδ2 T cells were stimulated with HMB-PP (10 nM) or zoledronate (5 μM) for 18 hours at 37°C and metabolic analysis was performed using the Seahorse XFp Analyser after sequential addition of oligomycin, FCCP, antimycin and rotenone and 2-DG. (A) Representative OCR trace of unstimulated, HMB-PP and zoledronate stimulated Vδ2 T cells from a healthy donor analysed on the same day. (B) Comparison of the basal respiration in unstimulated, HMB-PP and zoledronate stimulated Vδ2 T cells (C) Comparison of the maximal respiration in unstimulated, HMB-PP and zoledronate stimulated Vδ2 T cells from healthy donors. (D) Comparison of ATP-linked respiration in unstimulated, HMB-PP and zoledronate stimulated Vδ2 T cells from healthy donors and cancer patients. Bars show the means (n = 4). Samples were compared using a one-way ANOVA followed by a Kruskal–Wallis test analysis. *P < 0.05.*

We also measured the expression of the activation marker CD69 and nutrient receptor for iron uptake CD71 on Vδ2 T cells isolated from freshly-isolated PBMC from healthy donors and stimulated for 18 hours with HMB-PP or zoledronate. Figure 5.11 shows that both types of stimulation resulted in a modest upregulation of CD69 and CD71 expression by Vδ2 T cells. There was some variability between experiments and more repeat experiments are required to validate findings.

Taken together, these data suggest that HMB-PP activate Vδ2 T cells, and cause them to undergo metabolic changes, with increases in both the rates of glycolysis and OxPhos when compared with resting Vδ2 T cells. In contrast, Zoledronate was relatively poor in inducing robust metabolic changes.

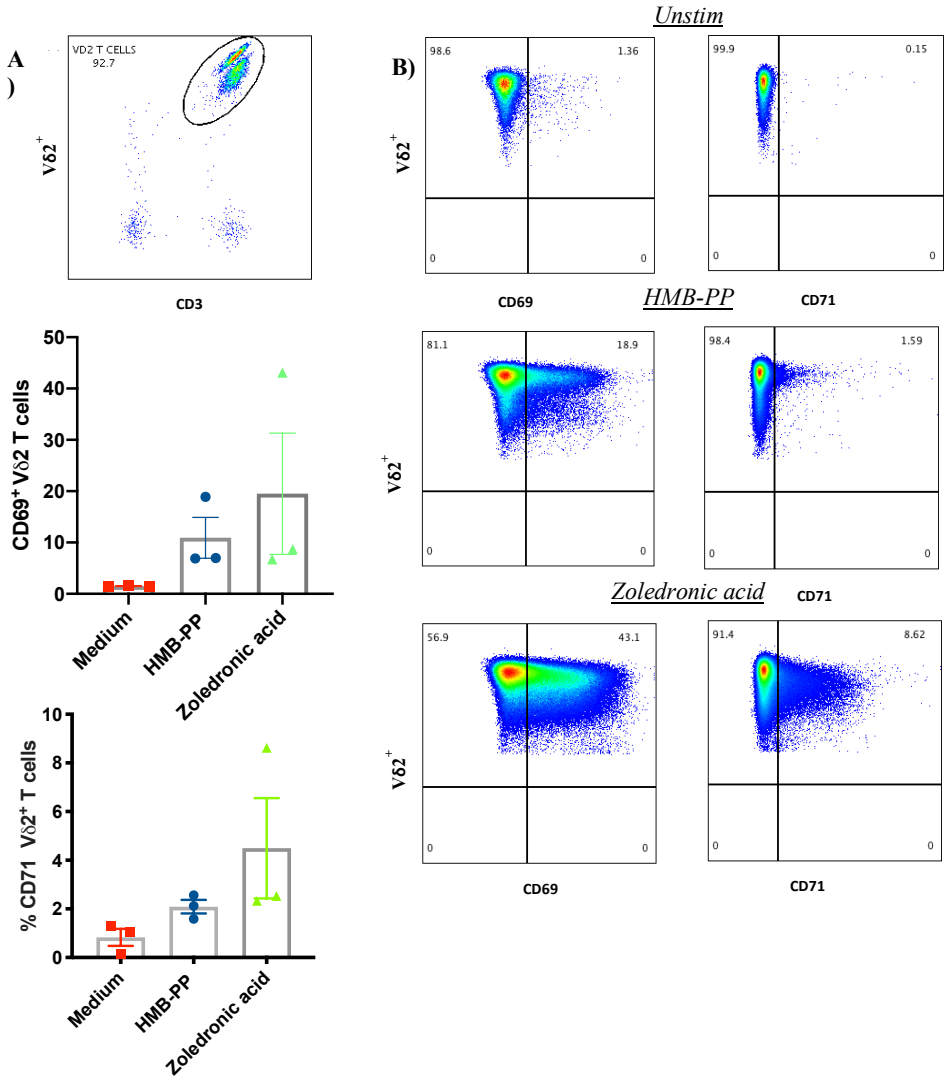

Figure 5. 11 Vδ2 T cells from healthy donors upregulate CD69 and CD71 normally in response to stimulation.

Freshly isolated Vδ2 T cells from healthy donors were stained with mAbs specific for CD3, Vδ2 TCR, CD69 or CD71 immediately after isolation or after stimulation with medium alone, HMB-PP (10nM) or zoledronate (5 μM) for 18 hours at 37°C. The expression of CD69 and CD71 by Vδ2 T cells was then analysed by flow cytometry. (A) Representative flow cytometry dot plot showing the purity of Vδ2 T cells. (B) Representative flow cytometry dot plots showing CD69 or CD71 expression by Vδ2 T cells from a healthy control subject in response to stimulation. The left panels show the expression of CD69 by gated *Vδ2 T cells*, while the right panels show the expression of CD71 by gated *Vδ2 T cells*. *The top panels show unstimulated Vδ2 T cells, whereas the middle and lower panels show HMB-PP and zoledronate stimulated Vδ2 T cells, respectively. (C) Frequencies of unstimulated and stimulated Vδ2 T cells from healthy control subjects (n = 3) that expressed CD69. (D) Frequencies of unstimulated and stimulated Vδ2 T cells that expressed CD71 (n = 3). Bars show means. Each dot represents 1 donor*. Samples were compared using a one-way ANOVA followed by a Kruskal–Wallis test.

5. 3 Discussion

Vδ2 T cells display a number of effector functions in innate and adaptive immunity. They can directly kill bacteria, virally infected cells and tumour cells, boost inflammation and wound healing, promote monocyte, neutrophils and dendritic cells survival, differentiation and activation, help B cells in the production of antibody, and prime $CD4^+$ and $CD8^+$ T cells (Chien et al., 2014; Kabelitz & He, 2012; Morita et al., 2007; Petrasca & Doherty, 2014; Vantourout & Hayday, 2013). Vδ2 T cells can also promote antigen presentation by other cells and can even themselves present peptide antigens to conventional $CD4^+$ and $CD8^+$ T cells (Davey et al., 2014; Devilder et al., 2006; Dunne et al., 2010; Dunne et al., 2010; Ismaili et al., 2002; Marcu-Malina et al., 2014). These multiple functions of Vδ2 T cells make them ideal potential candidates for immune therapies. Indeed, Vδ2 T cells are currently under investigation as immunotherapies for cancer in clinical trials (Berglund et al., 2018; Hoeres et al., 2018; Mao et al., 2019).

Vδ2 T cells can be expanded from PBMC by stimulation using the microbial metabolite, HMB-PP (Gober et al., 2003; Hintz et al., 2001) or by using aminobisphosphonates, such as zoledronate, to induce the accumulation of endogenous phosphoantigens in cells (Dieli et al., 2007). We found that stimulation of PBMC with HMB-PP or zoledronate and culturing them in the presence of IL-2 led to dramatic expansions of Vδ2 T cells, which reached purities of up to 80% within 2 weeks, without enrichment by sorting. However, activation of Vδ2 T cells with IL-12 and IL-15 or with anti-CD3 and anti-CD28 mAbs did not promote Vδ2 T cell expansion. In order to indicate if expanded Vδ2 T cell are metabolically active, we measured the expression of the nutrient uptake receptors

CD71 and CD98. Our data showed that both receptors were induced on Vδ2 T cells within 7 days of stimulation, before returning to basal levels by day 21. Interestingly, while stimulation by CD3 and CD28 ligation or using IL-12 and IL-15 did not induce Vδ2 T cell proliferation, these modes of activation induced moderate upregulation of CD71 and CD98. These data suggest that Vδ2 T cells upregulate metabolism in response to activation *in vitro*.

Activated Vδ2 T cells can upregulate and enhance their effector functions and produce cytokines such as IFN-γ and TNF-α (Domae et al., 2017.; Eberl et al., 2009). We investigated the effects of re-stimulation of Vδ2 T cells expanded using zoledronate and re-stimulated with HMB-PP, IL-12/15, zoledronate, anti-CD3 mAb/anti-CD28 mAb or PMA/ionomycin. Expanded Vδ2 T cells did not upregulate granzyme B expression in response to any of the stimuli. Interestingly, we found that a high percentage of HMB-PP and PMA/Ionomycin-activated Vδ2 T cells expressed IFN-γ, but did not produce IL-4 or IL-17 within 4 h. Furthermore, Vδ2 T cells did not produce IFN-γ, IL-4 or IL-17 upon 4 h stimulation with zoledronate, IL-12/15, anti-CD3 mAb/anti-CD28 mAb. These results suggest that zoledronate treatment is optimal for expanding Vδ2 T cells, whereas HMB-PP optimally activates their effector activities.

Cellular metabolism has a crucial role to play in the activation of immune cells (Kedia-Mehta & Finlay, 2019; Loftus & Finlay, 2016; O'Neill et al, 2016). Indeed, metabolic changes to lymphocytes have been demonstrated to be critical in determining the types of effector activities. The mechanisms by which energy metabolism dictates immune

function currently being explored. Metabolic reprogramming of immune cells occurs in response to environmental changes, such as antigenic stimulation, in order to generate energy to support the effector functions. An increase in glucose metabolism with high rates of both glycolysis and OxPhos is frequently observed upon cell activation (O'Neill et al., 2016). Furthermore, distinct metabolic pathways are frequently used by immune cells with distinct functions (Loftus & Finlay, 2016). For instance, naïve, effector, memory and regulatory T cells have distinct energy requirements and use distinct pathways of energy metabolism (Delgoffe & Powell, 2015). In naïve T cells, for example, small amount of glucose, glutamine and fatty acid are used as fuels and are metabolised by OxPhos to generate energy (Buck et al., 2015). Upon activation, they adjust their cellular metabolism to fit their functions. Indeed, a significant increase in aerobic glycolysis metabolic rate takes place in NK cells, proliferating T cells, in B cells dendritic cells neutrophils and M1 macrophages engaged in their effector functions (Kelly & O'Neill, 2015; Slattery & Gardiner, 2019). For instance, cytokine production by effector lymphocyte subsets such as NK cells and $CD8^+$ T cells is reliant on glycolytic metabolism actually, both glycolysis and OxPhos increase (Buck et al., 2015). High rates of glucose and glutamine consumption by effector T cells requires the induction of aerobic glycolysis in the cytoplasm and the TCA cycle in the mitochondria (Finlay et al., 2012; Swamy et al., 2016; R. Wang et al., 2011). Th1 cells, however, mostly use aerobic glycolysis, whereas both memory T cells and Treg utilize lipid oxidation to provide the energy required to support their long life span (Michalek et al., 2011; O'Sullivan et al., 2014). There is also some data emerging to suggest that macropahges may preferentially utilise fatty acid oxidation for energy during sepsis, indicating altered metabolism of innate immune cells during pathogenic situations such as sepsis

(Vachharajani & McCall, 2019). Therefore, it is essential to understand immune cell metabolic needs and how this can trigger and control their function, leading to immunotherapies developments. This study is the first to explore the metabolic pathways used by Vδ2 T cell using Seahorse technology.

We first investigated the metabolic profiles of Vδ2 T cells using a variety of approaches as we dealt with technical and biological optimisations. We first expanded Vδ2 T cells, which were sorted on a flow cytometric cell sorter to obtain high purities. However, we found that re-stimulated Vδ2 T cells had lower levels of glycolysis and OxPhos than non-re-stimulated cells. This may reflect an induction of Vδ2 T cell exhaustion as a result of repeated activation or potential cytotoxicity issues. To overcome this problem, we next used Vδ2 T cells sorted from small fresh blood samples from healthy donors. However, using these cells as starting material, only small changes in ECAR and OCR were observed as a result of stimulation, because insufficient numbers of cells were obtained.

Finally, we used Vδ2 T cells isolated using positive selection magnetic beads separation from 500 ml of fresh blood. When these cells were analysed using Seahorse extracellular flux analysis, increases in both glycolysis and OxPhos were observed in activated Vδ2 T cells. Our data showed that HMB-PP-activated Vδ2 T cells exhibited higher rates of glycolysis when compared to resting Vδ2 T cells, suggesting that they have upregulated their metabolism upon stimulation. Basal OxPhos and capacity were increased in HMB-PP and zoledronate-activated Vδ2 T cells upon stimulation as seen with other lymphocyte subsets which upregulate their metabolism in response to stimulation

(Donnelly & Finlay, 2015). We show that Vδ2 T cells increasing basal glycolysis rates in response to activation, similar to NK cells and MAIT cells (Assmann et al., 2017; O'Brien et al., 2019). Interestingly, HMB-PP induced larger increases in glycolytic capacity and OxPhos rates of Vδ2 T cells compared to zoledronate stimulated Vδ2 T cells. Thus, while zoledronic acid preferentially induces Vδ2 T cell proliferation, it appears that HMB-PP stimulation induces greater metabolic changes, consistent with its superior ability to induce cytokine production by these cells.

Data is starting to emerge on macrophages and monocytes and metabolic reprogramming towards increased use of fatty acid oxidation during sepsis. Our data on healthy Vδ2 T cell starts to add to our expanding knowledge of the complex regulation of other innate immune cells. This is a first step to defining what is normal for Vδ2 T cell. This will act as a benchmark for defining dysregulation during disease. A switch towards increased use of lipid oxidation pathways could be interrogated using Seahorse technology and pharmacological inhibitors of fatty acid oxidation. Caution is needed as we proceed as some of commonly inhibitors e.g. etomoxir have been described to have off-target effects which may impact on conclusions (Yao et al., 2018).

In summary, this chapter has compared the proliferation, cytotoxic potential, cytokine production, nutrient receptor expression and energy metabolism in Vδ2 T cells activated in different ways. Our findings indicate that zoledronate is required for optimal expansion of Vδ2 T cells and HMB-PP is best at activating certain effector functions of Vδ2 T cells. Furthermore, HMB-PP activated Vδ2 T cells underwent metabolic

changes to increase the rates of glycolysis and OxPhos. In contrast, Zoledronate was relatively poor at activating metabolic pathways in Vδ2 T cells. These results have shown that it is possible to expand Vδ2 T cells with zoledronate and then activate their effector functions with HMB-PP, steps towards a therapeutic strategy (Fig 5.12).

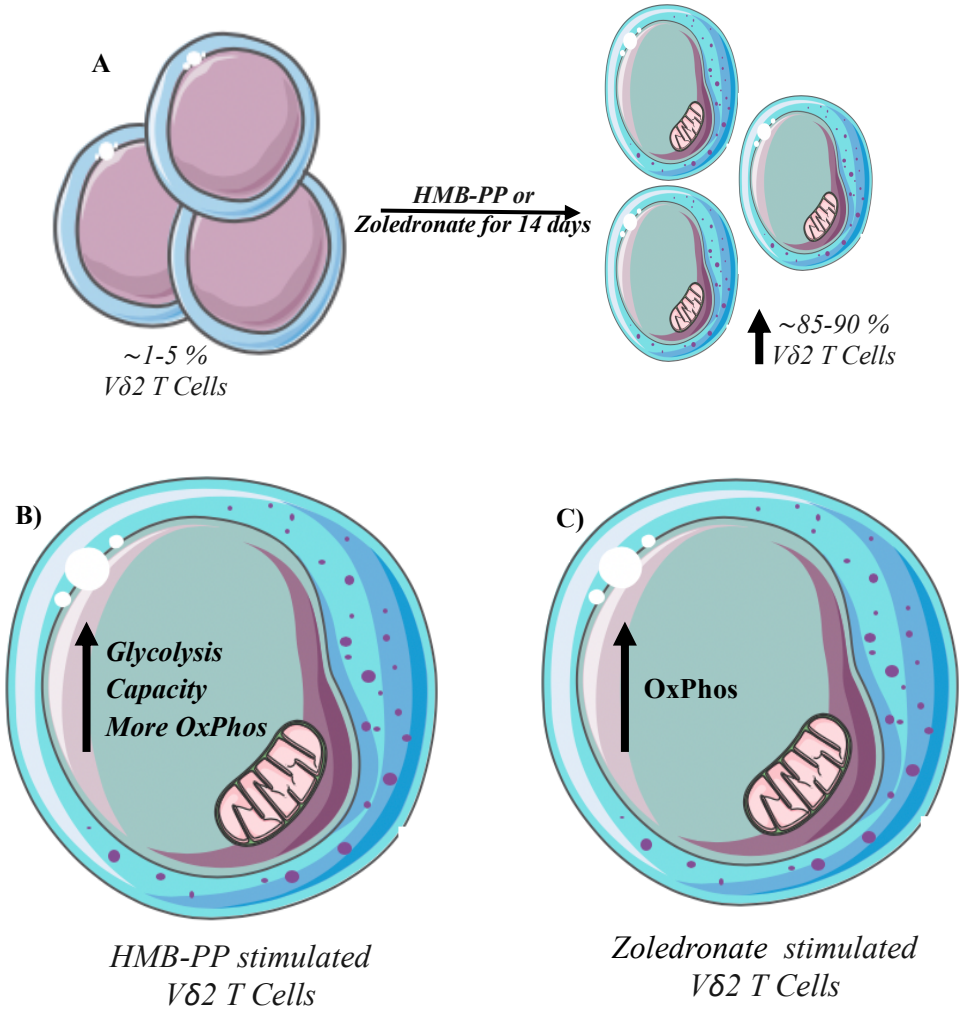

Figure 5. 12 Summary of the metabolic changes that occur to Vδ2 T cells upon activation in vitro.

PBMC or isolated Vδ2 T cells from healthy control subjects were stimulated with medium alone, HMB-PP (10nM) or zoledronate (5 μM) for 14 days. (A) Vδ2 T cell frequencies before and after stimulation for 14 days. (B-C) HMB-PP and zoledronate activated Vδ2 T cells undergo metabolic changes to increase glycolysis and OxPhos.

Chapter 6 - Final Discussion

6. 1 Final Discussion

NK cells and Vδ2 T cells mediate a rapid response to eradicate infection and tumour cells directly by secreting granzymes, perforin and pro-inflammatory cytokines such as IFN-γ (Monserrat et al., 2012, Rodriguez-Zapata et al., 1996) or indirectly by activating and regulating other adaptive immune cells (Colonna 2017, de Pablo et al., 2014, Walzer et al., 2005). Thus, NK cells and Vδ2 T cells have promising applications for immunotherapy for cancer, microbial infection and sepsis. There is an urgent unmet clinical need for early biochemical-immunological diagnosis of sepsis (Ono et al., 2018; Van der Poll et al., 2017). In the current study, we investigated if sepsis-induced immunosuppression can lead to defective NK cell and Vδ2 T cell effector functions and metabolism.

Immune cells traffic through various tissues, each with varying nutrient and oxygen availability. Hence, these cells must adjust their metabolism to survive and function. In many illnesses like cancer (Martinez-Outschoorn et al., 2017), metabolic syndrome and sepsis (Venet et al., 2017), the availability of nutrients are distinct from normal situations. Immune cells have different demands of nutrient and oxygen that are essential and for their survival and functions (Pearce et al., 2013). Metabolism is key to adjusting cell function by producing energy and building blocks required for a cell to efficiently accomplish their functions (Assmann & Finlay, 2016). In homeostatic conditions, lymphocytes such as NK cells have limited energy or biosynthetic requirement, and they use glucose via glycolysis and oxidative phosphorylation (OxPhos) to meet their energy requirement via ATP synthesis (Gardiner & Finlay, 2017). Studies have previously shown how NK cell metabolism is crucial for their functions

(Donnelly et al., 2014; Keppel et al., 2015). Activation of NK cells for 18 hours with cytokines leads to upregulation of glycolysis and OxPhos, with mainly a shift towards glycolysis (Donnelly et al., 2014; Keating et al., 2016). As a result of this shift, energy and biosynthetic intermediates are produced to meet NK cells' needs to perform their functions. Indeed, activated NK cells have increased mTORC1 activity and enhance their glycolytic flux. It has been demonstrated that mTORC1 signalling maintains glycolytic reprogramming of NK cells and controls their effector functions including IFN-γ and granzyme B production (Donnelly et al., 2014; Keating et al., 2016). Inhibition of glycolysis or OxPhos leads to inhibition of NK cell functions such as IFN-γ and cytotoxicity (Donnelly et al., 2014; Keating et al., 2016). However, little is known about how sepsis affects human NK cell metabolism and function. Therefore, in the current study, we characterized human NK cells frequencies, phenotypes, function, nutrients uptake, and cellular and mitochondrial metabolism in patients with sepsis to better understand how metabolism contributes to their function or dysfunction during disease (chapter 3). The first aim of this study was to investigate the frequencies and phenotypes of circulating NK cells in peripheral blood from patients with sepsis to determine if they altered their function and metabolism. We have shown that circulating NK cells frequencies in peripheral blood from our cohort of patients with sepsis are similar to those in healthy control subjects. The most significant differences that we observed were that NK cells from sepsis patients have a reduced frequencies of the $CD56^{bright}$ subset, as also reported by Toro *et al.* (2013). Freshly-isolated NK cells from these patients displayed similar activation requirements, functions, and metabolism to those in healthy donors. NK cells from patients with sepsis had increased CD69 and and upreglated levels of the metabolism markers CD71 and CD98 in response to IL-12 and IL-15 stimulation,

however, no changes in granzyme B expression observed. This is in contrary to previous reports of an increase in granzymes B levels in patients with sepsis (Napoli et al., 2012; Zeerleder et al., 2005). The production of IFN-γ by NK cells from sepsis patients was reduced compared to healthy donors. A similar inhibition was also previously reported in patients with sepsis (Boomer et al., 2012; Souza-Fonseca-Guimaraes et al., 2012). As mentioned previously, mTORC1 regulates NK cell activation, metabolism and function. An interesting finding was a reduction of mTORC1 activity in NK cells from patients with sepsis, which parallels the impaired production of IFN-γ. The mitochondrial structure and functions of NK cells were also investigated. NK cells from patients with sepsis displayed similar levels of mitochondrial mass, ATP synthase, mROS and cROS to healthy controls, indicating that NK cells from patients with sepsis had no sign of mitochondrial dysfunction. The results observed in this study suggest that NK cells from patients with sepsis upregulate their function and metabolism similar to healthy donors, with a selective defect in mTORC1 and IFN-γ. Thus an impairment in the antimicrobial activity of NK cells may contribute to the immunosuppression that is characteristic of sepsis or NK cells apoptosis and is correlated with mortality or development of secondary infection (Wesselkamper et al., 2008).

Vδ2 T cells are innate immune cells with multiple effector activities in the immune system and have been tested as an innovative immunotherapeutic target for several diseases (Li et al., 2015). Thus, targeting these immune cells through an immunomodulatory or immunotherapeutic technique may provide a novel approach toward the control of sepsis. In that sense, it was essential first to investigate the basic

biology and cellular metabolism of Vδ2 T cells in both healthy individuals and patients with sepsis (chapter 4). We carried out metabolic and functional analyses to show the impact of sepsis on Vδ2 T cells. We showed that patients with sepsis had significantly lower number of Vδ2 T cells and reduced expression of the activation marker CD69 compared to healthy donors. These results may be associated with the severity of sepsis and the immunosuppression that occurs in these patients. Previous studies also reported impairments in γδ T cells and their functions in septic patients and how these correlated with the severity of sepsis (Andreu-Ballester et al., 2013;Liao et al., 2017). In terms of metabolic analysis, the only difference observed was a higher expression of the amino acid transporter subunit CD98 in patients with sepsis compared to healthy individuals. However, no difference was observed in mTORC1 activity and CD71 expression in Vδ2 T cells from patients with sepsis. Therefore, further stimulation for Vδ2 T cells was performed to indicate any changes that might occur in Vδ2 T cells functions and metabolism. For our metabolic and functional studies, we stimulated Vδ2 T cells from patients and healthy individuals with IL-12/15 or HMB-PP. All of these stimuli are known to induce Vδ2 T cell activation and the production of cytokines, chemokines and cytolytic molecule (Bonneville et al., 2010; Hayday, 2009; Khan et al., 2014; Vantourout & Hayday, 2013). We found that stimulation of Vδ2 T cells from patients with sepsis and healthy individuals with IL-12/15 induced the expression of CD69 leading to a robust upregulation of metabolic markers CD71, but not CD98. Also, HMB-PP-stimulated Vδ2 T cells upregulated the expression of the activation marker CD69 on Vδ2 T cells in both patients with sepsis and healthy individuals. Although Vδ2 T cells from healthy individuals increased IFN-γ production in response to either IL-12/15 or HMB-PP stimulation, the production of IFN-γ was impaired in Vδ2 T cells from

patients with sepsis upon activation with the same stimuli. Similar to what has been previously reported, we observed a decrease in IFN-γ production of by γδ T cells in septic patients compared to healthy controls (Liao et al., 2017). This may be a result of increased apoptosis and immunosuppression during sepsis due to chronic activation (Chung et al., 2006; Correia et al., 2016; Poggi et al., 2004; Venet et al., 2005). Moreover, HMB-PP did not induce the expression of CD71 or CD98 in both patients with sepsis and healthy individuals. Also, no changes were observed in granzyme B, mitochondrial mass and ATP syntheses expression. Vδ2 T cells from sepsis patients had similar levels of mitochondrial ROS and cellular ROS to those in healthy individuals, suggesting that the mitochondria of these cells metabolically function with no evidence of mitochondrial stress and mitochondrial dysfunction. Our data revealed that stimulation with IL-12/15 or HMB-PP upregulated nutrient receptor expression by Vδ2 T cells from healthy individuals which was associated with a marked increase in IFN-γ production. In patients with sepsis, IL-12/IL-15 stimulation of Vδ2 T cells resulted in upregulation of nutrient receptor expression by Vδ2 T cells but not IFN-γ production. A possible explanation is that an excessive expression of PD-1 (programmed death receptor-1), CD279, PD-L1 (programmed receptor ligand 1) or CD274 on lymphocytes which was previously reported in sepsis decreased immune function (IFN-γ production, granzyme B, and CD107a expression), and accelerated death or exhaustion of Vδ2 T cells (Patera et al., 2016; Shao et al., 2016; Wilson, 2018).

In data not shown in the current study, we noted that a high population of granulocytes in PBMC of patients with sepsis, which were present at very low frequencies in control

subjects. These cells displayed a high side scatter, similar to granulocytes, but unlike other granulocytes, they were not pelleted by centrifugation over Lymphoprep™, used to prepare PBMC, giving them the name 'low density granulocytes'. These cells have been described in patients with other immune-mediated diseases and appear to have phenotypes of immature neutrophils (Ui Mhaonaigh et al., 2019). It has been suggested that the frequency of immature granulocytes may serve as a marker to indicate a microbial infection in critically ill patients, in particular sepsis (Park et al., 2011; Van der Geest et al., 2014).

Overall, our study on NK cells and Vδ2 T cells in patients with sepsis had several limitations. First, the number of samples and sample volume were low. Further investigation of a larger cohort of patients and larger amount of blood to perform further metabolic and functional analysis is required. Second, the stage of sepsis in patient cohorts and time point is a crucial key that should be taken into consideration to follow immune system change in sepsis. Third, the type of bacteria that cause sepsis and whether they belong to Gram-negative or Gram-positive families that should be included to gain a better understanding of host immune response under sepsis.

Metabolic reprogramming plays an essential role in the selective activation of various effector functions of macrophages, neutrophils, dendritic cells, NK cells, T cells and B cells. Investigating Vδ2 T cellular immunometabolism in healthy individuals and during infection and sepsis may identify therapeutic approaches targeting Vδ2 T cells for sepsis. We investigated the changes in cellular metabolism and their effector functions of Vδ2

T cells upon treatment with HMB-PP, zoledronate and IL-12/IL-15. We have shown that HMB-PP and especially zoledronate generate high purity Vδ2 T cell lines from PBMC. HMB-PP activation induced moderate upregulation of the nutrient receptor CD71 and CD98 by expanded Vδ2 T cells on day 7, presumably to meet the energy and metabolite requirements of cell proliferation and effector function.

The nature of the stimulus used for Vδ2 T cell activation has an important influence on their effector functions and cytokine profiles (Domae et al., 2017.; Eberl et al., 2009). We found that activation of zoledronate-expanded Vδ2 T cells with HMB-PP and PMA/ionomycin increased IFN-γ production, whereas stimulation with zoledronate, IL-12/IL-15, anti-CD3/CD28 mAb did not induce the production of IFN-γ, IL-4 or IL-17. Therefore, it is possible that zoledronate is ideal for inducing proliferation and expansion of Vδ2 T cells whereas HMB-PP is optimal for inducing cytokine production.

Immune cells have different demands of nutrient and oxygen that are essential and for their survival and functions (Pearce et al., 2013). Therefore, in order to understand Vδ2 T cells metabolic needs and if this can trigger and control their function, leading to immunotherapies developments, we explored for the first time the metabolic profile of Vδ2 T cell using Seahorse technology. Before subjecting Vδ2 T cells to metabolic analysis, Vδ2 T cells were purified from PBMC by positive magnetic bead separation and stimulated overnight with HMB-PP or zoledronate. We observed that when glycolysis was inhibited by adding 2DG, ECAR shuts down almost completely, indicating that resting and activated Vδ2 T cells utilise glycolysis. HMB-PP activated Vδ2 T cells had higher rates of ECAR, basal glycolysis and glycolytic capacity compared to resting Vδ2 T

cells, suggesting that they switch to aerobic glycolysis. We observed that Vδ2 T cells activated with zoledronate had a slightly higher basal level of ECAR compared to resting Vδ2 T cells, indicating that they are using glycolysis. Furthermore, when oligomycin was added OxPhos was inhibited in resting and activated Vδ2 T cells, indicating that these cells are also using OxPhos. While HMB-PP stimulated and zoledronate stimulated Vδ2 T cells exhibited a higher OCR rate compared to resting Vδ2 T cells, HMB-PP activation induces substantially more OxPhos. We show that activated Vδ2 T cells undergo metabolic changes similar to what reported in NK cells and MAIT cells, they increased their glycolysis and OxPhos in response to activation (Assmann et al., 2017; O'Brien et al., 2019).

Therapeutic approaches targeting metabolism of Vδ2 T cells have potential for the treatment of disease, including sepsis. It is likely that the metabolism of these and other immune cells will depend on the stage of sepsis and will change during progression to septic shock and immunosuppression. For instance, increased glycolysis, mTORC1 signalling, and OxPhos play a vital role in IFN-γ production. Furthermore, IFN-γ therapy during sepsis-associated mortality and immunosuppression enhances both the survival and healing from immunoparalysis (Takeyama et al., 2004). Hence this approach may be implemented for a patient displaying sepsis-associated immunosuppression and susceptibility to secondary or hospital-acquired infections.

To conclude, this study evaluated phenotypic, metabolic and functional properties of NK cells and Vδ2 T cells in healthy donors and in patients with sepsis. The results identify

phenotypic and functional changes to NK cells and Vδ2 T cells in sepsis patients and provide evidence to suggest that therapeutic activation of Vδ2 T cells may benefit these patients. The study also has provided a comparison of ways to expand Vδ2 T cells *in vitro*, using a variety of stimuli, and to activate these cells to produce cytokines. It has also enabled us to examine the metabolic requirements of Vδ2 T cells under different stimulatory conditions. This study identifies the importance of metabolism for Vδ2 T cells effector functions and further investigation should be carried out to underpin which metabolic mechanisms are the most crucial. With this knowledge, we must further investigate various stimuli implicated in the metabolic shift of Vδ2 T cells. These findings will help in the development of new approaches to manipulate Vδ2 T cells function to handle complicated conditions like inflammation, sepsis, infection and cancer. Vδ2 T cells are unique in that they can be easily expanded using conserved antigens, they can be selectively activated to carry out multiple functions, including cytotoxicity, cytokine and chemokine production, they can transactivate macrophages, neutrophils, dendritic cells and B cells and they can present peptide antigens to conventional T cells. Vδ2 T cell activators or *ex vivo* expanded Vδ2 T cells can transferred to humans without toxic effects and can modulate downstream immune responses to tumours. Therefore, these cells are attractive candidates for immunotherapy for sepsis. The present investigation also provides the first evidence that the therapeutic activities of Vδ2 T cells may in future be controlled by modulation of the metabolism of these cells.

6. 2 Future Direction

The results of the present investigation provide evidence that Vδ2 T cells may in future be targeted for the treatment of sepsis. As illustrated in Figure 6.1, we propose a potential immunotherapy for sepsis using autologous or allogeneic Vδ2 T cells. These cells can be expanded *in vitro* using zoledronate to generate high purity Vδ2 T cell lines. They can then be re-stimulated using a variety of activators to induce differentiation into effector cells, whose function and metabolism can be modulated in vitro, before they are adoptively transferred to patients. However, it should be noted that further analysis of Vδ2 T cell numbers, functions and metabolism in different stages of sepsis is required before these cells can be tested in humans.

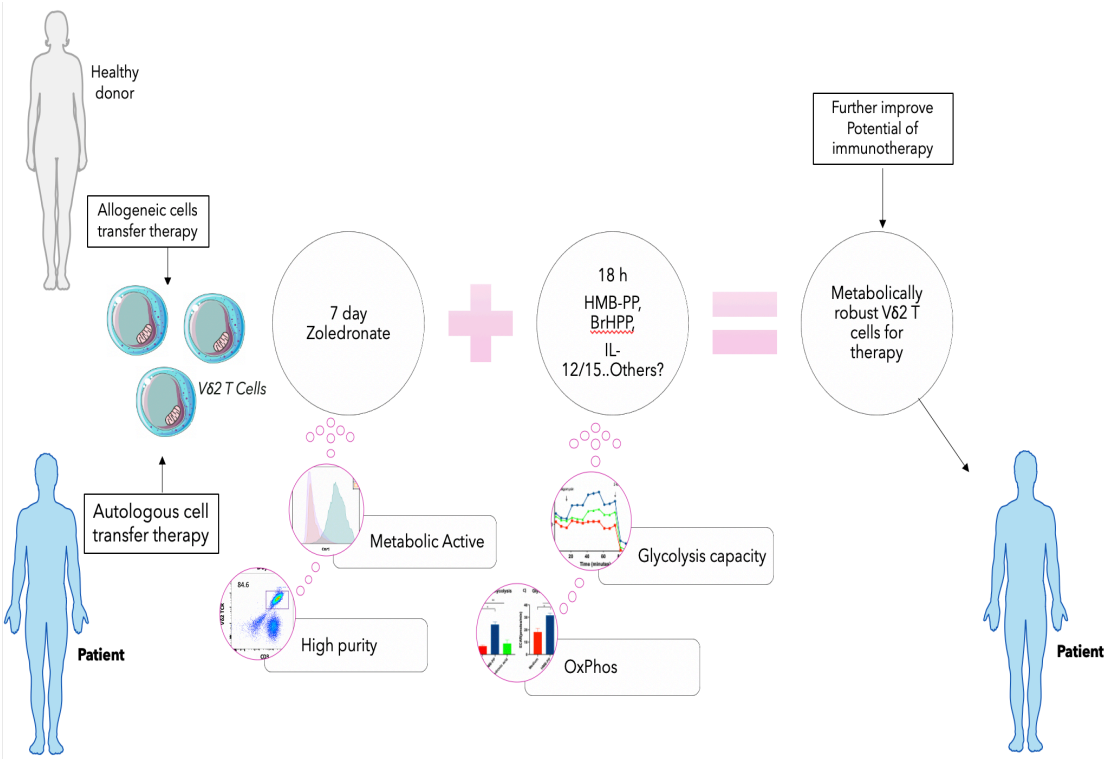

Figure 6. 1 A potential immunotherapy using Vδ2 T cells.

Autologous cell transfer therapy includes using a patient's personal cells, while allogeneic Vδ2 T cells transfer therapy includes using cells from another healthy donor or cord blood Vδ2 T cells lines. These Vδ2 T cells can be controlled ex vivo pharmacologically such that they have improved their functions and then infused into the patient. PBMC or isolated Vδ2 T cells from healthy control subjects can be stimulated with zoledronate (5 µM) for 7 days resulting in higher purities of Vδ2 T cells which are metabolically active. Expanded autologous or allogeneic Vδ2 T cells will then activated with various stimuli such as HMB-PP, BrHPP or IL-12/15, inducing them to undergo metabolic changes to increase glycolysis and OxPhos. These Vδ2 T cells may be better suited to mediate their functions for therapeutic application.

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