Adaptive and Innate T_H1 and T_H17 Responses in Elderly Hospitalised Patients with Infection and Sepsis

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by

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Declaration

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Abstract

Introduction: Sepsis carries a significant burden to health care. Almost a third of ICU patients have sepsis on admission or develop it during their admission. Sepsis carries a high mortality and affects all age groups but becomes more common with a rising mortality in the ageing population. Sepsis accounts for up to 63% of acute medical admissions to ICU for the age group of >60. Current treatment remains limited to antibiotics and good supportive care, as the pathophysiology of sepsis remains somewhat of a conundrum, and outcomes remain largely unchanged over the last few decades. Thus an improved understanding of the pathophysiology of sepsis is a prerequisite to the adoption of novel treatments into clinical practice. This study sought to examine the T_H1 and T_H17 response in adaptive and innate immunity in patients with sepsis. T_H1 response to produce interferon-γ (IFN-γ) is classically stimulated by IL-12 and facilitated by the transcription factor T-Bet. T_H17 response to produce IL-17A is classically stimulated by IL-23 and facilitated by the transcription factor RORγt.

Methods: A prospective observational study of T cell immunophenotypes, followed by cell stimulation to assess the function of these cells (expression of transcription factors T-Bet and RORγt, and expression of IFN-γ and IL-17A) was performed. Flow cytometry was used extensively. The study compared 30 controls, 30 patients with infection but not sepsis, and 42 patients with sepsis. The patients with infection were sampled over 2 time points a week apart, and patients with sepsis were sampled on 4 time points a week apart to assess changes over time. Cell differentiation status was also examined. Patients who were immunocompromised were excluded.

Results for adaptive T cell immunity: Circulating $CD4^+$ T cells from patients with infection predominantly expressed effector-memory or terminally differentiated phenotypes, while $CD4^+$ T cells from patients with severe sepsis predominantly expressed naive phenotypes ($p<0.0001$). $CD4^+$ T cells expressing IL-23 receptor were less frequently found in patients with sepsis compared to patients with infection alone ($p=0.007$). RORγt expression by $CD4^+$ T cells was less frequent in patients with sepsis ($p<0.001$), whereas T-Bet expressing $CD8^+$ T

cells, that did not express RORγt, were less frequent in the sepsis patients. HLA-DR expression by monocytes was lower in patients with sepsis. In septic patients fewer monocytes expressed IL-23.

Results for innate T cell immunity: Mucosal-associated invariant T (MAIT) cell counts were lower in the septic group ($p = 0.002$) and the infection group ($p < 0.001$) than in the control group. MAIT cell T-Bet expression in the infection group was greater than in the septic group ($p = 0.012$). MAIT RORγt expression in the septic group was lower than in the control group ($p = 0.003$). The natural killer (NK) cell counts differed in the three groups ($p < 0.001$), with lower NK cell counts in the septic group ($p < 0.001$) and in the infection group ($p = 0.001$) than in the control group. The NK cell counts increased in the septic group in the 3 weeks following the onset of sepsis ($p = 0.028$).

In lymphocyte stimulation experiments, fewer NK cells expressed T-Bet in the septic group than in the infection group ($p = 0.002$), and fewer NK cells expressed IFN-γ in the septic group than in the control group ($p = 0.002$).

Natural killer T (NKT) cell counts were lower in the septic group than both the control group ($p = 0.05$) and the infection group ($p = 0.04$). Fewer NKT cells expressed T-Bet in the septic group than in the infection group ($p = 0.004$). Fewer NKT cells expressed RORγt in the septic group than in the control group ($p = 0.003$). Fewer NKT cells expressed IFN-γ in the septic group than in both the control group ($p = 0.002$) and the infection group ($p = 0.036$).

Conclusion: Persistent failure of adaptive T cell activation was observed in patients with sepsis. Sepsis was associated with attenuated $CD8^+$ T_H1 and $CD4^+$ T_H17 based lymphocyte response. In sepsis failure of T lymphocyte activation may in turn be related to maladaptive responses in antigen presenting cells.

The clinical presentation of infection and or sepsis in patients is linked with a mosaic of changes in the innate lymphocyte T_H1 and T_H17 phenotypes.

The manipulation of the T lymphocyte phenotype offers a potential avenue for immune modulation in patients with sepsis and warrants further studies.

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List of publications

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Contents

Figures

Tables

Abbreviations

ADMA	Asymmetrical Dimethyl Arginine
APC	Antigen Presenting Cell
ARDS	Acute Respiratory Distress Syndrome
Bcl-6	B cell lymphoma 6
CD	Cluster of Differentiation
CM	Central Memory
CRP	C reactive protein
CTL	Cytotoxic T Lymphocyte
DAMPS	Danger-Associated Molecular Patterns
DC	Dendritic Cell
DDAH II	Dimethylarginine Dimethylaminohydrolase II
DNA	Deoxyribonucleic Acid
EB/AO	Ethidium Bromide/Acridine Orange
EDTA	Ethylenediaminetetraacetic acid
EM	Effector Memory
Fc	Fragment crystallizable
FCS	Foetal Calf Serum
FOXP3	Forkhead Box P3
FSC	Forward Scatter Channel
GATA-3	GATA Binding Protein 3
GCS	Glasgow Coma Scale
HLA	Human Leukocyte Antigen
HMBG1	High-Mobility Group Box 1
I	Ionomycin
ICU	Intensive Care Unit
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNKT	invariant natural killer T
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MAIT	Mucosal-Associated invariant T
MAP	Mean Arterial Pressure
µg	Microgram
µl	Microlitre
MHC	Major Histocompatibility Complex
mls	Millilitres
N	Naïve
NK	Natural Killer
NKT	Natural Killer T
nm	Nanometre

NO	Nitric Oxide
PAMPs	Pathogen-Associated Molecular Patterns
PBA	Phosphate Buffered Saline with Bovine Serum Albumin and sodium azide
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PMA	Phorbol Myristate Acetate
PRRs	Pattern-Recognition Receptors
REC	Research Ethics Committee
RNA	Ribonucleic Acid
RORγt	Retinoic Acid-Related Orphan Receptor gt
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute Medium
SJH	St James's Hospital
SOFA	Sequential Organ Failure Assessment
SSC	Side Scatter Channel
T-Bet	T-Box expressed in T cells
TCR	T Cell Receptor
TD	Terminally Differentiated
T_{FH}	Follicular Helper T Cells
TGF	Transforming Growth Factor
T_H	Helper T
TLRs	Toll-Like Receptors
TNF	Tissue Necrosis Factor
TRAIL	TNF-related apoptosis-inducing ligand
T_{reg}	Regulatory T Cell
U	Unit
UV	Ultraviolet
Vδ	V delta
γδT	Gamma Delta T

Chapter 1

1 Introduction

1.1 Sepsis

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. Organ dysfunction is measured using the Sequential Organ Failure Assessment (SOFA) score and is present when the SOFA score rises by 2 points or more (Table 1.1). Septic shock is a subdivision of sepsis which carries a high hospital mortality of more than 39%, and is present in 10.4% of patients admitted to Intensive Care Units (ICU) [2]. Septic shock is present when the mean arterial blood pressure requires support by vasopressors to maintain a mean arterial blood pressure greater than 65mmHg in the absence of hypovolaemic shock and when the arterial lactate is equal to or greater than 2 [1].

Sepsis remains a significant burden of medical care for hospital medicine and accounts for more hospital admissions than stroke or myocardial infarction [3]. Globally 29.5% of ICU patients have sepsis on admission or develop it during their admission [4]. Despite improvements in recognition and timely treatment, sepsis is frequently lethal; sepsis has a mortality of 25.8% in ICU [4], and a hospital mortality of 17% [5]. With improved supportive care, sepsis mortality rates in Australia decreased overtime from 35% in 2000 to 18.5% in 2012 [6]. Subsequently sepsis mortality rates have not decreased any further and the Irish in-hospital mortality rate in 2018 for sepsis was 20.3%, and the in-hospital crude mortality rate for septic shock was 41.6% [7]. Given the global incidence of sepsis, this represents a significant societal burden.

Sepsis affects all age groups but becomes more common in the ageing population. Sepsis accounts for up to 63% of acute medical admissions to ICU for the age group of >60 [8]. Sepsis is more common in the elderly and mortality rises with age [9]. In the past 40 years the world population of over 60 year olds has more than doubled from 382 million in 1980 to 962 million in 2017 and is predicted to double again in the next 30 years, when it is predicted to reach nearly 2.1 billion by 2050 [10]. Clearly the burden of sepsis continue to increase in hospitals and ICUs around the world. In order to reduce this burden novel sepsis therapies must evolve; therapies based upon an increased understanding of the pathophysiology of sepsis that may lead to improved outcomes.

Table 1-1 Sequential Organ Failure Assessment (SOFA) score.

	SOFA Score				
Variables	0	1	2	3	4
Respiration PaO_2/FiO_2 ratio (mmHg)	>400	≤400	≤300	≤200 with respiratory support	≤100 with respiratory support
Coagulation Platelets ($x10^3/\mu L$)	>150	≤150	≤100	≤50	≤20
Liver Bilirubin (µmol/L)	< 20	20-32	33-101	102-204	>204
Cardiovascular Hypotension (mmHg)	MAP ≥ 70	MAP < 70	Dobutamine use	Epinephrine or norepinephrine ≤0.1µg/kg/min	Epinephrine or norepinephrine >0.1µg/kg/min
Central Nervous System (GCS)	15	13-14	10-12	6-9	<6
Renal Creatinine (µmol/L)	<110	110-170	171-299	300-440	>440

MAP = Mean Arterial Blood Pressure, GCS = Glasgow Coma Scale

1.1.1 Sepsis Treatment

There have been very few advancements in the treatment of sepsis. The only current therapy to show improved survival is early administration of antibiotics [11] and advances in supportive care. It has been shown that the mortality rate rises for every hour delay in the administration of appropriate antibiotics in patients with septic shock [12, 13]. Protein C [14], early goal-directed therapy [15], and low dose steroids [16]; once thought to be beneficial, have more recently proved to be worthless [17-20]. Trials of different vasoactive medications [21-23], antithrombin III therapy [24], intensive insulin therapy [25, 26], and recombinant tissue factor pathway inhibitors [27] have not improved mortality. Some treatments have been associated with harm such as the use of starch containing fluid [26, 28]. The use of steroids, although not having an effect on mortality, has been shown to reduce vasopressor requirements in patients with septic shock [29]. For now the mainstay treatment of sepsis is early appropriate antibiotics, correction of hypovolaemia, source control, and organ support.

The pathophysiological events during sepsis in humans is poorly understood. Sepsis is alternatively hypothesised as a hyperinflammatory state or an immune suppressed state, or that the heterogenous presentation of sepsis is due to a combination of both the aforementioned. As the pathophysiology is becoming better understood molecules are being developed to affect different mediators of sepsis. These include cytokines, cell receptors, transcription factors, coagulation factors, and apoptosis pathways. Therapies to reduce

inflammation and therapies that stimulate innate and adaptive immune responses are currently being studied and being developed.

1.1.2 Sepsis Pathophysiology

When a pathogen invades the human host it displays pathogen-associated molecular patterns (PAMPs), which include endotoxins, lipids (e.g. lipopolysaccharide), bacterial DNA, viral RNA, proteins (e.g. flagellin), and peptidoglycans. These PAMPs bind to receptors on cells of the immune system, particularly monocytes and macrophages. These receptors are called the Pattern-Recognition Receptors (PRRs) which includes a family of receptors called the Toll-Like Receptors (TLRs).

There are 10 members of the TLR family and these receptors are found on innate cells (macrophages, dendritic cells, and natural killer cells), adaptive immune cells (some T cells and B cells), and on non-immune cells such as endothelial cells.

PRRs also bind with Danger-Associated Molecular Patterns (DAMPs) which are produced by the host from cells which are damaged or under stress such as heat-shock proteins, high-mobility group box 1 (HMGB1), and S100 proteins.

These PAMPs and DAMPs bind to the PRRs and stimulate the innate immune response. These herald an inflammatory response which is mediated by the secretion of pro-inflammatory cytokines, particularly Tissue Necrosis

Factor α (TNF-α), Interleukin (IL)-1, IL-2, IL-6, IL-8, and Interferon γ (IFN-γ). There are anti-inflammatory cytokines released as well including IL-10. Conventional wisdom holds that there is an initial pro-inflammatory phase closely followed by an anti-inflammatory phase particularly orchestrated by the adaptive immune cells. However these phases often coincide, and it is thought that sepsis is a result of under or overexpression of one of these phases. Septic shock is thought to be related to pro-inflammatory cytokines such as TNF-α and IL-1 causing vasodilation. However there are also theories that vasodilation may be due to endothelial nitric oxide (NO) expression and that multiorgan failure may be a consequence of dysregulation of the NO synthesis inhibitor asymmetrical dimethyl arginine (ADMA) and NO metabolite dimethylarginine dimethylaminohydrolase II (DDAH II). The dysregulation causing loss of the protective properties of NO and loss of organ vascular autoregulation, leading to impaired blood flow with endothelial and visceral damage. ADMA levels have been shown to correlate with sepsis severity and mortality [30, 31]. NO also regulates immune function and is expressed by macrophages and affects adaptive immune response. Therefore NO inhibition or interference with its synthesis could cause immune dysregulation and increase sepsis mortality [32, 33]. In sepsis, patients tend to become oedematous secondary to capillary leak of fluid from the intravascular space to the extravascular space which also contributes to hypotension. This is due to endothelial dysfunction and damage to the glycocalyx [34]. Angiopoietin-2 is a vasoactive cytokine which is thought to play an important role in the increased permeability of the endothelium [35].

1.2 Human Immune System

The human immune system comprises the innate and adaptive systems. The innate is the early immune response to infection and the adaptive is later. The innate immune system is the first line of defence. The adaptive response requires exposure to a presented antigen to develop an immune response overtime. This response typically takes approximately one week on the first exposure to reach maximal response and three days on the second exposure to reach maximal response.

The innate cells include epithelial and endothelial cells as a barrier, phagocytes (neutrophils and monocytes/macrophages), antigen presenting cells (macrophages, dendritic cells), natural killer (NK) cells, eosinophils, mast cells, innate lymphoid cells, complement, and other immune proteins such as C reactive protein (CRP). Once a pathogen gets through the first line of barriers the main line of defence is through the phagocytic cells. Antigen presenting cells (APCs) produce cytokines and chemokines to initiate inflammation at the site of bacterial infection.

In the case of viral infection NK Cells kill the viral infected cells. NK Cells produce IFN-γ which in turn activates macrophages. NK cells can be identified by surface expression of CD56 in the absence of CD3, with most NK cells also expressing CD16. CD16 is a low affinity receptor for the Fc portion of IgG and thus is involved in antibody mediated cytotoxicity of cells. If the pathogen then reaches the blood stream it encounters the complement system circulating in

blood. The complement system then opsonizes the pathogen for phagocytosis, but can also lyse pathogens.

The adaptive immune response is more specific and develops in response to exposure to antigens and then responds to subsequent re-exposures with the same antigens with a quicker more efficient response thanks to the formation of memory cells. There are two types of adaptive immunity and those are humoral immunity and cell-mediated immunity. Humoral immunity involves B lymphocytes producing antibodies primarily to extracellular microbes and toxins. Antibodies have a very diverse effector mechanism and very diverse antigen receptors. Cell-mediated immunity involves T lymphocytes. T cells primarily deal with intracellular infections but are also involved in dealing with extracellular pathogens by recruiting leukocytes and interacting with B cells in production of antibodies. T cells recognise foreign peptides bound to cell surface proteins called major histocompatibility complex (MHC). T cells only react to antigens bound to MHC molecules on antigen presenting cells (APCs) and not to antigens in free fluid or on pathogens. T cells either help phagocytosis or induce cell death of an infected cell. T cell receptors are specific for MHC molecules.

There are two classes of MHC molecules on human cells. Class I MHCs are expressed on all cells with a nucleus and comprise three isotypes HLA-A, HLA-B and HLA-C. MHC molecules are highly polymorphic and every cell can express six different class I MHCs. Class II MHCs are expressed mainly on the antigen presenting dendritic and macrophage cells and on B cells. Humans inherit three or four alleles from their parents and thus these cells can express more than 8 class II MHC isotypes. These are DPA and DPB, DQA and DQB, DRA and DRB. Thus

these cells express MHC complexes named HLA-DP, HLA-DQ, and HLA-DR respectively. Cytotoxic ($CD8^+$) T cells are class I-restricted and thus kill cells that present pathogen antigens or tumour antigens on their cell surfaces via MHC class I molecules. Helper ($CD4^+$) T Cells are class II-restricted and thus interact with few types of cells. When they interact with dendritic cells, acting as antigen presenting cells, the naïve T cells differentiate into mature helper T cells. Then these mature helper T cells interact with macrophages inducing their function to increase phagocytosis and elimination of extracellular pathogen. The mature helper T cells can also interact with the class II MHC molecules on B cells to promote antibody production and to help eliminate extracellular pathogens. MHC class I expression is upregulated by cytokines such as IFN-α, IFN-β, and IFN-γ. These cytokines are produced during the early innate immune response priming infected nucleated cells to be recognised by cytotoxic T cells. IFN-γ also upregulates class II MHCs in antigen presenting cells.

1.3 T Cells

T cells are produced in the bone marrow and then mature in the thymus. T cells can be identified for immunophenotyping by the cluster of differentiation (CD) 3 molecule present on its cell surface. Thus T cells are identified as $CD3^+$ cells. There are two main types of T lymphocytes. There are adaptive T cells and innate T cells. Adaptive T cells mainly consist of helper T cells and the cytotoxic T cells. Helper T cells secrete cytokines in response to being stimulated. Cytotoxic T cells destroy cells that present viral or tumour antigens on the cell

surface. Further subsets of helper T cells exist which include T_H1, T_H2, T_H17, regulatory T cells (T_{reg}), and follicular helper T cells (T_{FH}).

Innate T cells include natural killer T cells, invariant natural killer T cells, gamma-delta T cells, and mucosal-associated invariant T (MAIT) cells. While adaptive T cells recognise peptide antigens presented by MHC molecules, innate T cells recognise non-peptide antigens; such as lipids, phosphates and other metabolites; presented by other antigen-presenting molecules such as CD1, Butrophilin 3A1, and MR-1.

Antigen presenting cells present antigens to the T cells to initiate the adaptive response, in particular dendritic cells and macrophages do so. A dendritic cell can influences a helper T cell to become an IFN-γ producing cell (T_H1) or an IL-17 producing cell (T_H17) via the selective secretion of cytokines.

1.3.1 Helper T Cells

Helper T cells can be identified for immunophenotyping by the CD4 molecule present on its cell surface. Thus helper T cells are identified as $CD3^+CD4^+$ cells. They express antigen receptors called αβ T cell receptors (TCRs). When $CD4^+$ T cells are activated they produce IL-2 which stimulates clonal expansion of T Cells specific to that antigen. These naïve T cells then become effector cells. Effector cells can secrete other cytokines and migrate to the sites of infection. Some $CD4^+$ T cells recruit leukocytes and aid in phagocytosis and others help B cells produce immunoglobulin (Ig) E to activate eosinophils.

1.3.1.1 T_H1 Cells

T_H1 cells are a subset of $CD4^+$ effector cells. These cells produce IFN-γ, IL-2, and TNF-α. The main transcription factor in these cells is T-Box expressed in T cells (T-Bet). Its primary role is to activate macrophages to increase phagocytosis activity. They also promote the cytotoxic activities of $CD8^+$ T cells and NK cells. They are found at the site of infection, to which they migrate due to the chemokine receptors CCR5 and CXCR3. These chemokines are produced by innate immune cells. Endothelial cells at the site of inflammation express E-selectin and P-selectin for which T_H1 cells express ligands which assist movement of the cells into areas of inflammation. IFN-γ itself amplifies differentiation into T_H1 cells and inhibits differentiation into T_H2 or T_H17 cells subtypes. T_H1 cells are particularly effective against intracellular pathogens. Innate immune reactions to intracellular infection causes IL-12, IL-18, and IFN-γ to be produced which stimulates helper T cell differentiation into the T_H1 subtype. T-Bet is the transcription factor that directs the production of IFN-γ, but also IFN-γ has a positive feedback to increase T-Bet activity.

1.3.1.2 T_H2 Cells

T_H2 cells are a subset of $CD4^+$ effector cells. These cells produce IL-4, IL-5, and IL-13. The main transcription factor in these cells is GATA binding protein 3 (GATA-3). The main role of these cells is in response to parasite infection, and they play a role in B cell differentiation and antibody production. T_H2 cells express the chemokine receptors CCR3, CCR4, and CCR8. These chemokines are usually found at high levels at sites of helminth infections or allergic reactions. It is thought that IL-4 is produced by mast cells and other innate cells to stimulate differentiation towards the T_H2 subset. IL-4 then produced by T_H2 cells has a positive feedback and amplifies differentiation of Helper T cells into this subtype, while suppressing T_H1 and T_H17 differentiation. T_H2 cells induce IgE, mast cell, and eosinophil mediated immunity.

1.3.1.3 T_H17 Cells

T_H17 cells are a subset of $CD4^+$ effector cells. These cells produce IL-17, IL-21, and IL-22. The main transcription factor in these cells is retinoic acid-related orphan receptor γt (RORγt). T_H17 cells express the chemokine receptors CCR6. CCR6 binds to the chemokine CCL20, which is produced by macrophages and some tissue cells. IL-21 produced by T_H17 cells exerts a positive feedback and amplifies differentiation of Helper T cells into this subtype. This T_H subset has a

role in attracting leukocytes and inducing inflammation in response to extracellular bacteria (such as Klebsiella and Citrobacter) and fungi (such as Candida). It also plays a role in autoimmunity and it has a role in anti-inflammation. Dendritic cells produce IL-1 and IL-6 which are inflammatory cytokines and directs differentiation of helper T cells to the T_H17 subset. The dendritic cells produce IL-23, this is important in proliferation and maintenance of the T_H17 population[36]. Transforming growth factor β (TGF-β), an anti-inflammatory cytokine, promotes T_H17 differentiation. TGF-β, IL-6, and IL-1 activate the transcription factors RORγt and STAT3 which in turn are responsible for the effects of this cellular subtype.

IL-17 is a family of 6 cytokines. T_H17 cells produce IL-17A through to IL-17F of which IL-17A is thought to be the more important cytokine in immunity. IL-17 receptors are found on many cell types but their effects are largely unknown. T_H17 are the most important helper T cells in recruiting neutrophils to the site of tissue infections. IL-17 stimulates the production of chemokines and cytokines to mobilise neutrophils and monocytes to the area of inflammation where neutrophils phagocytose bacteria and fungi. IL-17 stimulates production of defensins which are antimicrobial peptides belonging to the innate immune system. IL-22, produced by T_H17 lymphocytes, is a cytokine which maintains endothelial integrity and production of antimicrobial peptides. It is important in the first line of defence of the innate immune system which is the barrier defence of the epithelium. IL-22 can also induce inflammation by stimulating production of chemokines by epithelial cells.

1.3.1.4 Regulatory T Cells (T_{reg})

T_{reg} cells are $CD4^+$. They also express αβ TCRs. The main transcription factor in these cells is Forkhead Box P3 (FOXP3). The immune response is inhibited by T_{reg} cells. Helper T cell and cytotoxic T cell activity are downregulated by producing transforming growth Factor β (TGF-β) and IL-10. It is thought that T_{reg} cells may suppress dendritic cell and B cell activity also. T_{reg} cells may also exhibit immune suppression by cytolysis with perforin or granzymes. T_{reg} cells may also consume IL-2 and hence deprive immune cells of IL-2 stimulation [37].

1.3.1.5 Follicular Helper T Cells (T_{FH})

Follicular helper T cells are a specialised subset of helper T cells. They migrate to the germinal centres of lymph node follicles where they interact with B cells. The main transcription factor in these cells is B cell lymphoma-6 (BCL-6). They produce IL-21 to support production and differentiation of B cells into antibody-producing plasma cells. They also aid in class-switching of B cells.

1.3.2 Cytotoxic T Cells

Cytotoxic T lymphocytes (CTLs) can be identified as $CD3^+CD8^+$ lymphocytes. Activated $CD8^+$ T Cells cause cell death of infected cells. They express $\alpha\beta$ TCRs.

Naïve CTLs can interact with antigens but need to differentiate and proliferate to be able to display activity. Specialised dendritic cells internalise viruses and cross-present the antigens via MHC class I to the naïve CTL. Helper T cells may be needed in where innate immune response is weak such as in latent infections. Helper T cells can stimulate CTL differentiation via the secretion of cytokines IL-2, IL-12, IFN-γ, IL-15, and IL-21. Differentiated CTLs have lysosomes containing proteins such as perforin, granzymes, and cytokines. These lysosomes release their contents and destroy targeted cells. Differentiation relies on the transcription factors T-Bet and eomesodermin to produce these lysosomes.

$CD8^+$ effector T cells can produce IFN-γ similar to T_H1 cells. $CD8^+$ T cells can also produce cytokines that are typically associated with T_H1, T_H2, and T_H17 cells; and have been termed Tc1, Tc2 and Tc17 cells. $CD8^+$ T cells can also differentiate into memory cells.

1.3.3 T Cell Differentiation

Dendritic cells express foreign peptides via the MHC molecules on their cell surfaces. They present them to T cells in lymphoid tissue (lymph nodes and spleen) where naïve T cells are circulating through. APCs also produce other molecules on their cell surface in response to a foreign antigen called co-stimulators. Only once the MHC complex presenting a foreign peptide is present with a co-stimulator on the APC cell surface will a naïve T cell respond. Thus there must be intact APC function to stimulate T cell differentiation. Macrophages and B cells also present antigens to T cells. Macrophages present antigens to helper T cells at the site of infection which in turn further activates the macrophage to enhance pathogen eradication.

A naïve T cell is a mature T cell that has yet to be stimulated by its antigen. Naïve T cells express IL-7 receptors as IL-7 promotes their survival. Naïve cells express a protein called CD45RA, whereas effector and memory cells express CD45RO. Naïve T cells also have a high amount of the chemokine receptor CCR7 (or CD197) which homes the naïve T cell to lymph nodes via chemokines in the high endothelial venules, just as it does for dendritic cells. Once a naïve T cell is activated to differentiate into an effector T cell, it can then mediate pathogen elimination. MHC and antigen recognition is required for the naïve T cell to mature and differentiate along with a co-stimulator as previously mentioned. This ensures that the differentiated T cells will only recognise MHCs bound with an antigen. These differentiated T cells will only recognise the specific antigen with that MHC complex type and results in what is called MHC restriction. IL-2 is

produced when the naïve helper T cell is activated. IL-2 stimulates proliferation and differentiation of the naïve T cell. IL-2 also stimulates T_{reg} cells and thus also suppresses immune response. Naïve T cells then proliferate into effector and memory T cells. The expanding population of effector and memory T cells are antigen-specific. This process is called clonal expansion.

Effector helper T cells can then produce a wide array of cytokines. Effector cytotoxic T cells develop cytoplasmic granules to be able to destroy infected cells. Most effector T cells have a short life span and they do not have IL-7 receptors. Effector cell expression of CCR7 is much reduced so that the T cell remains in the circulation and a change in chemokine receptor expression attracts it to chemokines expressed at infected sites.

Memory T cells are T cells which may survive for long periods of time and express high levels of IL-7 receptors. In humans effector and memory T cells can be hard to distinguish. There are two subsets of memory cells: central memory and effector memory T cells. Central memory T cells express high levels of CCR7 which homes them back to lymphatic tissue providing a longer term response whereas effector memory T cells produce low levels of CCR7 but they do have other chemokine receptors which tend to home them to peripheral tissue where they can produce cytokines in response to antigen stimulation. Central memory T cells have limited effector functions but proliferate quickly in response to repeat exposure to an antigen. Effector memory T cells produce cytokines or become cytotoxic in response to repeat antigen exposure. They have limited proliferation capacity.

The function of the differentiated cells is determined by gene expression and transcription factors. Effector helper T cells can express IFN-γ, IL-4 and IL-17 which depends on their transcription factors T-Bet, GATA-3, and ROR

respectively. These effector helper T cells are T_H1, T_H2, and T_H17 respectively. These subsets of helper T cells have different function and cytokine production in response to different pathogens.

1.3.4 Innate T Cells

Innate T cells are not MHC-restricted and recognise antigens by MHC-like antigen-presenting molecules, such as CD1 and MR1. Innate T cells include natural killer T cells, gamma-delta T cells, and mucosal-associated invariant T (MAIT) cells. These cells can undergo expansion without prior antigen exposure [38].

1.3.4.1 Natural Killer T Cells

Natural killer T (NKT) cells are a group of cells that express αβ or γδ TCRs and NK cell markers such as CD56 and CD161. Some NKT cells express CD4 or CD8 while others express neither ($CD4^-CD8^-$). NKT cells can be activated through their TCR or through cytokine receptors. They are cytotoxic T cells that can be

rapidly activated without the need for prior antigen priming. They are also rapid and potent producers of cytokines that are usually produced by T_H1, T_H2, T_H17, and T_{reg} cells; and therefore play important roles in polarising adaptive immune responses. Two classes of NKT cells are studied in the present investigation. The first is a subset of T cells that express an invariant TCR α-chain (Vα24Jα18) which recognises glycolipid antigens presented by the MHC class I-like antigen presenting molecule CD1d. These cells are termed 'invariant NKT (iNKT) cells' in this thesis. iNKT cells generally account for 0.01-1% of peripheral T cells but are enriched in tissues, such as the liver and adipose tissue. They play important roles in anti-tumour immunity and protection against infectious and autoimmune disease and are under investigation as cellular therapies for cancer. The second group of NKT cells express diverse TCRs and include MHC-restricted and CD1d-restricted T cells, including some iNKT cells. These cells are defined by the co-expression of CD3 and CD56, and account for about 5% of circulating T cells but are enriched in the liver. In this thesis NKT cells are defined as $CD56^+$ T Cells and iNKT cells are defined as $Vα24Jα18^+$ T cells.

1.3.4.2 Mucosal-Associated Invariant T Cells

MAIT cells are specific for microbial riboflavin-derivative antigens presented by the MHC class I-like protein MR1. MR1 binds to metabolites of Vitamin B and thus reacts to pathogens that metabolise Vitamin B including a wide range of bacteria and yeast. MAIT cells have limited TCR variability but they

can be activated by TCR and non-TCR pathways. MAIT cells react rapidly, similar to the other cells of the innate immune system. With TCR activation MAIT cells proliferate and produce cytokines and develop a cytotoxic effector function. Most MAIT cells produce TNF and IFN-γ but some of the population can produce IL-17A and IL-22. MAIT cells can co-express RORγt and T-Bet transcription factors. MAIT cells express cytokine receptors for IL-1, IL7, IL-12, IL-15, IL-18, and IL-23. These cytokines can stimulate MAIT cells. Different combinations of cytokine stimulation induces the MAIT cell to produce different cytokines [39]. Most interpretation of MAIT function in infection is derived from studying mice. There are very few studies of MAIT cell response in infection in humans.

1.3.4.3 Gamma Delta T Cells

Gamma delta T (γδT) cells express γδ TCRS and normally account for approximately 5% of circulating T cells. They can recognise non-protein antigens without the involvement of class I or class II MHC molecules. γδT cells recognise antigens in the form of lipids and a number of metabolites. The function of γδT cells is poorly understood, they produce perforin, granzymes, TNF-related apoptosis-inducing ligand (TRAIL) and a number of cytokines such as IFN- γ, TNF-α, and IL-17 [40]. There is great variation in the distribution of γδT cells in different species. γδT cells are subdivided based on their TCR V region. The subdivisions include Vδ1, Vδ2, and Vδ3 T cells. Vδ2 cells make up the majority of circulating γδT cells in humans. The other two subsets are found at low

frequencies in circulating blood in humans but they make up a large proportion of the lymphocytes found in epithelial regions in the skin, respiratory, and gastrointestinal tracts[41]. The Vδ1 T cell receptor can recognise the MHC class I chain-related proteins A and B (MICA and MICB) expressed by virus-infected and tumour cells; and can recognise CD1c and CD1d molecules presenting lipid antigens. The Vδ2 T cell receptors recognise (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an intermediate in the non-mevalonate pathway of isoprenoid synthesis found in some bacteria and protists. The antigens recognised by Vδ3 T cells are poorly described, but one study has shown that some recognised lipid antigens presented by CD1d [42]. γδ T cells produce cytokines and induce cytotoxicity in an infected cell [43]. Vδ1 and Vδ2 secrete chemokines and cytokines which stimulate antigen presenting cells, phagocytic cells, B cells, and other T cells. Little is known of Vδ3 cells but they have been shown to induce maturation of dendritic cells and B cells.

1.4 B Cells

B cells respond to foreign protein antigens once signalled to do so by helper T cells. These B cells differentiate mainly into IgM antibody-secreting plasma cells, which can then class-switch with the action of helper T cells to produce IgG, IgA, and IgE. Helper T cells are also involved in affinity maturation of antibodies to improve the humoral immune response. Immunoglobulins can bind to pathogens to stop them infecting cells, can bind to pathogens and present them to macrophages, neutrophils, mast cells, eosinophils and NK cells,

and can activate the complement system. Memory B cells are produced and enhance reaction to subsequent infections from the same pathogens in the future.

1.5 Cytokines

Cytokines are signalling proteins secreted by cells that induce or inhibit activity of the cells of the innate and adaptive immune system. Cytokines influence the cell once bound to the receptor for that cytokine. Chemokines are a subset of cytokines that encourage migration of immune cells.

1.5.1 Cytokines in Sepsis

As part of the innate response macrophages and dendritic cells produce TNF, IL-1, and IL-6. These cytokines act locally and cause inflammation. TNF is produced in response to PAMPs and DAMPs. Septic shock is thought to be mediated by a combination of cytokines but in particular TNF-α [44]. If TNF is produced in large enough quantities it is thought to be responsible for some of the clinical findings in septic shock. It can inhibit myocardial contractility leading to a septic cardiomyopathy; It can cause a disseminated intravascular coagulopathy by stimulating endothelial cell expression of tissue factor. Endotoxins leading to TNF production via TLRs as well as other cytokine

production such as IL-1, IFN-γ, IL-12 can lead to septic shock. However, TNF-α also plays a protective role in sepsis and is involved in limiting the spread of infection. There has been interest in the use of the use of anti-TNF-α treatments in septic shock with mixed results and has not lead to the use of these therapies in septic shock, although further exploration of TNF-α blockade with monoclonal antibodies is likely to be undertaken [45].

IL-1 is produced by many cells including macrophages, neutrophils, epithelial and endothelial cells. IL-6 production from monocytes and endothelial cells is stimulated by PAMPs, IL-1, and TNF. TNF and IL-1 cause pyrexia by acting at the hypothalamus. IL-1 and IL-6 act at the liver to produce acute phase proteins such as CRP. While IL-6 has pro-inflammatory properties is also has anti-inflammatory properties by inhibiting release of IL-1 and TNF-α. IL-12 is produced by dendritic cells and phagocytes. IL-12 stimulates T_H1 cellular differentiation and natural killer cells to produce IFN-γ. The role of IL-12 in sepsis is not fully understood. IFN-γ is an important cytokine in the response to intra-cellular infection and macrophage function. While IFN-γ is detectable in sepsis IFN-γ has been shown to improve macrophage function in sepsis in murine models [46].

In 1996 the production of inflammatory cytokines in response to infection as a cause of sepsis was called a cytokine storm [47]. This term was abandoned when it was subsequently shown that anti-inflammatory cytokines are produced in tandem to counteract the effects of pro-inflammatory cytokines and may be responsible for development of sepsis through immunosuppression. These anti-

inflammatory cytokines include IL-10, TNF receptor antagonists, IL-1 receptor antagonists, IL-4, and transforming growth factor β [48].

The role of IL-17 in human sepsis is unknown. It has been studied in murine models of sepsis and found to be important in prevention of infection but some have hypothesised that it is raised in sepsis. IL-17 stimulates release of chemokines to attract neutrophils, it aids neutrophils in phagocytosis, and it is important in mucosal endothelial function. It is important in bacterial, viral, fungal, and parasitic infections [49].

1.6 Hypothesis

The working hypothesis for the research presented in this thesis is that in sepsis the T_H1 and T_H17 immune response is that of an underactive immune response rather than an overactive immune response as hypothesised in other works. Specifically the T_H17 response has been suggested as being a cause of sepsis in murine models [50] however we know that murine models of immunity often do not translate to human immunity. For example mice do not have Vδ2 cells and so it is difficult to compare innate immunity. IL-17A has been shown to be higher in bronchoalveolar lavages of patients with ARDS and associated with higher sepsis scores [51]. Crucially no work has been done to compare the human response to infection in patients who do not develop sepsis to those who do develop sepsis and the hypothesis was that the response to infection may have an increased T_H1 and T_H17 when compared to the response in sepsis. We

hypothesised that there may be a problem with T cell differentiation whether it be a problem with antigen presentation or an inherent issue with the T cells themselves.

1.7 Objectives

The objective is to examine the role of T_H1 and T_H17 lymphocyte responses in human infection and sepsis of elderly patients.

The method to meet this objective is to compare adaptive and innate T_H1 and T_H17 responses in controls, patients with infection but not sepsis, and patients with sepsis using flow cytometry. This is examined in the following ways:

1) To compare IL-12 and IL-23 production.

2) To compare monocyte HLA-DR expression to examine antigen presentation to T cells.

3) To compare frequency and numbers of T cell subsets.

4) To compare IL-12 receptor and IL-23 receptor expression on these T cells.

5) To compare differentiation status of these T cells.

6) To compare transcription factor expression of T-Bet and RORγt in these T cells to infer potential to produce IFN-γ and IL-17A respectively.

7) To stimulate T cells to assess their actual production and potential production of IFN-γ and IL-17A.

Ultimately the overall objective is to assess if there is a problem with T_H1 and T_H17 responses in sepsis to see if further studies are warranted to consider this immune response as a potential target for immunotherapy in sepsis.

Chapter 2

2 Materials and Methods

2.1 Materials

Several types of equipment, consumables, reagents, and fluorochrome-conjugated monoclonal antibodies were used for the experiments as part of this thesis. Equipment used in these experiments are listed in Table 2-1. Consumables used in these experiments are listed in table 2-2. Reagents used in these experiments are listed in table 2-3. The fluorochrome-conjugated monoclonal antibodies used in these experiments are listed in table 2-4.

Table 2-1 Equipment and software used

Equipment	Model	Company
Balance	Adventurer Pro	Ohaus, NJ, USA
Centrifuge	Eppendorf 5810 and 5415D	Eppendorf, Hamburg, Germany
Haemocytometer	Kova Slides	Hycor Biomedical, IN, USA
CO2 Incubator	Heracell 150i	Thermo Fisher Scientific, MA, USA
Flow Cytometer	FACSCanto™ II + FACS Diva Software	Becton Dickinson, Oxford
Flow Jo	v10.2	Treestar Incorporated, NJ, USA
GraphPad Prism	v6	GraphPad Software Incorporated, CA, USA
Laminar air flow Class II safety Cabinet	CleanAir MSC BSS6-2	Thermo Fisher Scientific, MA, USA
Laminar air flow Class II safety Cabinet	MSC 1.2	Thermo Electron LED Gmbh, Germany
Microscope	Inverted: IX51	Olympus Corporation, Japan
Multichannel pipette	Finnpipette F1	ThermoFisher Scientific, USA
Pipettes p20, p100, p1000	Labmate pro	HTL lab solutions, Poland
Vortex	Vortex Genie	Scientific Industries, NY, USA
Vortex	IKA vortex Genius 3	Sigma-Aldrich, UK
Water bath	Grant	Fisher Scientific, PA, USA

Table 2-2 Consumables

Consumable	Source	Catalogue No.
Biohazard bin liners	Fisher Scientific	11-889-5
Flow cytometry tubes – FACS Canto	Fisher Scientific	12650366
FACS Clean Solution	BD Biosciences, Oxford, UK	340345
FACS Flow Sheath Fluid	BD Biosciences, Oxford, UK	342003
FACS Shutdown Solution	BD Biosciences, Oxford, UK	334224
Nitrile Gloves, large	Fisher Scientific	19041171D
Maxisorp ELISA plates	Thermo-Scientific Nunc	442404
P10 pipettor tips	Sarstedt	70.1130
P200 pipettor tips	Sarstedt	70.760.002
P1000 pipettor tips	Fisher Scientific	FB78084
Plastic pipettes, 3ml	Sarstedt	86.1171.010
Plastic pipettes, 5ml	Corning	PN5E1
Sharps bins	Fisher Scientific	SAT-641-060M
Tubes, 0.5ml	Sarstedt	72.730.006
Tubes, 2ml	Sarstedt	72.694.006
Tubes, 5ml	Sarstedt	62.558.201
Tubes, 15ml	Sarstedt	62.554.502
Tubes, 50ml	Sarstedt	62.547.254
Tissue Paper	Fisher Scientific	CMC-716-021F
Waterbath Treatment	Sigma-Aldrich	S5525

Table 2-3 Reagents

Reagent	Source	Catalogue No.
Acridine Orange	Sigma-Aldrich	A6014
BD FACS™ lysing solution	BD Biosciences	349202
Bovine Serum Albumin	Fisher Scientific	BPE-9703-100
Brefeldin A	eBioscience	00-4506-51
Cytometer setup and tracking research beads	BD Biosciences	655050
Ethanol 95%	Sigma-Aldrich	
Ethidium Bromide	Alfa Aesar	1239-45-8
Foetal Bovine Serum, Hyclone	Thermo Scientific	SH3 071.03
FoxP3 Staining Buffer Set	Miltenyi Biotec	130-093-142
Hepes	Gibco	15630056
Ionomycin	Sigma-Aldrich	IO634
Lightning-Link® Fluorescein Labelling Kit	Innova Biosciences Ltd	707-0030
Lipopolysaccharide	Sigma-Aldrich	
Live/Dead Fixable Aqua Dead Cell Stain	Thermofisher	L34957
Live/Dead Fixable Near Infrared Dead Cell Stain	Thermofisher	L34976
Lymphoprep™	Biosciences	1114547
OneComp beads	eBioscience	01-1111-42
Paraformaldehyde 4% in PBS	Sata Cruz, USA	Sc-281692
Penicillin/Streptomycin	Gibco	15140122
Phosphate Buffered Saline, sterile	Gibco	14190094
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	P1535
RPMI medium 1640 (1x) + Glutamax™-I	Gibco	61870010
Saponin	Sigma-Aldrich	S7900
Sodium Azide	Sigma-Aldrich	S8032

Table 2-4 Fluorochrome-conjugated monoclonal antibodies

Manufacturer	Antibody (Anti-)	Species Reactivity	Clone
Miltenyi Biotec *Surrey, UK*	CD4-Viobright FITC	Human	REA623
	CD197 PerCP-Vio700	Human	REA546
	CD56-Viobright FITC	Human	AF12-7H3
	CD19-APC-Viobright	Human	LT19
	CD45RA-PE-Vio770	Human	REA562
	IL-12R β2-APC	Human	REA333
	CD3-APC-Vio770	Human	REA613
	CD27-VioBright FITC	Human	M-T271
	CD27-FITC	Human	REA499
	TCR-Vδ1-PerCP-Vio 700	Human	REA173
	TCR-Vδ2-VioBlue	Human	123R3
	TCR-Vδ2-VioBlue	Human	REA771
	CD161-VioBright FITC	Human	REA631
	CD161-FITC	Human	191B8
	CD8 (BW135/80)-APC-Vio770	Human	BW 135/80
	CD3-VioGreen	Human	REA613
	TCR-V α7.2-VioBlue	Human	REA179
	CD3-VioBlue	Human	BW264/56
	iNKT-PE-Vio770	Human	6B11
	CD14-FITC	Human	TÜK4
	CD14-FITC	Human	REA599
	CD25-VioBright FITC	Human	4E3
	CD4-PerCP-Vio700	Human	REA623
	CD127-PE-Vio770	Human	REA614
	IFN-γ-PE	Human	REA600
	ROR γ (t)-PE	Human	REA278
	HLA-DR-PerCP-Vio700	Human	REA805
	CD56-PerCP-Vio700	Human	REA196
	CD16-PE-Vio770	Human	REA423
	CD8-PE-Vio770	Human	REA734
	IL-12(p35/p70)-APC	Human	REA121
	IL-17A-APC	Human	CZ8-23G1
	T-BET-APC	Human	REA102
	CD19-VioBlue	Human	LT19
R&D Systems Minnesota, US	IL-23R PE	Human	218213
	IL-23 p19 PE	Human	727753
Beckman Coulter, High Wycombe, UK	Vδ3 FITC		Polyclonal, conjugated in house

2.2 Methods

2.2.1 Ethical Approval

This research was carried out in accordance with the Declaration of Helsinki ethical guidelines for medical research involving human subjects. Ethical approval was granted by the St James's Hospital and Adelaide, Meath and National Children's Hospitals Research Ethics Committee (SJH/AMNCH). The REC reference is "2015-03 Chairman's Actions (9)." All specimens were collected after patient information leaflets were distributed and with prior informed written consent, from patients attending St James's Hospital, or from healthy age- and sex-matched donors. Where patients did not have capacity to consent, assent was provided by the next of kin and patients were subsequently consented when they regained capacity.

2.2.2 Stages of this Study

The study was comprised of two stages. The first stage was an immunophenotyping study to examine the phenotype of the T cells circulating in blood in the different groups of patients and controls. The first stage concentrated the state of differentiation of the cells and on the presence of IL-23 receptor and IL-12 receptor. The second stage of the study used in vitro stimulation of PBMCs to examine the response of these cells to stimulation. This

primarily focused on expression of cytokines IL-23 and IL-12 by antigen presenting cells which stimulate the T_H17 and T_H1 responses respectively. T cell expression of cytokines IFN-γ and IL-17A and T cell expression of transcription factors RORγt and T-Bet was examined. The study was performed over a two-year period from 2016 to 2018.

2.2.3 Subjects

Demographic data and clinical information were collected from the subjects (Table 3-1). For the purposes of this study we examined three groups of patients. The first group of interest were patients with sepsis. The second group of patients were patients with infection but not sepsis. The third group were controls. It was of particular interest to see if there was a difference in immune response between the patients with infection on the wards and the patients with sepsis in intensive care.

In the first stage of the study, immunophenotyping, a total of 42 septic patients, 30 patients with infection but not sepsis, and 30 controls were recruited. The patients in the sepsis group were recruited from the general intensive care unit (ICU) in St James's Hospital, according to the Sepsis 3 clinical criteria [1]. Blood samples were taken within 72 hours of admission and weekly thereafter for a further 3 samples or until death or discharge from hospital. Mortality was defined as mortality while in ICU but was also recorded as mortality in that hospital admission. The occurrence of secondary infections was

recorded. The patients in the infection group were recruited from the general hospital wards in collaboration with the clinical microbiology service. Blood samples were taken within 72 hours of positive cultures and a second blood sample was taken a week later unless the patient died or was discharged from hospital. The healthy control group was recruited from the community and the subjects did not have any current or recent infections. One blood sample was taken for each control subject.

In the second stage of the study, cell stimulation, blood samples were taken at one time point within a median of 5 days of onset of illness in the septic group, within 3 days of confirmation of infection in the infection group, and without infection in the control group.

Exclusion criteria in both parts of the study for all three groups were applied which included pre-existing immunodeficiency, immuno-modulating medications including steroids, chronic infection, malignancy, pre-existing liver disease, and any haematological disease.

2.2.4 Immunophenotyping Study

3mls of blood was collected in an EDTA Vacutainer™ tube for each patient. Samples were brought to the laboratory and processed immediately. Fluorochrome-conjugated monoclonal antibodies (mAb) specific for human cell-surface receptors, transcription factors and cytokines and their sources are listed in Table 2-4. Cocktails of up to 8 mAbs for flow cytometry were made up

in 50µl PBA buffer (Phosphate Buffered Saline with 1.5% Bovine Serum Albumin and 0.02% sodium azide). 100 µls of fresh whole blood was added to a flow cytometry tube per antibody panel. 50µl of LIVE/DEAD Fixable Aqua Dead Cell Stain (Molecular Probes) diluted in PBS (Phosphate Buffered Saline) was added to the blood, vortexed, and incubated in the dark for 15 minutes at room temperature. The antibody cocktail of interest was then added to the sample, vortexed, and incubated for 15 minutes in the dark at room temperature. Red cells were then lysed by adding 1ml of BD FACS™ Lysing Solution to each tube, vortexing the tube, and incubating in the dark for 15 minutes at room temperature. Cells were washed with 1ml of PBA, the sample was centrifuged at 1500rpm for 7 minutes. The supernatant was discarded. The samples were then washed with 2mls PBA per tube, vortexed, and centrifuged at 1500 rpm for 7 minutes. The supernatants were discarded and the samples were resuspended with a small amount of PBA, vortexed, and then acquired immediately with a BD FACSCanto™ II flow cytometer. The samples were kept cool and acquisition was completed within 2 hours. One patient sample in the infection group was not possible to analyse most likely due to a very high bilirubin level causing autofluorescence. Absolute cell counts were determined from cell frequencies and whole blood counts obtained at the time of blood sample collection.

2.2.5 Ex Vivo Cell Stimulation Study

15mls of blood was collected in EDTA Vacutainers™ for each patient. Samples were brought to the laboratory and processed immediately. They were processed under sterile conditions in a biosafety cabinet with sterile materials. Peripheral blood mononuclear cells (PBMCs) were prepared (see section 2.2.5.1). PBMC cell count was then performed (see section 2.2.5.2).

Wells intended for stimulating cells with CD3/CD28 were prepared in a 96 well flat bottomed microtiter plate >24 hours prior to the experiment. 0.05 µl of pure functional grade CD3 mAb (clone OKT3; Miltenyi Biotec) and 0.05 µl of pure functional grade CD28 mAb (clone 15E8; Miltenyi Biotec) in 100 µl of phosphate buffered saline were added to each well and incubated at 37°C and 5% CO_2. These wells were then aspirated and washed once with 100 µl PBS and aspirated prior to adding the cells for stimulation.

0.5 million cells were added to each well of the 96 well flat bottomed microtiter plate in 200µl of RPMI. Wells intended for stimulation with Phorbol Myristate Acetate (PMA) and Ionomycin had 50ng/ml PMA and 1 µg/ml Ionomycin added to them. Wells intended for lipopolysaccharide (LPS) stimulation had 10 ng/ml LPS added to them.

Wells intended for intracellular staining for IL-23, IL-12, IL-17A, and IFN-γ had 10 µg/ml Brefeldin A added to them to prevent cytokine release from the cells.

The cells were then stimulated for 5 hours at 37°C and 5% CO2.

Antibody panels were prepared for extracellular staining and made up to 50 µls with PBA per sample. Antibody panels were prepared for intracellular staining and made up to 50 µl with 0.2% saponin (in PBA buffer) for IL-12, IL-23, IL-17A, and IFN-γ per sample. Antibody panels for intracellular staining of RORγt and T-Bet were made up to 50 µl with Permeabilization Buffer (Miltenyi FoxP3 Staining Buffer Set).

Cells from the wells were transferred to flow cytometry tubes. 1ml phosphate buffered saline was added to the tubes and they were centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and they were resuspended in 100µl of Thermofisher LIVE/Dead Fixable Near IR Dead Cell Stain, vortexed, and incubated for 15 minutes in the dark at room temperature. 1ml PBA was added and centrifuged for 5 mins at 1500 rpm. The supernatant was discarded and the cells were resuspended in the 50 µl extracellular antibody panels, vortexed, and incubated for 15 minutes in the dark at room temperature. 1 ml PBA was added to the tubes and centrifuged at 1500rpm for 5 minutes. The supernatant was discarded.

For intracellular staining of RORγt and T-Bet the Miltenyi FoxP3 Staining Buffer Set and protocol was used. The cells were fixed by resuspending in 1 ml of Fixation/Permeabilisation solution, vortexing and incubating for 30 minutes. 1ml PBA was added and centrifuged for 5 minutes at 1500rpm. 1ml of Permeabilisation Buffer, diluted 1/10 in water was added, vortexed, and then centrifuged at 1500rpm. The supernatant was discarded and the T-Bet and RORγt antibodies were added, vortexed, and incubated at 4°C for 30 minutes in the dark. 1ml cold permeabilisation buffer was added and centrifuged at

1500rpm for 5 minutes. The supernatant was discarded and the cells were fixed by resuspending in 1% paraformaldehyde (PFA) for 10 minutes in the dark. 1ml of PBA was then added and centrifuged, the supernatant was discarded, and the cells were resuspended in a small amount of PBA. The samples were kept cold and acquired immediately with a BD FACSCanto™ II flow cytometer.

For intracellular staining of IL-12, IL-23, IL-17A, and IFN-γ the cells were fixed by resuspending in 0.5mls 4% PFA and incubating in the dark for 10 minutes. 1ml of PBA was added and the samples then centrifuged for 5 minutes at 1500rpm. The supernatant was discarded and the samples were resuspended in 0.2% Saponin and incubated in the dark for 10 minutes to permeabilise the cells. These samples were then centrifuged at 1500rpm for 5 minutes. The supernatant was discarded. The intracellular cytokine monoclonal antibody panels were added, vortexed, and incubated in the dark for 20 minutes. 1 ml PBA was then added and centrifuged for 5 minutes at 1500rpm. The supernatant was then discarded and the cells were resuspended in 0.5mls 1% PFA for 10 minutes. 1ml PBA was added and the samples were then centrifuged for 5 minutes at 1500rpm. The supernatant was discarded and the cells were resuspended in a small amount of PBA. The samples were kept cold and acquired immediately with a BD FACSCanto™ II flow cytometer.

2.2.5.1 Peripheral Blood Mononuclear Preparation

PBMCs were prepared by standard density gradient centrifugation over Lymphoprep™. 15 ml of blood was diluted 1:1 with 1% Foetal Calf Serum (Phosphate buffered saline with 1% foetal calf serum). This was then layered on top of 10 ml Lymphoprep™. The sample was then centrifuged at 400g for 25 minutes with the brake off. The buffy coat was collected with sterile pastettes and topped up to 10mls with PBS containing 1% Foetal Calf Serum (FCS). This was centrifuged for 8 minutes at 1500 rpm. The supernatant was discarded. The sample was topped up again with 10mls 1% FCS and centrifuged for 8 minutes at 1500 rpm. The supernatant was discarded. The cells were resuspended in 3-4mls of complete RPMI medium (RPMI 1640 containing GlutaMAX, 10% HyClone FBS, 50mg/mL streptomycin, 50U/mL penicillin, 2.5µg/ml amphotericin B - fungizone, and 25mM HEPES.

2.2.5.2 Counting Of Viable Cells

The final cell suspension was counted on a haemocytometer. The cells to be counted were diluted by a factor of 20 as follows: 10 µl of the solution was stained with 190 µl ethidium bromide/acridine orange (EB/AO) solution to detect dead and live cells respectively. This was then applied to a Glasstic slide and viewed through a UV microscope. Under UV light live cells stain green with

the acridine orange and dead cells stain orange from the ethidium bromide. EB/AO solution is made up of 10 µg/mL ethidium bromide and 30 µg/mL acridine orange in PBS. The average number of live cells were counted in 2 large squares, then multiplied by the dilution factor, and then by a factor of 10^4 to give the cell count/ml of the final cell suspension.

2.2.6 Principles of Flow Cytometry

A flow cytometer is an apparatus that passes a single cell through a chamber crossing a laser beam. As the cell passes through the laser beam the light will scatter in different directions. By labelling cells with fluorescent antibodies one can expose the cell to a particular laser wavelength to excite that particular fluorescent antibody, and if present on the cell light will be emitted by the fluorochrome and detected by the flow cytometer. Multiple laser wavelengths can be used for the detection of multiple fluorescent antibodies on a cell and detected by emission filters. Therefore a single cell can be interrogated to find out more about its functional and physical characteristics.

Fluid called sheath fluid is run through the flow cytometer. It runs in a laminar flow. Cells are aspirated from the sample tubes and injected under pressure into the centre of the laminar fluid. Hydrodynamic focusing causes the cells to travel in single file in the direction of the sheath fluid. This is how cells are passed individually through a chamber for analysis.

Multiple lasers are passed through the chamber. The BD FACSCanto™ II flow cytometer was used for these experiments. It uses 3 lasers to excite fluorochromes. It uses an argon (blue) laser at 488nm, a HeNe (red) laser at 633nm, and a violet laser at 405nm. It has 8 fluorescence channels and thus allows up to 8 attributes to be examined per cell passing through the chamber. The argon laser allows for excitation and detection of green fluorescence (FITC), yellow fluorescence (PE), red fluorescence (PerCP or PerCP-Cy5.5), and infra-red fluorescence (PE-C7). The HeNe laser allows for excitation and detection of red fluorescence (APC) and Infra-Red fluorescence (APC-Cy7). The Violet laser allows for excitation and detection of blue fluorescence (Pacific Blue), and Cyan fluorescence (AmCyan). The BD FACSCanto™ II flow cytometer can detect up to 10,000 events per second.

There are three types of detectors in the chamber. There is one detector placed in directly in front of the laser source (forward scatter channel or FSC), there is one detector placed 90 degrees to the side of the laser source (side scatter channel or SCC), and the third type of detector is the fluorescence detectors. When a cell passes through a laser it will scatter light in multiple directions. The FSC will give information about the size of the cell passing through the chamber. The SCC will give information about the shape and the complexity of the cell. Thus when combining FSC and SCC properties of cells it is possible to see different populations of cells based on their size and complexities. The fluorescence detectors will detect the light emitted by the fluorescent-labelled antibodies or dead cell stains. Emission filters allow for detection of multiple fluorochromes at once from a cell with multiple labels on

it. These detectors can also quantify the amount of the fluorescence emitted from the stimulated fluorochrome also.

The fluorescent signal is then converted to a digital form and presented graphically by software on a computer and allows for analysis of the cells.

2.2.7 Fluorochrome Conjugated Antibodies used for Immunophenotyping

For this experiment 6 panels were devised to immunophenotype T cells in whole blood. See Table 2-4 for full details of antibodies used. The first panel (Table 2-5) examined T cells as $CD3^+$, helper T cells as $CD3^+CD4^+$, and cytotoxic T cells as $CD3^+CD8^+$. It was possible to examine the differentiation state of these cells with CD197 (also known as CCR7) and CD45RA. Differentiation status was defined as: naive ($CD45RA^+$, $CD197^+$), central memory ($CD45RA^-$, $CD197^+$), effector memory ($CD45RA^-$, $CD197^-$), or terminally differentiated ($CD45RA^+$, $CD197^-$). It was then possible to look at IL-23 receptor and IL-12 receptor on these cells.

Table 2-5 Panel 1

Laser Filter	Laser	Colour	Panel 1
530/30	FL1	Viobright FITC	CD4
585/45	FL2	PE	IL-23R
670-735	FL3	PerCP-Vio700	CD197
780/60	FL4	PEVio770	CD45RA
660/20	FL5	APC	IL-12R
780/60	FL6	APC Vio770	CD8
450/50	FL7	Vioblue	CD3
510/50	FL8	Amcyan/Viogreen	Dead Cell Stain

The second panel (Table 2-6) examined $CD56^+$ T cells as $CD3^+CD56^+$, which as mentioned previously defined NKT cells in this thesis. It also examined B Cells as $CD3^-CD19^+$ and natural killer cells as $CD3^-CD56^+$. This allowed for enumerating cells as total lymphocyte counts were known. B cells, T cells, and NK cells make up more than 98% of circulating lymphocytes allowing for approximation of the total count of these cells. Again, differentiation status and IL-23 receptor and IL-12 receptor could be examined.

Table 2-6 Panel 2

Laser Filter	Laser	Colour	Panel 2
530/30	FL1	Viobright FITC	CD56
585/45	FL2	PE	IL-23R
670-735	FL3	PerCP-Vio700	CD197
780/60	FL4	PEVio770	CD45RA
660/20	FL5	APC	IL-12R
780/60	FL6	APC Vio770	CD19
450/50	FL7	Vioblue	CD3
510/50	FL8	Amcyan/Viogreen	Dead Cell Stain

The third panel (Table 2-7) examined γδT cells, specifically subdivisions include Vδ1 ($CD3^+$ $Vδ1^+$), Vδ2 ($CD3^+Vδ2^+$). It looked at the differentiation status of these cells. Differentiation status was determined as naive ($CD45RA^+$, $CD27^+$), central memory ($CD45RA^-$, $CD27^+$), effector memory ($CD45RA^-$, $CD27^-$), or terminally differentiated ($CD45RA^+$, $CD27^-$). It was then possible to examine the IL-23 receptor and IL-12 receptor status of these cells.

Table 2-7 Panel 3

Laser Filter	Laser	Colour	Panel 3
530/30	FL1	Viobright FITC	CD27
585/45	FL2	PE	IL-23R
670-735	FL3	PerCP-Vio700	TCR Vdelta1
780/60	FL4	PEVio770	CD45RA
660/20	FL5	APC	IL-12R
780/60	FL6	APC Vio770	CD3
450/50	FL7	Vioblue	TCR Vdelta2
510/50	FL8	Amcyan/Viogreen	Dead Cell Stain

The fourth panel (Table 2-8) examined MAIT cells ($CD3^+V\alpha7.2^+CD161^+$). It allowed to look at a subpopulation for MAIT $CD8^+$ T cells. It allowed examination of differentiation of these cells and IL-23 receptor and IL-12 receptor status. Due to constraints of 8 colour detectors dead cell stain was omitted from this panel. All other panels confirmed negligible dead cells due to all samples being processed immediately upon drawing of fresh blood.

Table 2-8 Panel 4

Laser Filter	Laser	Colour	Panel 4
530/30	FL1	Viobright FITC	CD161
585/45	FL2	PE	IL-23R
670-735	FL3	PerCP-Vio700	CD197
780/60	FL4	PEVio770	CD45RA
660/20	FL5	APC	IL-12R
780/60	FL6	APC Vio770	CD8
450/50	FL7	Vioblue	TCR Vα7.2
510/50	FL8	Amcyan/Viogreen	CD3

The fifth panel (Table 2-9) intended to examine iNKT cells and Vdelta 3 cells. Anti-Vdelta 3 antibody was made in FITC fluorochrome using a Lightning-Link Rapid Conjugation System by Innova Biosciences as per company protocol. There were minimal iNKT cells and minimal Vδ3 Cells in circulating blood of septic patients, patients with infection, and controls. This panel was omitted from publications as there were insufficient/minimal cells to analyse. It was confirmed that the iNKT antibodies worked on a separate iNKT cell line outside of the remit of this experiment to ensure that it was not an issue with the antibodies.

Table 2-9 Panel 5

Laser Filter	Laser	Colour	Panel 5
530/30	FL1	Viobright FITC	Vdelta3
585/45	FL2	PE	IL-23R
670-735	FL3	PerCP-Vio700	
780/60	FL4	PEVio770	iNKT
660/20	FL5	APC	IL-12R
780/60	FL6	APC Vio770	
450/50	FL7	Vioblue	CD3
510/50	FL8	Amcyan/Viogreen	Dead Cell Stain

The sixth panel (Table 2-10) examined T_{reg} cells ($CD3^+CD4^+CD25^+CD127^-$) and the status of IL-23 receptors and IL-12 receptors on these cells.

Table 2-10 Panel 6

Laser Filter	Laser	Colour	Panel 6
530/30	FL1	Viobright FITC	CD25
585/45	FL2	PE	IL-23R
670-735	FL3	PerCP-Vio700	CD4
780/60	FL4	PEVio770	CD127
660/20	FL5	APC	IL-12R
780/60	FL6	APC Vio770	
450/50	FL7	Vioblue	CD3
510/50	FL8	Amcyan/Viogreen	Dead Cell Stain

Flow cytometry data was analysed using FlowJo software. Single stains were used for compensation controls and fluorescence minus one (FMO) controls were used to help set gates.

2.2.8 Fluorochrome Conjugated Antibodies used for Cell Stimulation

The first panel (Table 2-11) was designed to look at B cells and monocytes ($CD14^{+}$). Specifically looking at production of IL-23 and IL 12, and to compare monocyte HLA-DR presence between the different groups.

Table 2-11 Panel A

Laser Filter	Laser	Colour	Panel A
530/30	FL1	Viobright FITC	CD14
585/45	FL2	PE	IL-23
670-735	FL3	PerCP-Vio700	HLA-DR
780/60	FL4	PEVio770	CD16
660/20	FL5	APC	IL-12
780/60	FL6	APC Vio770	Dead Cell Stain
450/50	FL7	Vioblue	CD19
510/50	FL8	Amcyan/Viogreen	CD3

The second and fourth panels (table 2-12) were to examine Vδ1 and Vδ2 cell production of cytokines IL-17A and IFN-γ and presence of transcription factors RORγt and T-Bet.

Table 2-12 Panels B and D

Laser Filter	Laser	Colour	Panel B	Panel D
530/30	FL1	Viobright FITC	CD4	CD4
585/45	FL2	PE	IFN-γ	RORγt
670-735	FL3	PerCP-Vio700	Vδ1	Vδ1
780/60	FL4	PEVio770	CD8	CD8
660/20	FL5	APC	IL-17A	T-Bet
780/60	FL6	APC Vio770	Dead Cell Stain	Dead Cell Stain
450/50	FL7	Vioblue	Vδ2	Vδ2
510/50	FL8	Amcyan/Viogreen	CD3	CD3

The third and fifth panels (table 2-13) were to examine MAIT cell and NKT cell production of cytokines IL-17A and IFN-γ and presence of transcription factors RORγt and T-Bet.

Table 2-13 Panels C and E

Laser Filter	Laser	Colour	Panel C	Panel E
530/30	FL1	Viobright FITC	CD161	CD161
585/45	FL2	PE	IFN-γ	RORγt
670-735	FL3	PerCP-Vio700	CD56	CD56
780/60	FL4	PEVio770		
660/20	FL5	APC	IL-17A	T-Bet
780/60	FL6	APC Vio770	Dead Cell Stain	Dead Cell Stain
450/50	FL7	Vioblue	Vα7.2	Vα7.2
510/50	FL8	Amcyan/Viogreen	CD3	CD3

The sixth Panel (Table 2-14) was designed to look at the differentiation status of Vδ1 and Vδ2 cells and to examine the cytokine expression of IFN-γ and IL-17A in each group.

Table 2-14 Panel F

Laser Filter	Laser	Colour	Panel F
530/30	FL1	Viobright FITC	CD27
585/45	FL2	PE	IFN-γ
670-735	FL3	PerCP-Vio700	Vδ1
780/60	FL4	PEVio770	CD45RA
660/20	FL5	APC	IL-17A
780/60	FL6	APC Vio770	Dead Cell Stain
450/50	FL7	Vioblue	Vδ2
510/50	FL8	Amcyan/Viogreen	CD3

The seventh panel (Table 2-15) was designed to examine the differentiation status of MAIT cells and their expression of transcription factors RORγt and T-Bet.

Table 2-15 Panel G

Laser Filter	Laser	Colour	Panel G
530/30	FL1	Viobright FITC	CD161
585/45	FL2	PE	RORγt
670-735	FL3	PerCP-Vio700	CD197
780/60	FL4	PEVio770	CD45RA
660/20	FL5	APC	T-Bet
780/60	FL6	APC Vio770	Dead Cell Stain
450/50	FL7	Vioblue	Vα7.2
510/50	FL8	Amcyan/Viogreen	CD3

Flow cytometry data was analysed using FlowJo software. Single stains were used for compensation controls and fluorescence minus one (FMO) controls were used to help set gates.

2.2.9 Antibody Optimisation

Prior to commencement of patient recruitment the quantity of antibody needed per sample was titrated and optimized in a series of experiments on healthy volunteers. The quantity of antibody in each cocktail that gave optimal staining was used to avoid under-staining and over-staining of samples.

In the immunophenotyping study in 100µl whole blood samples, the Miltenyi antibodies in table 2-4 were titrated in a series of experiments using 10µl, 5µl, 2.5µl, and 1µl. It was found that 2.5µl gave optimal staining except for the anti-CD197 antibody which required 10µl. The R&D Systems antibody for IL-23R was found to need the recommended 10µl. The Vδ3 antibody conjugated in house was found to need 5µl for optimal staining.

In the cell stimulation study in PBMCs 0.5 million cells were added per well. Fluorochrome conjugated antibodies were titrated for optimal staining of these cells. The antibodies in table 2-4 were titrated in a series of experiments using 10µl, 5µl, 2µl, and 1µl per 0.5 million PBMCs. The antibodies were found to need 5µl per well with the exception of HLA-DR antibody which required 1µl and CD8 antibody in PeVio770 which required 2µl. Miltenyi changed the concentration of antibodies per vial supplied for CD14, CD4, CD8, and Vδ2 during the course of the recruitment for the stimulation experiment; antibody quantities needed fort these were 1µl rather than 5µl.

2.2.10 Gating Strategy for Flow Cytometry

The flow cytometer was maintained in accordance with manufacturer recommendations. For each experiment compensation beads were run with the appropriate fluorochromes and fluorescence minus one (FMO) controls were done to determine gating positions. FSC threshold was set at 50k to exclude debris. Samples were acquired and analysed with FlowJo software. Initially FSC was plotted against SSC to determine the population of interest which was lymphocytes for the majority of these experiments except for the monocyte population for Panel A of the stimulation experiment. Next doublets were excluded by plotting FSC-Area against FSC-Height, followed by exclusion of dead cells by gating on live cells. Populations of cells such as $CD3^+$ cells could then be gated on and examined. Figure 2-1 A-G shows an example of a gating strategy. A shows gating on the lymphocyte population with FSC plotted against SSC. B shows exclusion of doublets. C shows gating on live cells. D shows CD3 plotted against CD56 and split into quadrants. Upper left quadrant shows $CD3^-CD56^+$ cells which are natural killer cells. Lower left quadrant shows cells that are $CD3^-CD56^-$. Lower right quadrant shows $CD3^+CD56^-$ cells which are all T cells that do not express CD56 on the cell surface. Upper right quadrant shows $CD3^+CD56^+$ cells which are $CD56^+$ T Cells. E shows gating on the natural killer cell quadrant. These cells can be subdivided into dim and bright natural killer cells depending on their surface density expression of CD56. F shows the expression of IFN-γ in unstimulated dim NK cells. G shows the expression of IFN-γ in stimulated dim NK

cells when stimulated with phorbol 12-myristate 13-acetate and ionomycin. Other examples of gating strategies can be seen in Chapter 3 and Chapter 4.

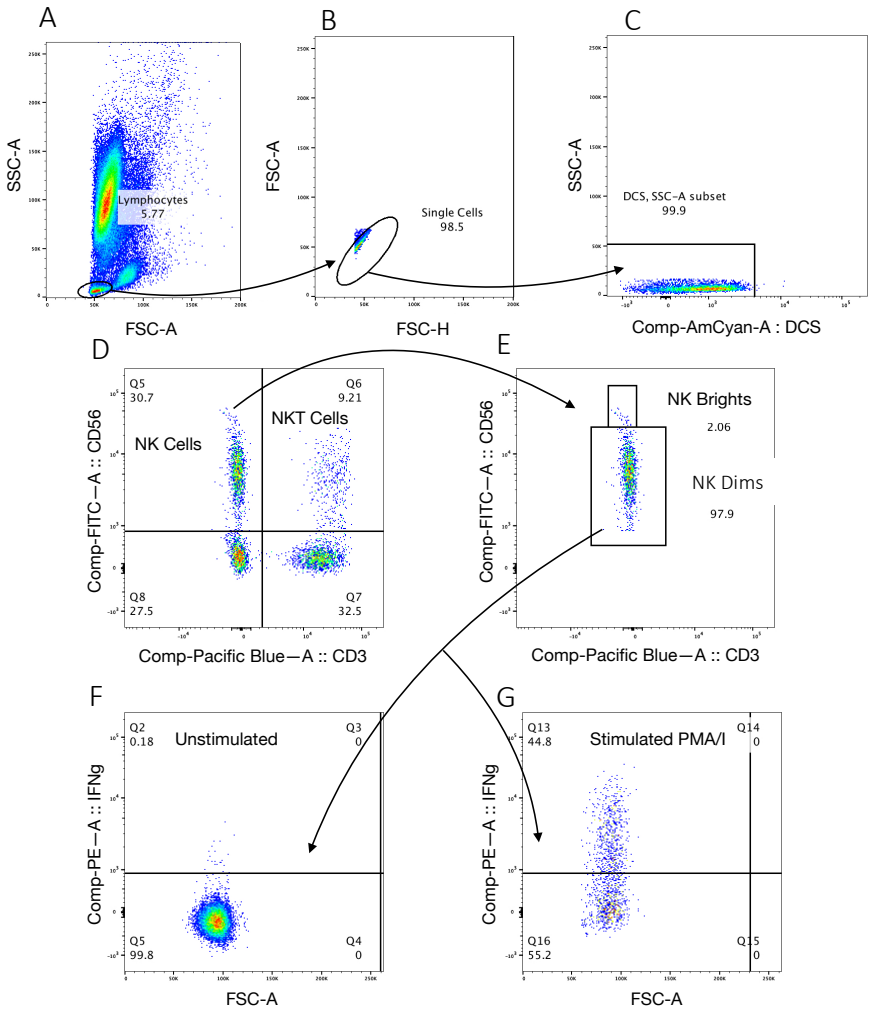

Figure 2-1 Gating Strategy

A; Gating on the lymphocyte population with FSC plotted against SSC. B; Exclusion of doublets. C; Gating on live cells. D; CD3 plotted against CD56 and split into quadrants. E; Gating on dim and bright natural killer cells. F; Expression of IFN-γ in unstimulated dim NK cells. G; Expression of IFN-γ in stimulated dim NK cells when stimulated with phorbol 12-myristate 13-acetate and ionomycin.

2.2.11 Statistical Analysis

The JMP® (SAS, North Carolina, USA) and SPSS® Statistical Software (IBM Corp, NY, USA) programs were used for the analysis of the statistics. Wilcoxon/Kruskal Wallis testing was employed to compare the differences between the three patient groups (represented by a capped line in the figures in Chapter 3 and 4). The *p* values for subsequent comparisons between the individual groups were Bonferoni adjusted for multiple comparisons (represented as a n-zigzag line in the figures in Chapter 3 and 4). Chi Square testing was employed to compare the categorical variables.

A mixed effects general linear regression model was employed to analyse repeated measurements. Where a significant change in a repeated measurement was detected, the repeated assays were compared with the initial assay values with Bonferoni adjusted *p* values for multiple comparisons.

Throughout the analysis, a *p* value of less than 0.05 was considered significant.

Chapter 3

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3.1 Title

Dysregulated T helper type 1 (T_H1) and T_H17 responses in elderly hospitalised patients with infection and sepsis.

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3.4 Abstract

3.4.1 Objective.

The role of T_H1 and T_H17 lymphocyte responses in human infection and sepsis of elderly patients has yet to be clarified.

3.4.2 Design.

A prospective observational study of patients with sepsis, infection only and healthy controls.

3.4.3 Setting.

The acute medical wards and intensive care units in a 1000 bed university hospital.

3.4.4 Patients.

32 patients with sepsis, 20 patients with infection, and 20 healthy controls. Patients and controls were older than 65 years of age. Patients with recognised underlying immune compromise were excluded.

3.4.5 Methods.

Phenotype, differentiation status and cytokine production by T lymphocytes were determined by flow cytometry.

3.4.6 Measurements.

The differentiation states of circulating $CD3^+$, $CD4^+$, and $CD8^+$ T cells were characterised as naive ($CD45RA^+$, $CD197^+$), central memory ($CD45RA^-$, $CD197^+$), effector memory ($CD45RA^-$, $CD197^-$), or terminally differentated ($CD45RA^+$, $CD197^-$). Expression of IL-12 and IL-23 receptors, and the transcription factors T-Bet and RORγt, was analysed in circulating T lymphocytes. Expression of interferon-γ and IL-17A were analysed following stimulation *in vitro*.

3.4.7 Results.

$CD4^+$ T cells from patients with infection predominantly expressed effector-memory or terminally differentiated phenotypes but $CD4^+$ T cells from patients with severe sepsis predominantly expressed naive phenotypes ($p<0.0001$). $CD4^+$ T cells expressing IL-23 receptor were lower in patients with sepsis compared to patients with infection alone ($p=0.007$). RORγt expression by $CD4^+$ T cells was less frequent in patients with sepsis ($p<0.001$), whereas T-Bet expressing $CD8^+$ T cells that do not express RORγt was lower in the sepsis patients.

HLA-DR expression by monocytes was lower in patients with sepsis. In septic patients fewer monocytes expressed IL-23.

3.4.8 Conclusion.

Persistent failure of T cell activation was observed in patients with sepsis. Sepsis was associated with attenuated $CD8^+$ T_H1 and $CD4^+$ T_H17 based lymphocyte response.

3.5 Introduction

Sepsis is a common disease worldwide, accounting for more fatalities than many common cancers[9], however elderly patients experience particularly high mortality rates[52]. Sepsis has been regarded as the clinical manifestation of a cytokine storm. However the animal models supporting this hypothesis do not accurately reflect human sepsis pathophysiology[53]. Recently the role of immunosuppression in the pathophysiology of sepsis in patients has been highlighted[54]. Septic patients exhibit a failure of both innate and adaptive immunity[55, 56] with increased apoptosis, impaired pathogen killing and decreased production of proinflammatory cytokines by a range of lymphocytes [57-59] [60-62]. Decreased antigen presentation by antigen presenting cells is associated with expression of inhibitory receptors on T cells and expansion of T regulatory (T_{reg}) cells [59, 63, 64].

Adaptive immune responses against microbial infections are controlled by $CD4^+$ T cells, which differentiate into distinct types of effector T cells. T_H1 cells are induced by IL-12 and are characterised by the expression of the transcription factor T-Bet and the cytokines interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), promoting macrophage and cytotoxic T cell activation and immunity against viruses and intracellular bacteria. T_H17 cells are induced by IL-1β, IL-6, transforming growth factor-β (TGF-β) and express the transcription factor RORγt and release IL-17A, IL-17F and IL-22. These cytokines promote immunity against extracellular bacteria and fungi by inducing neutrophil recruitment, antimicrobial peptide release, and the maintainence of

intestinal barrier function [65, 66]. $CD8^+$ T cells also show distinct phenotypes with some producing IFN-γ and others producing IL-17.

Previous studies have demonstrated that patients with sepsis show skewed $CD4^+$ T cells responses, polarised towards T_H2 and T_{reg} responses with depletion and impairment of T_H1 cells [63]. In our study, we used in vitro stimulation and flow cytometry to examine T_H1 and T_H17 pathway activation in circulating naive, central memory, effector memory and terminally differentiated $CD4^+$ and $CD8^+$ T cells from sepsis patients and control subjects. By quantifying IL-12 and IL-23 production by monocytes and IL-12 and IL-23 receptors, T-Bet and RORγt, and IFN-γ and IL-17A expression by $CD4^+$ and $CD8^+$ T cells, we identified defects in both T_H1 and T_H17 responses in septic patients. Furthermore, to discern sepsis specific molecular mechanisms from those associated with the occurrence of infection, we included an additional patient group with infection but without sepsis. Given adverse outcome in elderly patients with sepsis, and the occurrence of immune senescence in the elderly [67], this study was performed in elderly hospitalised patients.

3.6 Methods and Materials

3.6.1 Study Population

The St. James's hospital Research and Ethics Committee granted ethical approval for this study. Patients gave written informed consent, or when not possible, the next of kin assented to the study as per guidelines of National Consent Advisory group[68]. Blood samples, demographic data and clinically relevant information were collected from 42 patients in a septic group (32 for immune-phenotyping, and 10 for cell stimulation), 30 patients for the infection group (20 for immune-phenotyping, and 10 for cell stimulation), and 30 patients for the control group (20 for immune-phenotyping and 10 for cell stimulation). The study was performed from 2016 to 2018. Patients admitted to the intensive care unit (ICU) in St James's Hospital with septic shock, according to the Sepsis 3 definitions, were screened for inclusion in the study. Blood samples were taken within 72 hours of admission and weekly thereafter for a further 3 samples until death or discharge from hospital. Patients with infection were recruited from hospital wards in collaboration with the clinical microbiology service. In the infection group blood samples were taken within 72 hours of positive culture and a second blood sample was taken a week later unless the patient was discharged from hospital. One patient in the infection group was removed due to a very high bilirubin causing the flow cytometry to be uninterpretable, and so reducing the patient number to 19. The healthy control group was recruited

from the community; these subjects were attending a hospital phlebotomy service for community-based family practice and did not have any current or recent infections. Patient demographics are shown in Table 3-1.

Exclusion criteria included pre-existing immunodeficiency, immune modulating medications including steroids, chronic infection, malignancy, pre-existing liver disease, and any haematological disease.

Table 3-1 Demographic and Clinical Data

Clinical Data	Immunophenotype Study			Stimulation Experiment		
	Control	Infection	Septic	Control	Infection	Septic
n	20	19	32	10	10	10
Age	73 [69-78.25]	81.5 [70.25-87.25]	73.5 [68.75-79.25]	72 [67-74.5]	85 [68.75-87.5]	57.5 [53.75-69.25]
Male Gender	6 (30%)	11 (58%)	20 (62.5%)	5 (50%)	6 (60%)	4 (40%)
APACHE score	N/A	12.5 [8-16.5]	19 [16-24.5] p<0.0001	N/A	14 [10-14]	21.5 [16.25-24.5]
SAPS score	N/A	N/A	48 [37.75-54.5]	N/A	N/A	49 [38.25-55.75]
SOFA score on admission	N/A	3 [1.75-4]	7 [5.75-10] p<0.0001	N/A	2 [0-3]	10 [7.5-11.75]
SOFA score on day of first sample	N/A	1 [0.75-1.25]	7 [5-8.25]	N/A	1 [0-1]	8 [4.5-9.75]
Time to 1st sample from admission (days)	N/A	2.5 [2-3]	1.5 [0.75-2]	N/A	3 [2.5-4.5]	5 [4-6]
ICU duration (days)	N/A	N/A	14.5 [8.75-33.25]	N/A	N/A	17.5 [9.25-26]
Mortality in ICU	N/A	N/A	11 (34.4%)	N/A	N/A	1 (10%)
Mortality in Hospital	N/A	0	13 (40.6%)	N/A	1 (10%)	3 (30%)
Inotropic Support	N/A	0	30 (93.75%)	N/A	0	10 (100%)
Days on inotropes	N/A	0	7 [3-13]	N/A	0	7.5 [6-10.5]
Invasive ventilation	N/A	N/A	28 (87.5%)	N/A	N/A	9 (90%)
Days on invasive ventilation	N/A	N/A	14.5 [5-29.25]	N/A	N/A	8.5 [6.25-14.5]
P/F ratio (mmHg)	N/A	265.5 [231-392.25]	170 [135.75-240.5]	N/A	411.5 [386.5-436.5]	163 [129-205.75]
Muscle Relaxant infusion	N/A	N/A	11 (34.4%)	N/A	N/A	5 (50%)
Acute Kidney Injury KDIGO grade ≥1	0	6 (31.58%)	26 (81.25%)	0	2 (20%)	10 (100%)
Renal Replacement Therapy	0	0	16 (50%)	0	0	8 (80%)
Concomitant cardiac failure	0	0	6 (18.75%)	0	0	0
Stress dose steroids	0	1 (5.3%)	6 (18.75%)	0	1 (10%)	5 (50%)

Table 3-1 Demographic and Clinical Data continued.

Clinical Data		Immunophenotype Study			Stimulation Experiment		
		Control	Infection	Septic	Control	Infection	Septic
n		20	19	32	10	10	10
Source of Sepsis	Respiratory	N/A	5 (26.3%)	16 (50%)	N/A	3 (30%)	4 (40%)
	Abdominal	N/A	7 (36.8%)	11 (34.4%)	N/A	2 (20%)	3 (30%)
	Skin	N/A	0	4 (12.5%)	N/A	1 (10%)	1 (10%)
	Urine	N/A	6 (31.6%)	0	N/A	4 (40%)	2 (20%)
	Osteomyelitis	N/A	1 (5.3%)	0	N/A	0	0
	Mediastinitis	N/A	0	1 (3.1%)	N/A	0	0
Type of organism	Gram -ve organism	N/A	16 (84%)	9 (28%)	N/A	5 (50%)	5 (50%)
	Gram +ve organism	N/A	3 (16%)	9 (28%)	N/A	5 (50%)	2 (20%)
	Fungal	N/A	0	1 (3%)	N/A	0	0
	Empiric treatment	N/A	0	13 (41%)	N/A	0	3 (30%)
Secondary Infections		N/A	0	17 (53.1%)	N/A	0	5 (50%)
Lactate on admission		N/A	2.1 [1.15-3.57]	2.86 [2.27-4.09]	N/A	1.95 [1.6-3.1]	2.63 [1.81-3.36]
Comorbidities ≥ 1		19 (95%)	18 (94.7%)	26 (81.25%)	7 (70%)	9 (90%)	8 (80%)

Categorical data are presented as numbers with percentages in parentheses(). Data and parameters are presented as medians and interquartile ranges [Q1-Q3]. n, number of patients; APACHE score, Acute Physiologic Assessment and Chronic Health Evaluation; SOFA score, Sequential Organ Failure Assessment score; KDIGO Kidney Disease: Improving Global Outcomes; N/A , Not Applicable; P/F ratio, ratio of arterial oxygen partial pressure to fractional inspired oxygen.

3.6.2 Phenotypic Analysis of Circulating Leukocytes

Fresh whole blood was stained with a dead cell stain (LIVE/DEAD Fixable Aqua dead cell stain purchased from Molecular Probes, *Leiden, The Netherlands)* followed by fluorochrome-conjugated monoclonal antibodies (mAb) specific for cell surface expression of CD3 (clone REA613, BW264/56), CD4 (REA623), CD8 (BW135/80, REA734), CD14 (REA599, TÜK4), CD16 (REA423), CD25 (4E3), CD45RA (REA562), CD127 (REA614), CD197 (CCR7; REA546), HLA-DR (REA805), IL-12Rβ2 (REA333) and IL-23R (218213) (purchased from Miltenyi Biotec, Gladbach Bergische, Germany and R&D Systems, Abingdon, UK). Cells were stained with mAbs in PBS containing 1% bovine serum albumin and 0.02% sodium azide. Red cells were lysed with BD FACS™ Lysing Solution and analysed using a FACSCanto™ II flow cytometer (BD Biosciences) and FlowJo software (Treestar). Lymphocytes were gated on and any doublets or dead cells were excluded from the analysis. Single stained controls were used to set compensation parameters and fluorescence-minus-one controls were used to set gates. Cell frequencies were expressed as percentages of $CD3^+$ lymphocytes. Absolute numbers were determined from full blood cell counts.

dx.doi.org/10.17504/protocols.io.6zxhf7n

3.6.3 Stimulation Experiments

Peripheral blood mononuclear cells (PBMC) were prepared from fresh blood by density gradient centrifugation over Lymphoprep™ (Axis-Shield, Dundee, UK).

$0.5x10^6$ cells were stimulated for 5 hours with plate-bound mAbs specific for CD3 (OKT3) and CD28 (15E8), 50 ng/ml phorbol myristate acetate (PMA) with 1 µg/ml ionomcyin, or 10 ng/ml lipopolysaccharide (LPS). Wells intended for intracellular staining for IL-23, IL-12, IL-17A and IFN-γ contained brefeldin-A.

The cells were stained with dead cell stain (LIVE/DEAD Fixable Near IR dead cell stain purchased from Thermo Fisher Scientific, Massachusetts, US) followed by fluorochrome-conjugated antibodies for labelling cell surface markers. Cells were then fixed with 4% paraformaldehyde and permeabilised with 0.2% saponin before staining with mAbs specific for intracellular IL-12 (REA121), IL-23 (727753), IL-17A (CZ8-23G1), and IFN-γ (REA600). For intracellular staining of RORγt (REA278) and T-Bet (REA102) FoxP3 Staining Buffer Set was used. Once stained, the cells were fixed. The samples were acquired immediately with a BD FACSCanto™ II flow cytometer and analysed using FlowJo 10.4.2 software. The gating strategy used is shown in Fig 3-1.

dx.doi.org/10.17504/protocols.io.6zyhf7w

dx.doi.org/10.17504/protocols.io.6zzhf76

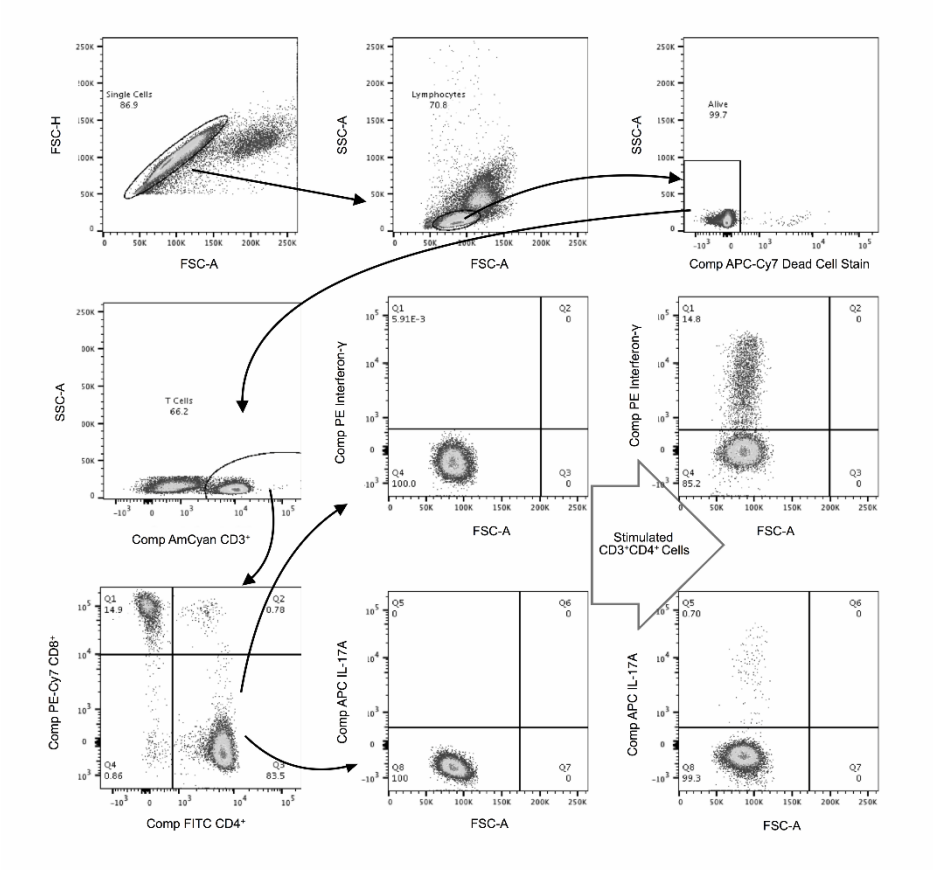


Figure 3-1 Gating Strategy of Flow Cytometry

Gating Strategy of Flow Cytometry showing $CD3^{+}CD4^{+}$ cells expressing Interferon-γ and Interleukin17a in unstimulated and stimulated cells. Cells stimulated with phorbol myristate acetate and ionomycin.

3.6.4 Statistical Analysis

All statistical analysis was performed with JMP® and SPSS® Statistical Software. Differences between the three groups were analysed for continuous variables by a Wilcoxon / Kruskal-Wallis test (capped line in figures), with pair-wise comparison (n-zigzag line in figures) and Bonferoni adjusted p values. Categorical variables were compared using a Chi-Square Test. Repeated assay were analysed using a mixed effects general liner regression model, all comparisons with admission values, with Bonferroni correction for multiple comparisons. Results were considered significant for p values lower than 0.05.

3.7 Results

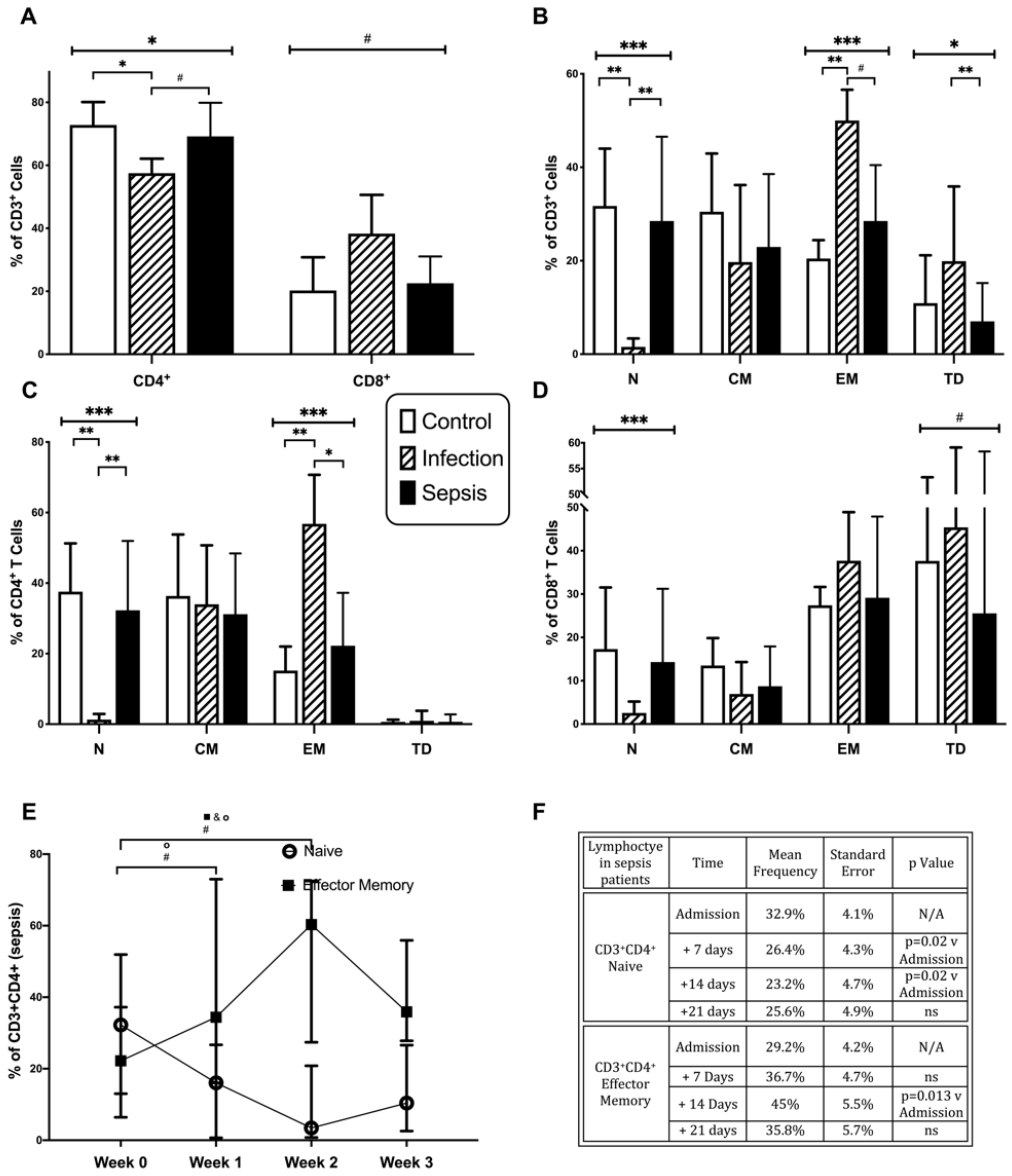
3.7.1 Demographics

The demographic characteristic of patients in this study, as outlined in Table 1, indicate that age and gender distribution was similar in all 3 groups. Patients with sepsis had greater Apache II scores ($p<0.0001$) and organ failure scores ($p<0.0001$) than patients with infection. In the phenotype study group no patients in the control or infection groups died whereas 13 (40%) of the sepsis group died.

3.7.2 $CD4^+$ and $CD8^+$ T Lymphocyte Differentiation in Patients with Infection and Sepsis

The differentiation status of total $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells was examined by flow cytometric analysis of CD45RA and CD197 expression. The percentage frequencies of naïve (N; $CD45RA^+$ $CD197^+$), central memory (CM; $CD45RA^-CD197^+$), effector memory (EM; $CD45RA^-CD197^-$) and terminally differentiated (TD; $CD45RA^+CD197^-$) T cells are shown in Fig 3-2 and the corresponding cell counts are shown in Fig 3-3. The frequencies of T cells expressing CD4 were significantly lower in patients with infection without sepsis, with concomitant increases in $CD8^+$ T cells (Fig 3-2A). Overall numbers of both $CD4^+$ and $CD8^+$ T cells were reduced in infection and sepsis patients (Fig 3-3A). The differentiation status of T cells differed across patient groups; with the frequencies of $CD3^+$ naïve lymphocytes being lower in patients with infection compared to control ($p<0.001$) and sepsis ($p<0.001$) groups; and the frequencies of EM T cells being greater in patients with infection compared to control ($p<0.001$) and sepsis ($p=0.02$) groups (Fig 3-2B). The percentages of $CD3^+$ TD cells were greater in patients with infection than in those with sepsis ($p=0.001$) (Fig 3-2B). The absolute numbers of naive T cells were lower in patients with sepsis than in controls ($p<0.0001$) and lower in patients with infection than in septic patients ($p<0.0001$) (Fig 3-3B). $CD3^+$ CM counts were lower in patients with infection ($p<0.0001$) and with sepsis ($p<0.0001$) compared to controls. $CD3^+$ EM counts were lower in septic patients ($p=0.014$) than in controls, while

$CD3^+$ TD counts were lower in patients with infection ($p=0.025$) and sepsis ($p<0.0001$) compared to controls.



Lymphoctye in sepsis patients	Time	Mean Frequency	Standard Error	p Value
CD3+CD4+ Naive	Admission	32.9%	4.1%	N/A
	+ 7 days	26.4%	4.3%	p=0.02 v Admission
	+14 days	23.2%	4.7%	p=0.02 v Admission
	+21 days	25.6%	4.9%	ns
CD3+CD4+ Effector Memory	Admission	29.2%	4.2%	N/A
	+ 7 Days	36.7%	4.7%	ns
	+ 14 Days	45%	5.5%	p=0.013 v Admission
	+ 21 days	35.8%	5.7%	ns

Figure 3-2 Lymphocyte Differentiation by Frequency.

A-D, Frequencies of CD3+, CD4+, and CD8+ Naive (N), Central Memory (CM), Effector Memory (EM), and Terminally Differentiated (TD) T Cells in control subjects (n=20), patients with infection (n=19), and patients with sepsis (n=32). Data represented as median with interquartile range. E-F, Frequencies of CD4+ Naïve and Effector Memory T Cells at admission (n=32), at 7 days (n=20), 14 days (n=14) and 21 days (n=15). SE= standard error of the mean. NS = non significant, N/A = Not applicable. All comparisons with admission values, with Bonferroni correction for multiple comparisons. (#=p<0.05;=p<0.01;**=p<0.001; ***=p<0.0001)*

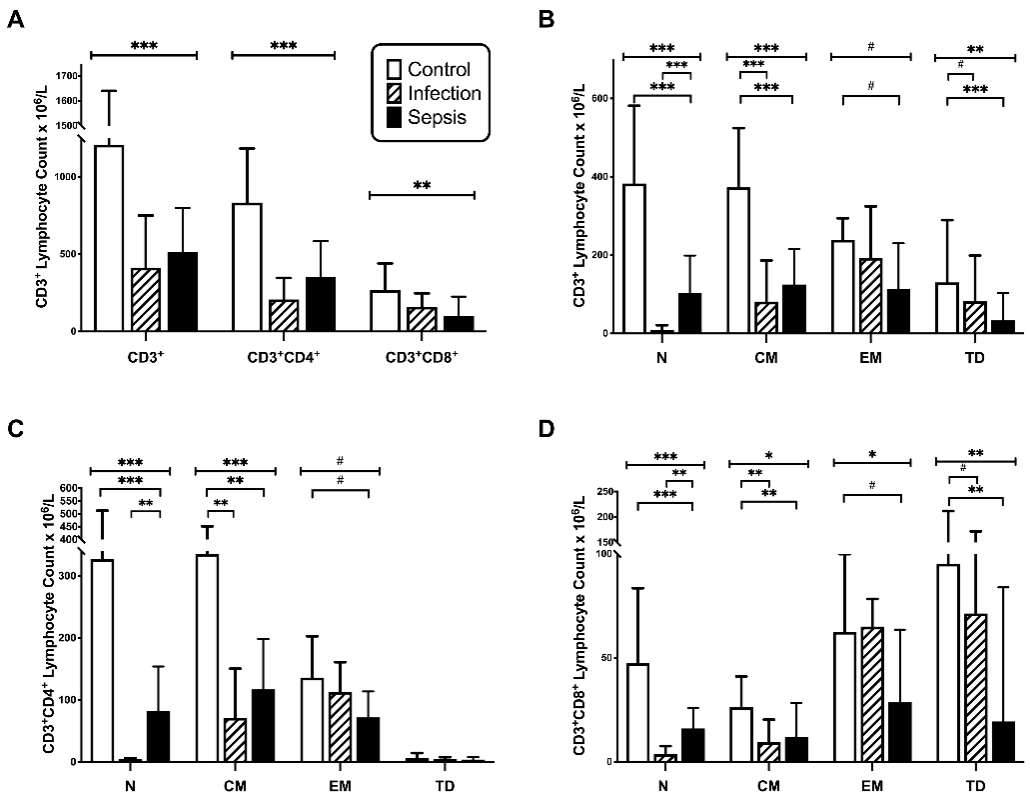


Figure 3-3 Lymphocyte Differentiation by cell count.

A-D. Absolute cell counts of CD3⁺, CD4⁺, and CD8⁺ Naive (N), Central Memory (CM), Effector Memory (EM), and Terminally Differentiated (TD) T Cells in control subjects (n=20), patients with infection (n=19), and patients with sepsis (n=32). Data represented as median with interquartile range. (#=p<0.05;=p<0.01;**=p<0.001; ***=p<0.0001)*

The percentages of $CD4^+$ T cells with naïve phenotypes was lower in patients with infection compared to the control ($p<0.001$) and sepsis ($p<0.001$) groups; whereas percentages of EM $CD4^+$ T cells was greater in patients with infection than in the control ($p<0.001$) and sepsis ($p=0.007$) groups (Fig 3-2C). A similar pattern was observed for $CD8^+$ lymphocyte differentiation, with the exception that higher frequencies of $CD8^+$ cells from all subject groups expressed TD phenotypes. (Fig 3-2D).

Absolute numbers of $CD4^+$ T cells were lower in patients with infection and sepsis compared to controls ($p<0.001$) (Fig 3-3C). Within the $CD4^+$ T cell compartment, naïve cell counts were lower in sepsis patients than in controls ($p=0.0001$) and lower with patients with infection than in those with sepsis ($p<0.001$). CM $CD4^+$ T cell counts were lower in patients with sepsis than in healthy controls ($p<0.001$) and lower in patients with infection than healthy controls ($p<0.001$). EM $CD4^+$ T cell counts were lower in patients with sepsis than healthy controls ($p=0.011$). $CD4^+$ TD lymphocyte counts were similarly low in all three groups (Fig 3-3C).

Similar results were observed with $CD8^+$ T cell counts, except that TD cells were more prominent among $CD8^+$ than $CD4^+$ T lymphocytes, and were lower in patients with infection ($p=0.018$) and sepsis ($p=0.001$) compared to controls (Fig 3-3D).

Thus, at the start of illness, both $CD4^+$ and $CD8^+$ T lymphocytes were activated in patients with infection, but not patients with sepsis. However, in the sepsis group, the percentage of $CD3^+CD4^+$ naive cells decreased over time ($p=0.025$) (Fig 3-2E). $CD4^+$ naive cells decreased in the two weeks after the onset of sepsis (Fig 3-2F). The percentage of $CD3^+CD4^+$ effector memory cells increased over time ($p=0.02$) (Figs 3-2E and 3-2F) with $CD4^+$ effector memory cells increasing two weeks after the onset of sepsis (Figs 3-2E and 3-2F). There was no change in $CD8^+$ activation over the same time.

3.7.3 IL-23 Production by Monocytes is Impaired in Patients with Sepsis

As previously reported, lower frequencies of $CD14^+$ monocytes expressed HLA-DR in patients with sepsis compared to control subjects ($p<0.0001$) (Fig 3-4E), and patients with infections only ($p=0.038$), potentially accounting for the observed failure of lymphocyte activation in patients with sepsis. Few $CD14^+$ monocytes expressed either IL-12 or IL-23 upon stimulation with LPS or PMA and ionomycin (Figs 3-4A and 3-4C). Unstimulated monocytes from septic patients exhibited lower expression of IL-23 than monocytes from control subjects ($p=0.038$) and patient's with infection ($p=0.021$) (Fig 3-4C), suggesting that T_H17 cell differentiation may be impaired in septic patients.

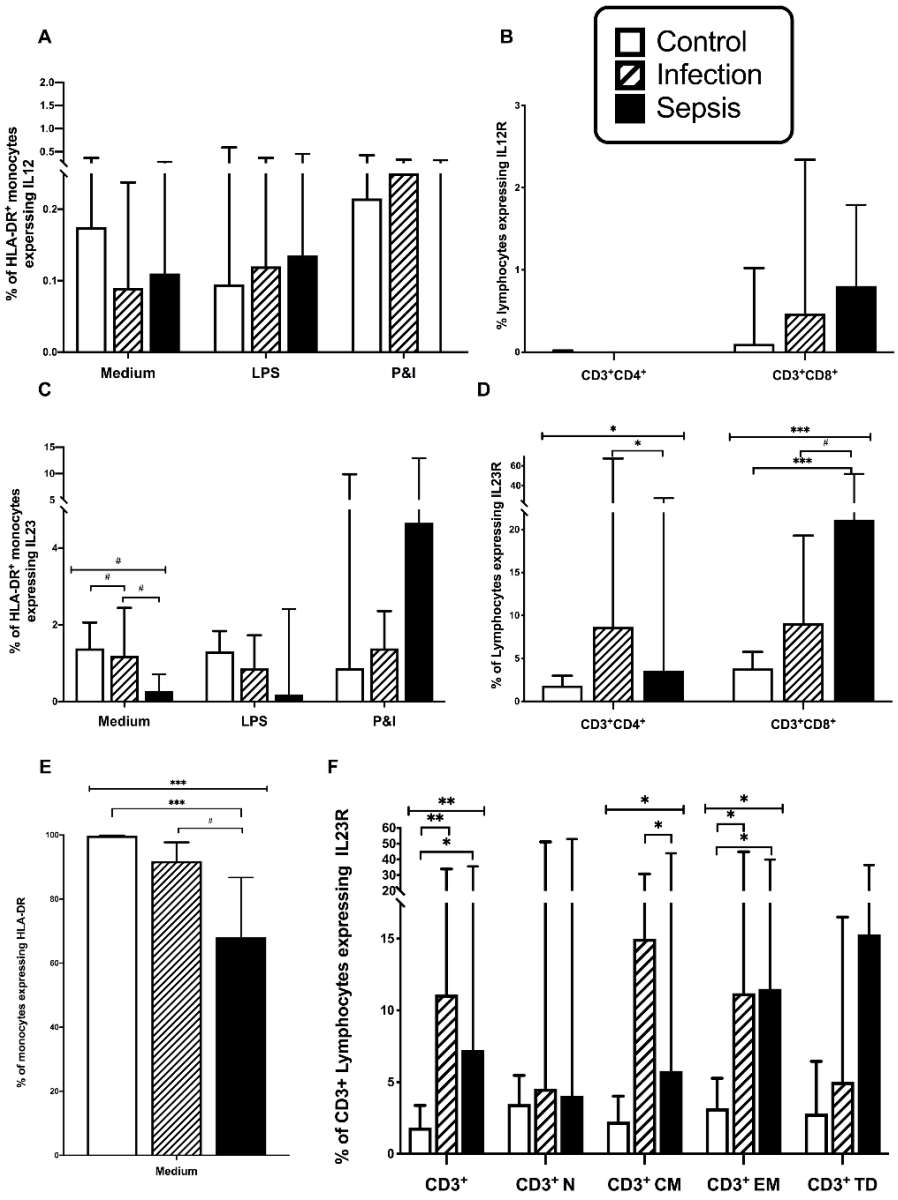


Figure 3-4 Monocyte expression of HLA-DR, IL-12, and IL-23 and lymphocyte expression of IL-12R and IL-23R.

A; HLA-DR+ monocytes expressing IL-12. B; T Lymphocytes expressing IL-12R. C; HLA-DR+ monocytes expressing IL-23. D; T Lymphocytes expressing IL-23R. E; Monocytes expressing HLA-DR+. F; T lymphocytes expressing IL-23R by differentiation status, ie Naive (N), Central Memory (CM), Effector Memory (EM), and Terminally Differentiated (TD). Control subjects (n=20 in T cell group, 10 in monocyte group), patients with infection (n=19 in T cell group, 10 in monocyte group), and patients with sepsis (n=32 in T cell group, 10 in monocyte group). Monocytes are unstimulated (medium) or stimulated with lipopolysaccharide (LPS) or phorbol myristate acetate (PMA) and ionomycin. Data represented as median with interquartile range. (#=p<0.05;=p<0.01;**=p<0.001; ***=p<0.0001)*

3.7.4 Transcription Factors in $CD3^+$ Cells

We next investigated the expression of the T_H1-associated transcription factor T-Bet and the T_H17-associated transcription factor RORγt by total T Cells, $CD4^+$, and $CD8^+$ T cells from the three subject groups. RORγt was found to be expressed by lower frequencies of T cells from patients with sepsis compared to patients with infections only ($p=0.003$) and control subjects ($p<0.0001$) (Fig 3-5D). RORγt expression by naïve T cells was similar in all 3 patient groups, but it was significantly lower in CM and EM T cells from septic patients compared to controls ($p<0.0001$) and lower in CM from sepsis compared to infected patients ($p=0.034$). (Fig 3-5B) Thus sepsis was associated with reduced expression of the T_H17 transcription factor RORγt in effector, central memory, and terminally differentiated $CD3^+$ lymphocytes.

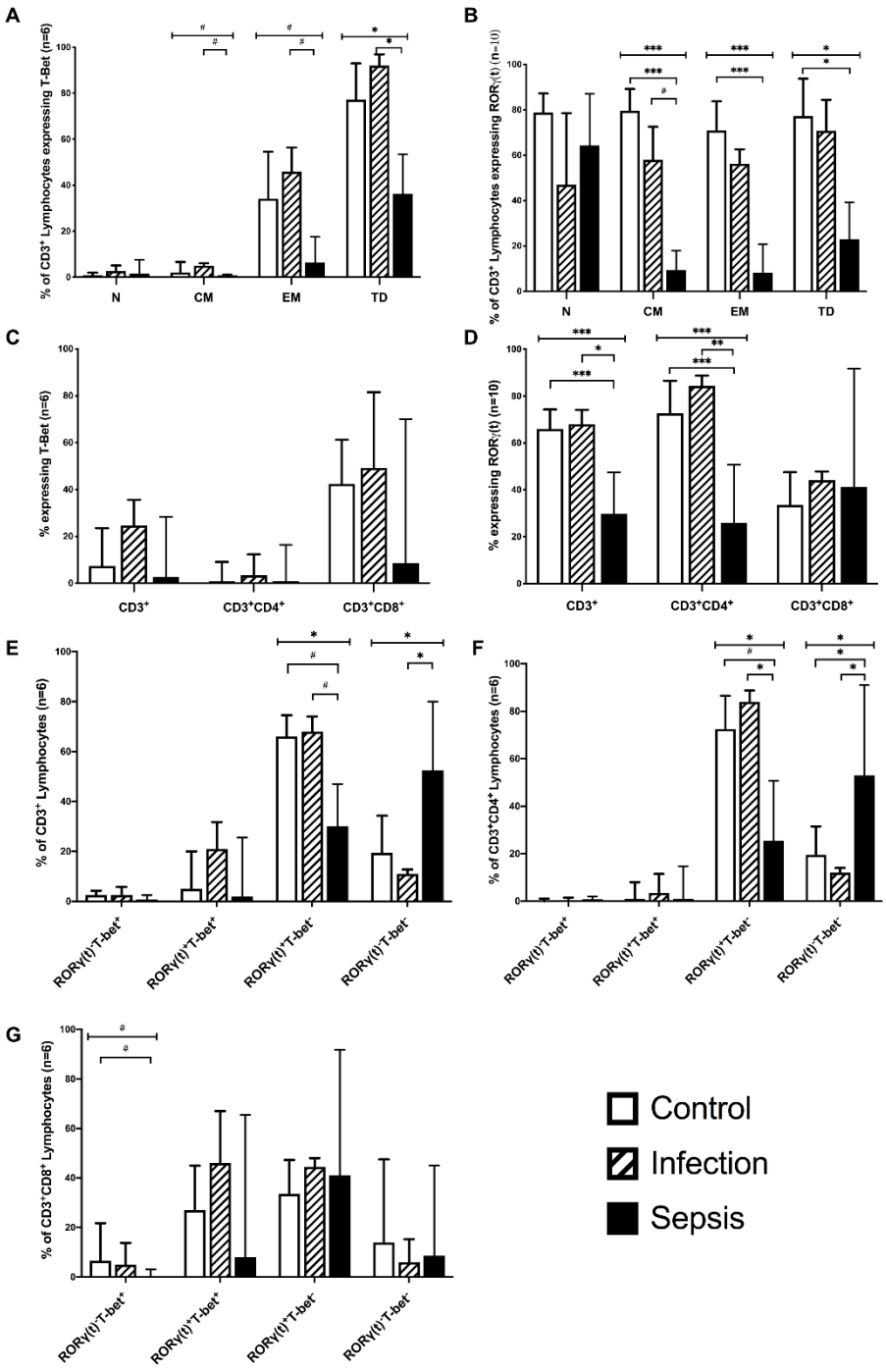


Figure 3-5 Frequency of RORγt and T-Bet expression in CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ Lymphocytes.

A-G. Expression of transcription factors RORγt and T-Bet in CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ Lymphocytes and in CD3⁺ Naive (N), Central Memory (CM), Effector Memory (EM), and Terminally Differentiated (TD) Cells. Co-expression of RORγt and T-Bet by CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ lymphocytes was also analysed in 4E-G. n, number of patients. Data represented as median with interquartile range. (#=p<0.05;=p<0.01;**=p<0.001; ***=p<0.0001)*

There was no significant difference in the expression of T-Bet by total T cells, $CD4^+$ and $CD8^+$ T cells (Fig 3-5C). T-Bet expression was similar in naïve T cells in all groups, but it was lower in CM (p=0.018), EM (p=0.019), and TD (p=0.003) T cells from sepsis patients compared to patients with infections only. Collectively these data suggests that sepsis is associated with a failure of T cell activation to both T_H1 and T_H17 phenotypes. An underlying lymphocyte anomaly seems unlikely in septic patients as T-Bet and RORγt transcription factor expression was similar in Naïve T cells from all three subject groups. The predominance of RORγt expression in $CD3^+$ lymphocytes with sepsis highlights the importance of the T_H17 response in sepsis.

3.7.5 Transcription Factors in $CD4^+$ and $CD8^+$ Cells

Most $CD4^+$ T cells from control subjects and patients with infections only expressed RORγt, with very few $CD4^+$ T cells expressing T-Bet (Figs 3-5C, 3-5D, and 3-5F). The expression of RORγt by $CD4^+$ T cells was significantly lower in septic patients than in infected patients (p=0.001) or healthy controls (p=0.0001).

$CD8^+$ T cells from control subjects and patients with infections only frequently expressed both T-Bet and RORγt, (Figs 3-5C and 3-5D). In patients with sepsis compared to control the expression of T-Bet was significantly lower

(p=0.02) in $CD3^+CD8^+$ cells that did not co-express RORγt. Co-expression of RORγt and T-Bet was similar in all three groups (Fig 3-5G).

The decreased expression of the T_H17 signature transcription factor in effector $CD4^+$ T lymphocytes of patients with sepsis, but normal RORγt expression in naïve $CD4^+$ T cells, suggests a failure of $CD4^+$ T_H17 differentiation in these patients. Similarly sepsis appears to be associated with a failure of $CD8^+$ T_H1 cell differentiation , rather than an underlying defect in lymphocyte functionality.

3.7.6 IL-12R and IL-23R Expression by T Cells from Patients with Sepsis

The T_H1 lymphocyte phenotype is characterised by surface expression of IL-12R, while surface expression of IL-23R characterises T_H17 lymphocytes. Few $CD3^+$ lymphocytes from healthy volunteers expressed either IL-12R or IL-23R (Figs 3-4B and 3-4D), which is consistent with existing literature in patients [69, 70]. However IL-23R expression was more frequent in patients with infection (p=0.001) and sepsis (p=0.002), compared with healthy controls (Fig 3-4F). IL-23R expression by naïve and TD T cells was similar in the three patient groups. However IL-23R expression was greater in CM T cells from patients with infection compared with sepsis (p=0.01) and was greater in EM T cells from patients with sepsis (p=0.008) and from patients with infection (p=0.003) compared with

healthy controls. This data points to a T_H17 immune response taking place in patients with infection which is attenuated in septic patients.

In $CD4^+$ T cells, IL-23R expression was greater in patients with infection than sepsis ($p=0.005$), while in $CD8^+$ T cells, IL-23R expression was greater in patients with sepsis than with patients with infection ($p=0.04$) and controls ($p<0.001$) (Fig 3-4D). Expression of the IL-12R was rare in $CD4^+$ and $CD8^+$ T cells and was similar in all patient groups (Fig 3-4B).

As expression of the IL-23R is a hallmark of T_H17 phenotype, our data suggest that immunity to infection is predominantly based upon a T_H17 response, rather than a T_H1 response. Furthermore as IL-23R expression was greater in $CD3^+$ and $CD3^+CD4^+$ lymphocytes of patients with infection, who did not have multiple organ failure, then an exaggerated T_H17 response in circulating lymphocytes does not appear to be the basis of sepsis induced organ failure. Lastly the T_H17 response was preserved in $CD8^+$ lymphocytes. As only 10% of $CD4^+RORγt^+$ T cells from patients with infection expressed IL-23R, this suggests that activation of a relatively small proportion of the potential pool of $CD4^+$ T_H17 cells is required to control infection.

3.7.7 Interferon-γ and IL-17A Production by Stimulated T Cells from Patients and Controls

$CD4^+$ and $CD8^+$ T cells from patients and healthy controls expressed little or no IFN-γ or IL-17A without *in vitro* stimulation (Figs 3-6A to 3-6D), which is consistent with existing literature [71]. However stimulation with PMA and ionomycin, induced IFN-γ production by significant numbers of $CD4^+$ and $CD8^+$ T cells and IL-17A by smaller numbers of $CD4^+$ T cells (Figs 3-6A to 3-6D). The frequencies of $CD4^+$ and $CD8^+$ T cells that produced IFN-γ or IL-17 in response to any mode of stimulation were similar using PBMC from healthy donors and patients with infection only or sepsis. These data suggest that $CD4^+$ and $CD8^+$ T cells from patients with sepsis are not defective in their ability to produce IFN-γ or IL-17, but may be failing to receive stimulatory and/or T_H1/ T_H17 polarisation signals from cells of the innate immune system.

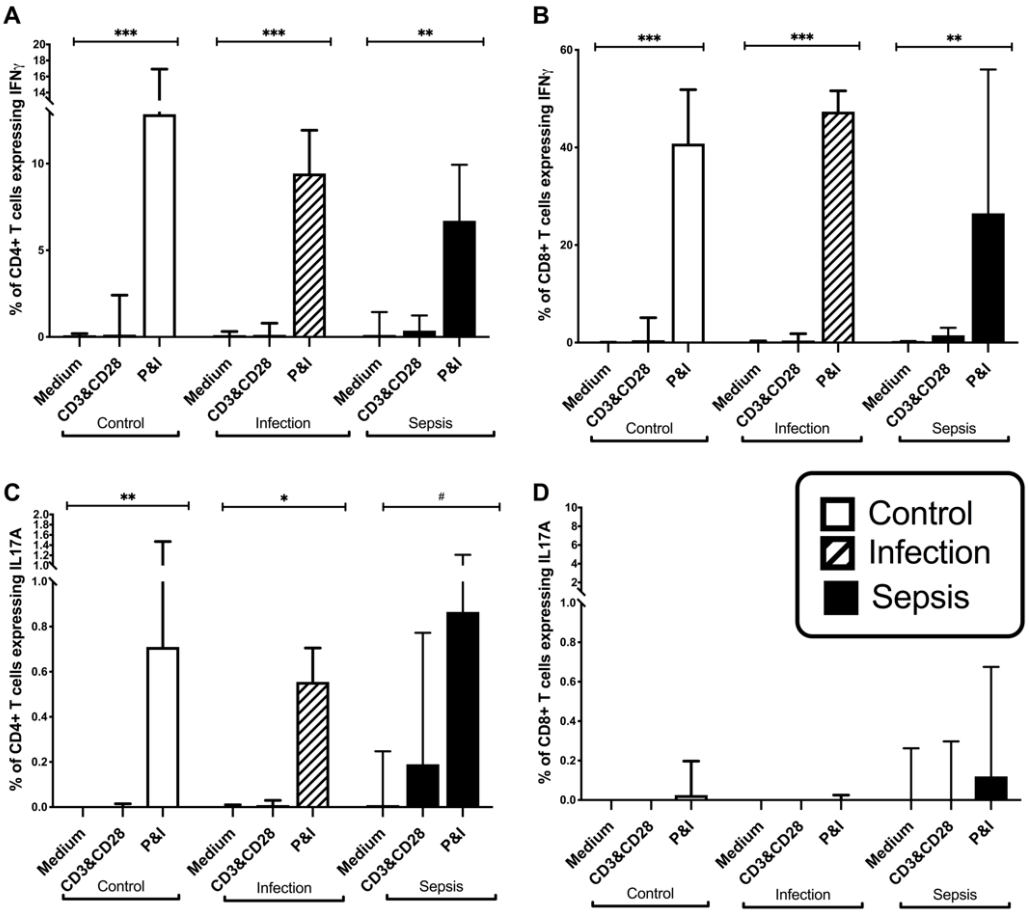


Figure 3-6 Frequency of IFN-γ and IL-17A expression on stimulated $CD3^+CD4^+$and $CD3^+CD8^+$ Lymphocytes

*A-D, Frequencies of Interferon-γ (IFN-γ) and IL-17A expressing $CD4^+$ and $CD8^+$ T Cells in control subjects (n=20), patients with infection (n=19), and patients with sepsis (n=32). Medium are unstimulated cells, CD3&CD28 is stimulation with CD3 and CD28 antibodies, P&I is stimulation with Phorbol Myristate Acetate and Ionomcyin. Data represented as median with interquartile range. (#p<0.05;*p<0.01;**p<0.001; ***p<0.0001)*

The frequencies of T_{reg} lymphocytes, defined by the $CD3^+CD4^+CD25^+CD127^-$ phenotype, were significantly different across patient groups, (p=0.008), being greatest in patients with sepsis, (median 11.7%, IQR 7.5-14.6%), than with infection (Median 7.8%, IQR 6-10%) and healthy controls (Median 7.7%, IQR 6.7-9.2%). (Figure 3.7 Not in original publication).

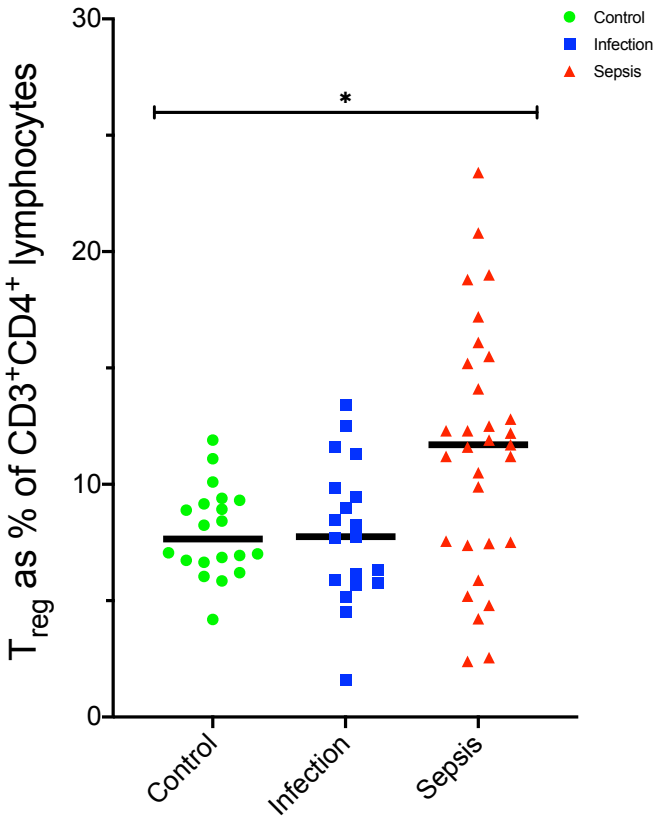


Figure 3-7 T_{reg} Cells as % of T Helper Cells

*Frequencies of T_{reg} Cells in control subjects (n=20), patients with infection (n=19), and patients with sepsis (n=32). Data represented as scattered plot with a line to show the median. (*p<0.01) This figure was not in the original paper due to restrictions on number of figures allowed for publication.*

3.9 Discussion

In this study the clinical presentation of infection was linked with specific attributes of host immunity; in septic patients there was a complex failure to differentiate T lymphocytes to an activated phenotype at onset of illness, in particular $CD8^+$ T_H1 cells and $CD4^+$ T_H17 cells, while simultaneously expanding an inhibitory population of T_{reg} cells. While septic patients eventually activated circulating T lymphocytes, this was only after a considerable delay.

In our study of human sepsis, expression of the signature T_H17 transcription factor and of surface IL-23R were reduced in $CD4^+$ lymphocytes of septic patients. Indeed patients who tolerated infection with relative impunity, without developing organ failure, elaborated a more robust T_H17 response, which may potentially be protective in this context. Accordingly the T_H17 lymphocyte response, may not account for systemic inflammation and multiple organ failure, as suggested in prior animal studies of sepsis [50]. The absence of IL-12 receptor expression in both $CD4^+$ and $CD8^+$ lymphocytes, which is consistent with existing human data suggests that immunity in elderly humans with sepsis is predominantly mediated by a T_H17 rather than a T_H1 response [70].

In human sepsis the relative importance of the T_H17 response in driving inflammation and or providing immunity is unclear. The IL-23/IL-17 axis is involved in systemic inflammation in murine models of infection[50]. However in humans, and in line with our findings, Ronit *et al* reported a decrease in T_H17 lymphocytes, with endotoxin induced systemic inflammation [72]. In contrast

Mikacenic *et al.* reported elevated IL-17 in the broncho-alveolar lavage fluid of patients with acute respiratory distress syndrome (ARDS), with IL-17 levels correlating with the severity of multiple organ failure [51]. Similarly Zan *et al.,* also studying patients with sepsis induced ARDS, reported elevated IL-17 in sepsis non-survivors [73]. Furthermore, Brunialti *et al*, reported that T lymphocytes expressing IL-17 were increased in patients with sepsis [74], and Maravista *et al*, reported IL-17 as the only cytokine produced in excess by shocked septic patients, [75]. In a separate and distinct context, a study of neonatal sepsis reported that mortality was linked with excess levels of IL-17[76]. However not all studies have found an association between IL-17 and outcome in sepsis [77] [78].

Crucially none of the aforementioned studies included patients with infection who did not develop sepsis, and thus did not adequately explore the link between T_H17 responses and sepsis outcomes. The present study, by including a cohort of patients with infection without sepsis, indicates that the T_H17 response may have an essential role in mediating protective immune responses in humans with infection.

This concept that human sepsis is fundamentally related to a failure to elaborate a robust adaptive immune response is consistent with the current appreciation of the significance of immune suppression in patients with sepsis [79]. This study suggests a specific failure of the $CD4^+$ effector memory lymphocyte mediated T_H17 response. This is plausible as IL-17 enhances polymorphonuclear chemotaxis and activation, and is crucially important in mediating mucosal immunity, specifically for systemic *Candida* infections, which

account for 20% of all infections in critically ill patients [80-82]. Furthermore IL-23 responsiveness of human $CD8^+$ memory lymphocytes decreases with age [83], and there is lower potential for inducible $CD4^+$/IL-23r with age [84] and this decrease in T_H17 responsiveness with age could potentially contribute to the adverse sepsis outcomes observed in the elderly.

As in other studies, HLA-DR expression by monocytes from septic patients was decreased [58]; which likely accounted for the observed failure of T lymphocyte activation. However, in T cell activation, there is an additive effect of co-stimulatory cytokines from the IL-12 family [85]. IL-12 drives $CD4^+$ T cell differentiation into IFN-γ-secreting T_H1 cells, whereas IL-23 in concert with IL-1β, IL-6 and TGFβ-1 drives activated $CD4^+$ differentiation to a T_H17/IL-17 secreting phenotype [86] [87]. In this study, IL-23 production by $CD14^+$ monocytes from septic patients was decreased which would inhibit a robust T_H17 response [85].

3.10 Limitations

This study was not designed or powered to detect an association between specific markers of a T_H17 adaptive immune response and either the occurrence of nosocomial infection, or the incidence of mortality in critically ill patients. A larger study will be required to test these associations and to test the utility of specific indices of T lymphocyte activation as clinical biomarkers of the risk for nosocomial infection and for sepsis mortality.

This analysis of circulating T lymphocyte activation and phenotype may not reflect the status of tissue bound lymphocytes. While including a group of patients with infection in the study design provides evidence of circulating T lymphocyte activation in patients with infection who do not develop sepsis, this still leaves questions regarding the activation of tissue bound lymphocytes largely unanswered. The results of this study suggest a link between T lymphocyte activation and the occurrence of sepsis in patients with infection, but does not differentiate between a causal and coincidental link. The observation of T lymphocyte activation in patients with infection provides at best inferential evidence of causal immune mediation of patients' response to infection. Lastly the study was designed to recruit patients with an infection or who had established sepsis and does provide insight to the earlier stages of illness.

However the results of this study clearly link delayed activation of $CD4^+$ T_H17 lymphocytes with sepsis in elderly humans and provide biomarkers for the occurrence of sepsis in the elderly that have the potential to inform subsequent studies of immune modulation in elderly patients with sepsis.

Chapter 4

4 Paper 2 Published in Vaccines.

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4.1 Title

Innate Lymphocyte T_H1 and T_H17 Responses in Elderly Hospitalised Patients with Infection and Sepsis

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4.3 Abstract

4.3.1 Background

The role of innate immunity in human sepsis must be fully clarified to identify potential avenues for novel immune adjuvant sepsis therapies.

4.3.2 Methods

A prospective observational study was performed including patients with sepsis (septic group), infection without sepsis (infection group), and healthy controls (control group) in the setting of acute medical wards and intensive care units in a 1000-bed university hospital. A total of 42 patients with sepsis, 30 patients with infection, and 30 healthy controls were studied. The differentiation states of circulating mucosal associated invariant T (MAIT) cells and natural killer T (NKT) cells were characterised as naive ($CD45RA^+$, $CD197^+$), central memory ($CD45RA^-$, $CD197^+$), effector memory ($CD45RA^-$, $CD197^-$), or terminally differentiated ($CD45RA^+$, $CD197^-$). The differentiation status of circulating gamma-delta T lymphocytes were characterised as naive ($CD45RA^+$, $CD27^+$), central memory ($CD45RA^-$, $CD27^+$), effector memory ($CD45RA^-$, $CD27^-$), or terminally differentiated ($CD45RA^+$, $CD27^-$). The expression of IL-12 and IL-23

receptors, the transcription factors T-Bet and RORγt, and interferon-γ and IL-17a were analysed.

4.3.3 Results

MAIT cell counts were lower in the septic group (p = 0.002) and the infection group (p < 0.001) than in the control group. The MAIT cell T-Bet expression in the infection group was greater than in the septic group (p = 0.012). The MAIT RORγt expression in the septic group was lower than in the control group (p = 0.003). The NK cell counts differed in the three groups (p < 0.001), with lower natural killer (NK) cell counts in the septic group (p < 0.001) and in the infection group (p = 0.001) than in the control group. The NK cell counts increased in the septic group in the 3 weeks following the onset of sepsis (p = 0.028). In lymphocyte stimulation experiments, fewer NK cells expressed T-Bet in the septic group than in the infection group (p = 0.002), and fewer NK cells expressed IFN-γ in the septic group than in the control group (p = 0.002). The NKT cell counts were lower in the septic group than both the control group (p = 0.05) and the infection group (p = 0.04). Fewer NKT cells expressed T-Bet in the septic group than in the infection group (p = 0.004). Fewer NKT cells expressed RORγt in the septic group than in the control group (p = 0.003). Fewer NKT cells expressed IFN-γ in the septic group than in both the control group (p = 0.002) and the infection group (p = 0.036).

4.3.4 Conclusion

The clinical presentation of infection and or sepsis in patients is linked with a mosaic of changes in the innate lymphocyte T_H1 and T_H17 phenotypes. The manipulation of the innate lymphocyte phenotype offers a potential avenue for immune modulation in patients with sepsis.

Keywords: sepsis; infection; human; immunity; lymphocyte; innate; natural killer; gamma delta; MAIT; T_H17

4.4 Introduction

Although sepsis accounts for more fatalities than many common cancers and is particularly lethal in the elderly [9], the pathophysiology of sepsis in humans is yet to be clearly defined. Animal models portraying sepsis as a cytokine storm do not accurately reflect the human sepsis pathophysiology [53]. Patients with sepsis predominantly demonstrate a failure of innate immunity and adaptive immunity [55, 56]. Human sepsis is characterised by impaired pathogen elimination, increased apoptosis, the reduced production of proinflammatory cytokines by a broad range of lymphocytes, and an emergence of regulatory T cells [57-62]. As a consequence, immunosuppression has recently become recognised as an obvious pivotal role in the pathophysiology of human sepsis [54].

We have recently shown that human sepsis is linked with a delayed failure to activate $CD3^{+}CD4^{+}$ lymphocytes to a T_H17 phenotype [88]. Innate lymphocytes play pivotal roles in T cell polarisation via the rapid secretion of T_H1, T_H2, T_H17, and regulatory T(T_{reg}) cytokines. In the presence of such a profound paresis of adaptive immunity, innate lymphocytes may assume a crucial role in the pathophysiology of human sepsis, and their role in generating host immunity should be clarified in order to identify potential avenues for novel immune adjuvant therapies and also to provide an enhanced suite of biomarkers of innate immunity in human sepsis.

Innate lymphocytes and T cells with innate properties such as mucosal associated invariant T (MAIT) cells and natural killer T (NKT) cells bridge the gap

between innate and adaptive immunity [89]. MAIT cells are the most numerous invariant T lymphocytes in the peripheral circulation and are also found in the gastrointestinal and respiratory tracts [90, 91]. MAIT cells are activated by the vitamin B-related products of bacterial metabolism, bound to the major histocompatibility complex (MHC)-1-like antigen-presenting molecule MR-1, and express cytokines such as interferon-γ (IFN-γ) and interleukin-17 (IL-17). Reduced MAIT cell numbers have been linked with pseudomonal infections in cystic fibrosis patients and with streptococcal infection in patients with septic shock [92, 93]. However, it is unclear whether the altered MAIT cell counts and phenotypes found in patients with infection are similar to those found in patients with overt sepsis.

Other innate lymphocytes, such as natural killer (NK) cells, natural killer T (NKT) cells, and gamma-delta T (γδ T) cells also have strong influence on the $T_H1/T_H2/ T_H17/T_{reg}$ balance of cytokine production. Whereas NK cells are stimulated through a variety of activating and inhibitory receptors, NKT cells respond to bacterial and autologous glycolipid antigens presented by CD1d. Both NK cells and NKT cells are important sources of early IFN-γ but can also be induced to produce other Th cytokines such as IL-17 [94-96], and these cells are variably reported as reduced or increased in patients with sepsis [97, 98]. Currently, little is known of the role of the NKT cell phenotype in human sepsis. γδ T cells are also important innate sources of T_H1 (IFN-γ and TNF-α), T_H2 (IL-4 and IL-13), T_H17 (IL-17), and T_{reg} (IL-10) cytokines. Human γδ T cells can be divided into three main subsets. The most abundant subset found in blood, Vδ2 T cells, recognise pyrophosphate antigens produced by some bacteria bound to

butryophilin 3A1, whereas Vδ1 and Vδ3 T cells can recognise glycolipids presented by CD1 and stress-inducible proteins found in tumour and virus-infected cells [99-101].

We used flow cytometry to examine the numbers, phenotypes, and activation status of circulating innate lymphocyte populations from healthy controls and patients with sepsis. We also investigated the potential of these cells to produce T_H1 and T_H17 cytokines by examining the IL-12 receptor and IL-23 receptor expression, respectively, and by measuring the ability of these cells to express the T_H1 and T_H17-associated transcription factors, RORγt and T-Bet, and produce IL-17 and IFN-γ following stimulation in vitro. In our study, we included an additional group of patients from the wards with infection. These patients did not have sepsis. We included this group to compare a normal immune response to infection with the immune response in sepsis, rather than just comparing to a control group with an immune system not challenged by infection. Given the prevalence of sepsis in the elderly and immune cellular senescence in elderly patients [67], this study focused on older hospitalised patients.

4.5 Methods and Materials

4.5.1 Study Population

Ethical approval was granted by the St. James's hospital Research and Ethics Committee (Ethic approval code 2015-03). Informed written consent was obtained from patients. When this was not possible, we obtained assent from the next of kin, as is permitted by the National Consent Advisory Group for the ethical conduct of research [68]. We collected samples of blood, clinical data, and appropriate information. Within the septic group (42 patients), there were 32 patients assigned to the phenotype group and 10 patients assigned to the stimulation group. Within the infection group (30 patients), there were 20 patients assigned to the phenotype group and 10 patients assigned to the stimulation group. Within the control group (30 age matched healthy donors), there were 20 donors assigned to the phenotype group and 10 donors assigned to the stimulation group. We conducted this study between 2016 and 2018.

Septic group: The Sepsis 3 definitions were used to identify patients with sepsis in the St James's Hospital intensive care unit [1]. The septic patients were then screened with the inclusion and exclusion criteria. If they met the inclusion criteria and consent was obtained, we then drew blood samples. These samples were obtained in the first 72 h of admission. This was time point "0". We then drew 3 more blood samples every 7 days unless the patient was discharged from hospital or died. These were time points "1", "2", and "3".

Infection group: The microbiology service identified patients with proven clinical infection but not sepsis. We recruited these patients from hospital wards in St James's Hospital. We drew blood within 72 h of a clinically significant positive culture. We also drew a second sample of blood 7 days later, unless the patient had been discharged or died. A patient in the phenotype infection group was removed, reducing the number to 19; this was due to hyperbilirubinemia causing uninterpretable flow cytometry.

Control group: Donors for this group were obtained from the community. The donors did not have any infective symptoms in the previous 8 weeks. We drew blood at one time point for this group.

Samples for the cell stimulation were only taken at the time point "0". The patient demographics (Table 4-1) have been reported in a previous study [88]. Our exclusion criteria are detailed in Table 4-2.

Table 4-1 Demographic and clinical data

Clinical Data	Immunophenotype Study			Stimulation Experiment		
	Control	Infection	Septic	Control	Infection	Septic
n	20	19	32	10	10	10
Age	73 [69–78.25]	81.5 [70.25–87.25]	73.5 [68.75–79.25]	72 [67–74.5]	85 [68.75–87.5]	57.5 [53.75–69.25]
Male Gender	6 (30%)	11 (58%)	20 (62.5%)	5 (50%)	6 (60%)	4 (40%)
APACHE score	N/A	12.5 [8–16.5]	19 [16–24.5] $p < 0.0001$	N/A	14 [10–14]	21.5 [16.25–24.5]
SAPS score	N/A	N/A	48 [37.75–54.5]	N/A	N/A	49 [38.25–55.75]
SOFA score on admission	N/A	3 [1.75–4]	7 [5.75–10] $p < 0.0001$	N/A	2 [0–3]	10 [7.5–11.75]
SOFA score on day of first sample	N/A	1 [0.75–1.25]	7 [5–8.25]	N/A	1 [0,1]	8 [4.5–9.75]
Time to 1st sample from admission (days)	N/A	2.5 [2,3]	1.5 [0.75–2]	N/A	3 [2.5–4.5]	5 [4–6]
ICU duration (days)	N/A	N/A	14.5 [8.75–33.25]	N/A	N/A	17.5 [9.25–26]
Mortality in ICU	N/A	N/A	11 (34.4%)	N/A	N/A	1 (10%)
Mortality in Hospital	N/A	0	13 (40.6%)	N/A	1 (10%)	3 (30%)
Inotropic Support	N/A	0	30 (93.75%)	N/A	0	10 (100%)
Days on inotropes	N/A	0	7 [3–13]	N/A	0	7.5 [6–10.5]
Invasive ventilation	N/A	N/A	28 (87.5%)	N/A	N/A	9 (90%)
Days on invasive ventilation	N/A	N/A	14.5 [5–29.25]	N/A	N/A	8.5 [6.25–14.5]
p/F ratio (mmHg)	N/A	265.5 [331–392.25]	170 [135.75–240.5]	N/A	411.5 [386.5–436.5]	163 [129–205.75]

Muscle Relaxant infusion		N/A	N/A	11 (34.4%)	N/A	N/A	5 (50%)
Acute Kidney Injury KDIGO grade ≥1		0	6 (31.58%)	26 (81.25%)	0	2 (20%)	10 (100%)
Renal Replacement Therapy		0	0	16 (50%)	0	0	8 (80%)
Stress dose steroids		0	1 (5.3%)	6 (18.75%)	0	1 (10%)	5 (50%)
Source of Sepsis	Respiratory	N/A	5 (26.3%)	16 (50%)	N/A	3 (30%)	4 (40%)
	Abdominal	N/A	7 (36.8%)	11 (34.4%)	N/A	2 (20%)	3 (30%)
	Skin	N/A	0	4 (12.5%)	N/A	1 (10%)	1 (10%)
	Urine	N/A	6 (31.6%)	0	N/A	4 (40%)	2 (20%)
	Osteomyelitis	N/A	1 (5.3%)	0	N/A	0	0
	Mediastinitis	N/A	0	1 (3.1%)	N/A	0	0
Type of organism	Gram– ve organism	N/A	16 (84%)	9 (28%)	N/A	5 (50%)	5 (50%)
	Gram+ ve organism	N/A	3 (16%)	9 (28%)	N/A	5 (50%)	2 (20%)
	Fungal	N/A	0	1 (3%)	N/A	0	0
	Empiric treatment	N/A	0	13 (41%)	N/A	0	3 (30%)
Secondary Infections		N/A	0	17 (53.1%)	N/A	0	5 (50%)
Lactate on admission		N/A	2.1 [1.15–3.57]	2.86 [2.27–4.09]	N/A	1.95 [1.6–3.1]	2.63 [1.81–3.36]
Comorbidities ≥ 1		19 (95%)	18 (94.7%)	26 (81.25%)	7 (70%)	9 (90%)	8 (80%)

Categorical data are presented as numbers with percentages in parentheses (). Data and parameters are presented as medians and interquartile ranges [Q1-Q3]. n, number of patients; APACHE score, Acute Physiologic Assessment and Chronic Health Evaluation; SOFA score, Sequential Organ Failure Assessment score; KDIGO Kidney Disease: Improving Global Outcomes; N/A, Not Applicable; p/F ratio, ratio of arterial oxygen partial pressure to fractional inspired oxygen.

Table 4-2 Exclusion Criteria

	Exclusion Criteria
1	Any haematological disease
2	Pre-existing liver disease
3	Pre-existing immunodeficiency
4	Immune modulating medications (incl. steroids prior to onset of sepsis)
5	Chronic infection
6	Malignancy

4.5.2 Immune Phenotyping of Circulating Lymphocytes

Freshly drawn whole blood was used for this analysis. Firstly, it was stained with a dead cell stain. The blood was then incubated with fluorochrome-conjugated monoclonal antibodies (mAb). These mAbs were used to identify cell types, differentiation status, and receptor expression. A BD FACS™ lysing solution was then used to lyse the erythrocytes. The samples were then acquired using a FACSCanto™ II flow cytometer (BD Biosciences). The flow cytometry analysis was performed using Treestar's FlowJo™ v10.4.2 software. Gating was conducted on lymphocytes, and analysis was performed once the doublets and dead cells were excluded. The compensation parameters were set using single stained controls. The gates were set using fluorescence-minus-one controls. Full blood counts were used to determine the cell counts.

The mAbs used were CD3 (BW264/56), CD56 (AF12-7H3, REA196), CD45RA (REA562), CD197 (CCR7; REA546), CD27 (M-T271, REA499), IL-12Rβ2 (REA333) and IL-23R (218213), Anti-TCR-Vδ1 (REA173), Anti-TCR-Vδ2 (123R3, REA771), CD161 (REA631, 191B8), Anti-TCR-Vα7.2 (REA179), and CD8 (BW135/80, REA734) (R&D Systems, Abingdon, UK and Miltenyi Biotec, Gladbach Bergische, Germany). The dead cells stain used was LIVE/DEAD Fixable Aqua dead cell stain, Molecular Probes, Leiden, The Netherlands.

The NK cells were identified as $CD3^{-}CD56^{+}$. The differentiation states of the circulating MAIT ($CD3^{+}CD161^{+}V\alpha7.2^{+}$) and NKT ($CD3^{+}CD56^{+}$) cells were characterised as naive ($CD45RA^{+}$, $CD197^{+}$), central memory ($CD45RA^{-}$, $CD197^{+}$), effector memory ($CD45RA^{-}$, $CD197^{-}$), or terminally differentiated ($CD45RA^{+}$, $CD197^{-}$). The differentiation states of the circulating γδ T cell lymphocytes were characterised as naive ($CD45RA^{+}$, $CD27^{+}$), central memory ($CD45RA^{-}$, $CD27^{+}$), effector memory ($CD45RA^{-}$, $CD27^{-}$), or terminally differentiated ($CD45RA^{+}$, $CD27^{-}$).

The details of the experiment protocols can be found on the following open access link: dx.doi.org/10.17504/protocols.io.bfvhjn36.

4.5.3 Lymphocyte Stimulation in Vitro

Freshly drawn blood was prepared by density gradient centrifugation over Lymphoprep™ (Axis-Shield, Dundee, UK) to produce peripheral blood mononuclear cells (PBMCs). The stimulation of 0.5 million cells was performed for 5 h using 50 ng/mL of phorbol myristate acetate with 1 µg/mL of ionomycin (PMA/I). Brefeldin-A was added to the wells intended for the intracellular staining of IFN-γ and IL-17A. After stimulation, a dead cell stain was added. The cell surface markers were then labelled with fluorochrome-conjugated mAbs. The fixation and permeabilization of the cells were performed with 4% paraformaldehyde and 0.2% saponin, respectively. The cells were then stained intracellularly with fluorochrome-conjugated mAbs specific for IFN-γ and IL-17a. The FoxP3 Staining Buffer Set (Miltenyi Biotec) was used in place of the paraformaldehyde and saponin above for the intra-cellular staining of T-Bet and RORγt. A cell fixation was performed again once they were stained. An immediate acquisition of the samples was performed. The acquisition and analysis were as per the immune phenotyping above in Section 4.5.2.

The mAbs used were as above in Section 4.5.2. and mAbs IFN-γ (REA600), IL-17A (CZ8-23G1), T-Bet (REA102), and RORγt (REA278). The dead cell stain used was LIVE/DEAD Fixable Near IR dead cell stain, Thermo Fisher Scientific, Massachusetts, US. The protocols for these experiments can be found at the following open access links: dx.doi.org/10.17504/protocols.io.bfvgjn3w and dx.doi.org/10.17504/protocols.io.bfvfjn3n.

For an analysis with flow cytometry, the gating strategy was as per Section 4.5.2 and an example of this can be seen in Figure 4-1.

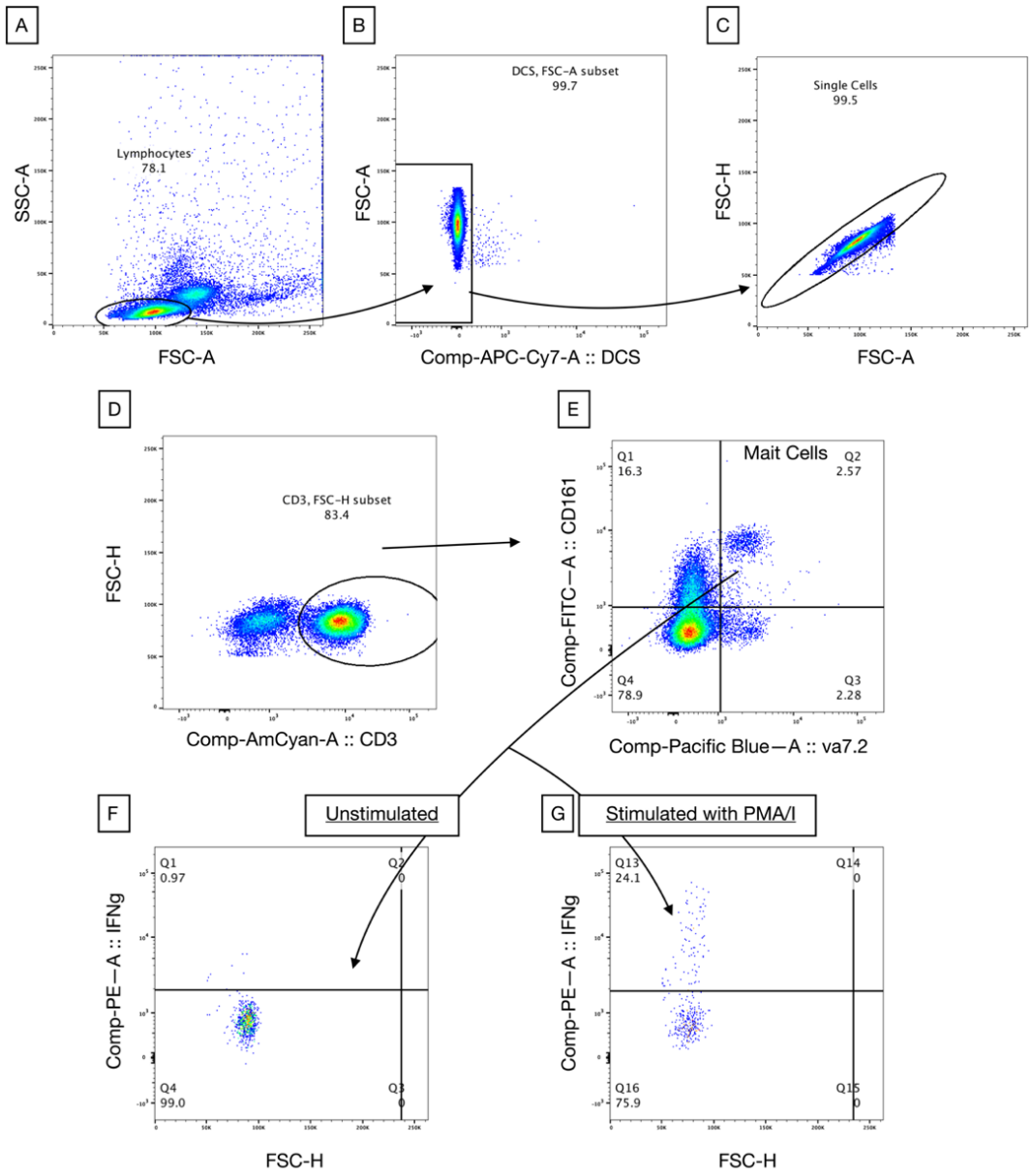

Figure 4-1 Flow cytometry gating strategy

*Flow cytometry analysis. Lymphocyte gating on PBMCs (**A**), with exclusion of dead cells (**B**) and exclusion of doublets (**C**). Gating on CD3⁺ cells (**D**) to show MAIT cells (**E**). MAIT cells were gated on showing interferon-γ (IFN-γ) expression in unstimulated (**F**) and stimulated (**G**) cells. Stimulation was performed with phorbol myristate acetate and ionomycin (PMA/I). DCS is Dead Cell Stain.*

4.5.4 Statistical Analysis

The SPSS® Statistical Software (IBM Corp, New York, USA) program was used for the analysis of the statistics. Wilcoxon/Kruskal Wallis testing was employed to compare the differences between the three patient groups (represented by a capped line in the figures). The *p* values for subsequent comparisons between the individual groups were Bonferoni adjusted for multiple comparisons (represented as a n-zigzag line in the figures). Chi Square testing was employed to compare the categorical variables.

A mixed effects general linear regression model was employed to analyse repeated measurements. Where a significant change in a repeated measurement was detected, the repeated assays were compared with the initial assay values with Bonferoni adjusted *p* values for multiple comparisons.

Throughout the analysis, a *p* value of less than 0.05 was considered significant.

4.6 Results

4.6.1 Demographics

Table 4-1 outlines the demographic data. The three groups had similar age demographics. There were more male than female patients in the immunophenotyping study. This is consistent with sepsis being a disorder of the elderly and more so in males [52]. The septic group had higher organ failure scores ($p < 0.0001$) and Apache II scores ($p < 0.0001$) than the infection group. In the immune phenotyping study, 13 (40%) patients in the septic group died, whereas mortality was 0% in the control and infection groups. The phenotype of the cells presented in this paper are those at the first time point unless otherwise stated.

4.6.2 MAIT Cells

The percentage of MAIT cells among the lymphocytes in peripheral circulation differed across the three patient groups ($p = 0.03$), with the percentage of MAIT cells being lower in the infection group compared with in the control group ($p = 0.03$) (Figure 4-2B). The percentage of MAIT cells expressing CD8 was similar in the three groups (Figure 4-2C). The absolute MAIT cell counts differed across the three groups ($p < 0.001$), with MAIT cell counts

being lower in the septic group (p = 0.002) and the infection group (p < 0.001) than in the control group (Figure 4-2D). In the septic group, the MAIT cell counts did not change over time (Figure 4-2E). Thus, the MAIT cells appear to be depleted in both sepsis and infection.

The percentages of naive MAIT cells differed across the three groups (p < 0.001) and were lower in the infection group (p < 0.001) and the septic group (p = 0.003) than in the control group (Figure 4-2F). Similarly, the percentages of central memory MAIT cells were different between the groups (p = 0.029), with lower percentages of central memory MAIT cells in the septic group than in the control group (p = 0.051) (Figure 4-2F). The percentages of effector memory and terminally differentiated MAIT cells were similar in the three groups. The decrease in the naive and central memory MAIT cells in patients with sepsis suggests that MAIT cells are activated both in patients with sepsis and with infection.

The percentage of MAIT cells expressing IL-12Rβ2 and IL-23R was similar in the three patient groups, with IL-23R expression being more prevalent than that of IL-12Rβ2 (Figure 4-3A, B). The absolute numbers of IL-23R expressing MAIT cells differed across the patient groups (p = 0.004), with the MAIT IL-23R$^+$ cell counts being lower in the septic group (p = 0.003) than in the control group (Figure 4-3C). The MAIT IL-23R$^+$ cell count in the septic group did not change over time (Figure 4-3D). The discordance between the percentage and absolute counts of MAIT IL-23R$^+$ cells in patients may arise from relative lymphopoenia and redistribution into extravascular compartments in patients with sepsis.

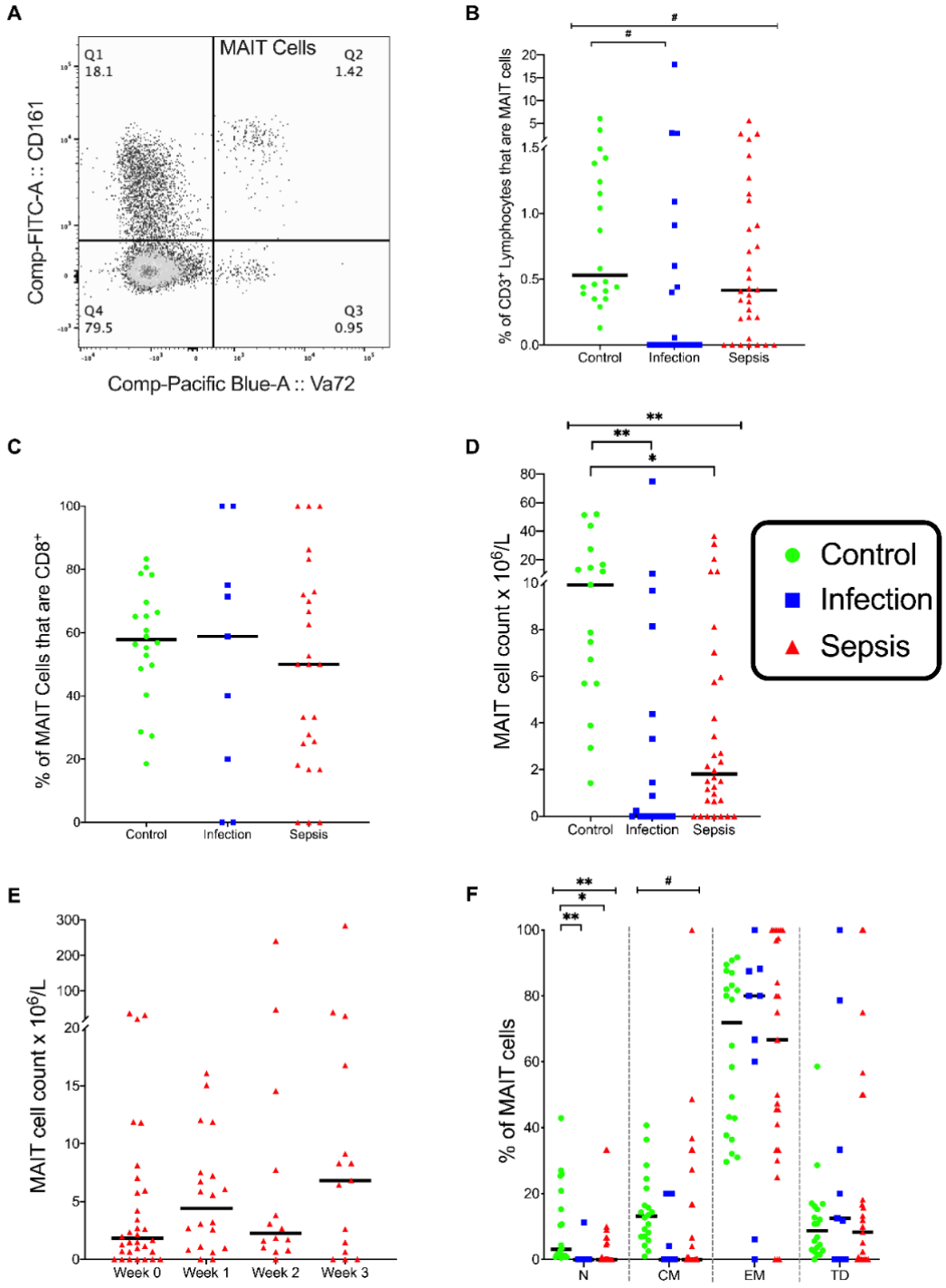

Figure 4-2 MAIT cell phenotypes

*(**A**) Flow cytometry plot showing MAIT cell population ($CD3^+CD161^+V\alpha7.2^+$) on gated $CD3^+$ lymphocytes. (**B**) Frequency of MAIT cells as a percentage of T cells ($CD3^+$ lymphocytes). (**C**) Frequency of MAIT $CD8^+$ cells as a % of MAIT cells. (**D**) Total MAIT cell count in circulating blood. (**E**) Total MAIT cell count over time in the septic group. (**F**) Frequencies of naive, central memory, effector memory, and terminally differentiated (N, CM, EM, TD, respectively) MAIT cells in circulating blood. Control group (n = 20), infection group (n = 19), and septic group (n = 32). Graphs are plotted with bars representing the median. # = $p < 0.05$; * = $p \leq 0.01$; ** = $p \leq 0.001$.*

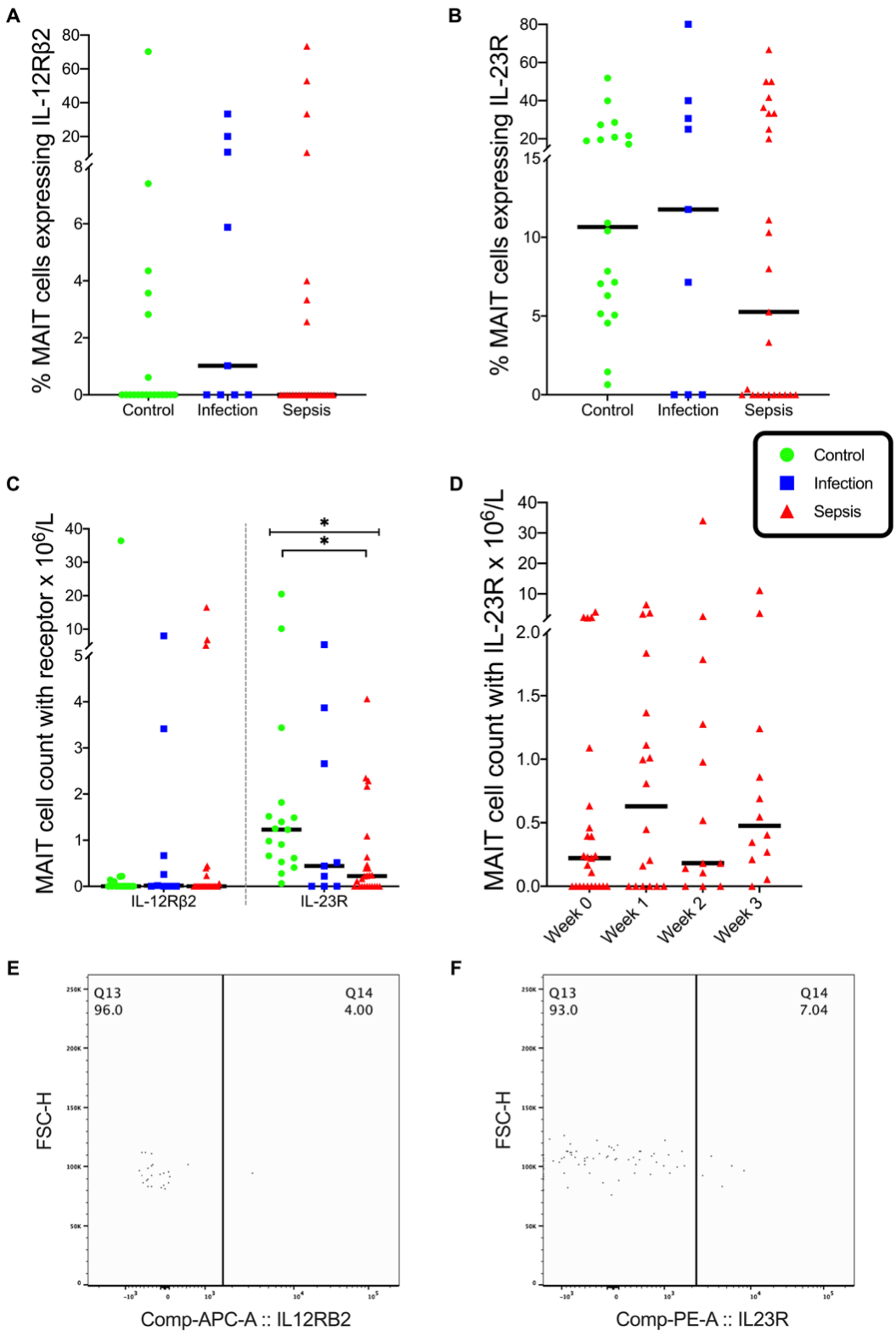

Figure 4-3 MAIT cell expression of IL-12R and IL-23R

*(**A**, **B**) Percentage of MAIT cells in circulating blood expressing IL-12 receptor (IL-12Rβ2) and IL-23 receptor (IL-23R). (**C**) MAIT cell count in circulating blood expressing IL-12Rβ2 and IL-23R. (**D**) MAIT cell count expressing IL-23R over time. (**E**) Flow cytometry plot on gated MAIT cells showing IL-12Rβ2. (**F**) Flow cytometry plot on gated MAIT cells showing IL-23 receptor. Control group (n = 20), infection group (n = 19), and septic group (n = 32). Graphs are plotted with bars representing the median. * = p ≤ 0.01.*

In the absence of stimulation, the T-Bet expression in MAIT cells differed across the three patient groups (p = 0.015), with T-Bet expression being greater in the infection group than in the septic group (p = 0.012) (Figure 4-4A). When the MAIT cells were stimulated with phorbol myristate acetate and ionomycin (PMA/I), the T-Bet expression was different in the three groups (p = 0.008), with the T-Bet expression being greater in the infection group than in the septic group (p = 0.006) (Figure 4-4A). In the absence of stimulation, the terminally differentiated MAIT cell expression of T-Bet was different in the three groups (p = 0.007), with T-Bet expression being greater in the infection group than in the septic group (p = 0.006) (Figure 4-4C). Thus, the MAIT cells expressed a predominant T_H1 phenotype in patients with infection but not in those with sepsis.

When MAIT cells were stimulated with PMA/I, the RORγt expression was different in the three groups (p = 0.004), with the RORγt expression being lower in the septic group than in the control group (p = 0.003) (Figure 4-4B). The RORγt expression in effector memory MAIT cells stimulated with PMA/I differed in the three groups (p = 0.008), with the RORγt expression being lower in the septic group than in the control group (p = 0.008) (Figure 4-4D). Thus, the MAIT cells of septic patients failed to express a T_H17 phenotype, which is consistent with the depletion of IL-23R expression in MAIT cells in patients with sepsis. MAIT cells stimulated with PMA/I exhibited a similar expression of IFN-γ and IL-17a in the three patient groups (Figure 4-4E). Thus, the clinical presentation of infection or sepsis is linked both with MAIT cell counts but also with the MAIT cell phenotype.

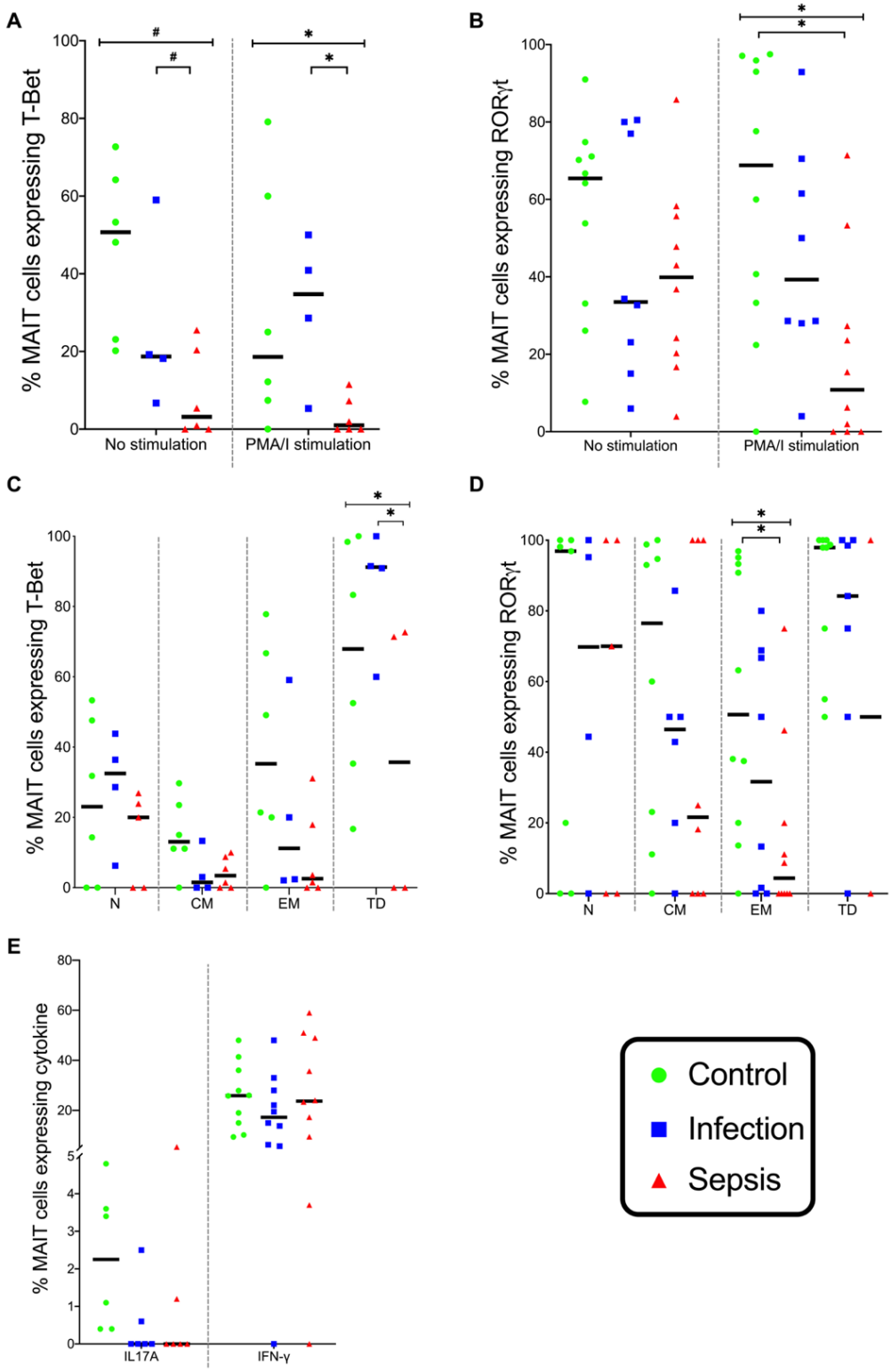

Figure 4-4 MAIT cell expression of RORγt, T-Bet, IL-17A, and IFN-γ

*(**A**) MAIT cell expression of T-Bet when stimulated with the medium alone or PMA/I. (**B**) MAIT cell expression of RORγt when stimulated with the medium alone or PMA/I. (**C**) Expression of transcription factor T-Bet in unstimulated naive, central memory, effector memory, and terminally differentiated (N, CM, EM, TD, respectively) MAIT cells. (**D**) Transcription factor RORγt expression in PMA/I-stimulated N, CM, EM, and TD MAIT cells. (**E**) MAIT cell expression of IL-17a and IFN-γ when stimulated with PMA/I. Control group (n = 10), infection group (n = 10), and septic group (n = 10). Graphs are plotted with bars representing the median. # = $p < 0.05$; * = $p \leq 0.01$.*

4.6.3 $CD3^+$ $CD161^+$ Lymphocytes

$CD3^+$ lymphocytes expressing $CD161^+$ include a composite population of lymphocytes capable of secreting IL-17, including both adaptive and innate lymphocytes such as MAIT and NKT cells [87, 102].

The percentage of $CD3^+CD161^+$ lymphocytes was similar in the three patient groups (Figure 4-5A). There was a significant difference in the percentages of naive $CD3^+$ $CD161^+$ lymphocytes in the three patient groups (p = 0.001); the percentages of naive $CD3^+CD161^+$ lymphocytes were lower in the infection group than either in the control group (p = 0.001) or the sepsis group (p = 0.004) (Figure 4-5B). There was a significant difference in the percentages of central memory $CD3^+CD161^+$ lymphocytes in the three groups (p = 0.001), with percentages of central memory $CD3^+CD161^+$ lymphocytes being lower in the infection group than in either the control group (p = 0.002) or the septic group (p = 0.012) (Figure 4-5B). The percentages of effector memory and terminally differentiated $CD3^+CD161^+$ lymphocytes were similar in all the patient groups (Figure 4-5B). The decrease in naive and central memory $CD3^+CD161^+$ lymphocytes in patients with infection reflects the activation of these lymphocytes, which was not present in patients with sepsis. As many of these $CD3^+CD161^+$ lymphocytes are adaptive and thus MHC-restricted lymphocytes, the $CD3^+CD161^+$ lymphocyte activation failure in patients with sepsis likely reflects the well-described downregulation of antigen presentation mechanisms in sepsis. This contrasts with innate non-MHC-restricted innate lymphocytes,

such as MAIT cells, that are activated equally in patients with sepsis and infection.

With regard to absolute counts, $CD3^+CD161^+$ lymphocytes increased in patients with sepsis from 106 +/− 15 (mean +/− standard error) to 211 +/− 64 (mean +/− standard error) over the course of the study ($p = 0.047$) (Figure 4-5C). The absolute number of effector memory $CD3^+$ $CD161^+$ lymphocytes ($p = 0.006$) and terminally differentiated $CD3^+CD161^+$ lymphocytes ($p = 0.004$) increased in the septic group in the three weeks after admission (Figure 4-5C).

In the unstimulated $CD3^+CD161^+$ lymphocytes, the T-Bet expression differed across the three groups ($p= 0.025$), with a greater T-Bet expression in the infection group than in the septic group ($p = 0.02$) (Figure 4-5D). In unstimulated $CD3^+CD161^+$ lymphocytes, RORγt expression differed across the three groups ($p = 0.004$), with a lower RORγt expression in the septic group than in the control group ($p = 0.003$) (Figure 4-5D). IL-17A and IFN-γ expression was similar in the $CD3^+CD161^+$ lymphocytes from the three patient groups when stimulated with PMA/I (Figure 4-5D). Thus, in a similar manner to MAIT cells, $CD3^+CD161^+$ lymphocytes from patients with infection exhibit an augmented T_H1 response, whereas the $CD3^+CD161^+$ lymphocytes of patients with sepsis exhibit a deficient T_H17 response.

Nine of the patients with sepsis died. Among the patients with sepsis, the percentage of all the lymphocytes that were $CD3^+$ $CD161^+$ lymphocytes was lower in patients who died ($n = 9$, median 3%, 95% confidence interval 1–7%)

versus in survivors (*n* = 23, median 9%, 95% confidence interval 7–17%) (Mann U Whitney test, *p* = 0.008) (Figure 4-5E).

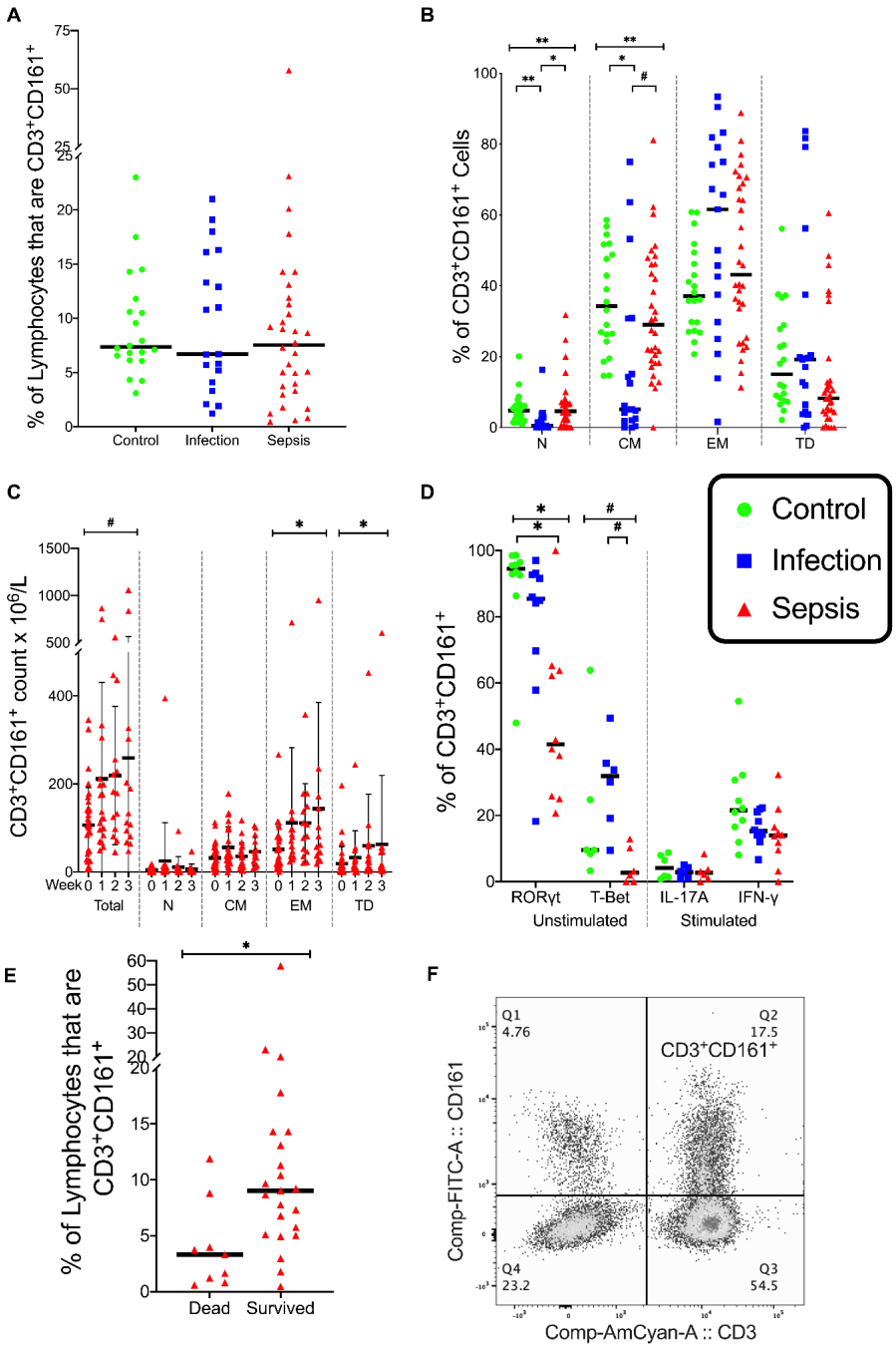

Figure 4-5 CD3⁺CD161⁺ lymphocytes

*(**A**) Frequency of CD3⁺CD161⁺ cells as a percentage of lymphocytes. (**B**) Differentiation status of CD3⁺CD161⁺ lymphocytes in the circulating blood. Naive, central memory, effector memory, and terminally differentiated (N, CM, EM, TD, respectively) differentiation states shown. (**C**) Total number of CD3⁺CD161⁺ lymphocytes and the number of CD3⁺CD161⁺ lymphocytes in their differentiated states in patients with sepsis over time. (**E**) Frequency of lymphocytes that are CD3⁺CD161⁺ in patients who survived compare to those that died. Control group (n = 20), infection group (n = 19), and septic group (n = 32). (**D**) RORγt and T-Bet expression in unstimulated CD161⁺ T cells and cytokines IL-17A and IFN-γ in stimulated CD161⁺ T cells with phorbol myristate acetate and ionomycin. Control group (n = 10), infection group (n = 10), and septic group (n = 10). (**F**) Flow cytometry plot of gated lymphocytes showing CD3⁺CD161⁺ cells. (**A**, **B**, **D**, **E**) Graphs are plotted with bars representing the median. (**C**) Data represented as mean with standard deviation. # = $p < 0.05$; * = $p \leq 0.01$; ** = $p \leq 0.001$.*

4.6.4 Natural Killer (NK) Cells

The percentage of NK cells within the pool of circulating lymphocytes was different across the three groups ($p = 0.002$), with proportionately fewer NK cells in the septic group than in the infection group ($p = 0.02$) and the control group ($p = 0.006$) (Figure 4-6B). In the septic group, the percentage of NK cells increased over the four study times ($p = 0.004$), from 5% (+/−0.7%) to 11.1% (+/−1.7) (Figure 4-6D).

The NK cell counts differed in the three groups ($p < 0.001$), with lower NK counts in the septic group ($p < 0.001$) and the infection group ($p = 0.001$) than in the control group (Figure 4-6C). The NK cell counts increased in septic patients over the four study time points ($p = 0.028$) (Figure 4-6E).

The majority of NK cells were $CD56^{dim}$ (NK Dim) cells. The expression of IL-23R in NK cells was similar across the three patient groups (Figure 4-7A). The expression of IL-12Rβ2 in NK cells differed significantly across the three groups ($p = 0.008$), with the NK cell IL-12Rβ2 expression lower in the septic group than in the control group ($p = 0.05$) and lower in the infection group than in the control group ($p = 0.01$) (Figure 4-7A). The number of NK IL-12Rβ2 lymphocytes differed across the three groups ($p = 0.002$), with fewer NK IL-12Rβ2 lymphocytes in the septic group ($p = 0.006$) and the infection group ($p = 0.003$) than in the control group (Figure 4-7B). However, the expression of IL-12Rβ2 by NK cells was so small that it is unlikely to be of clinical significance.

NK cells stimulated with PMA/I showed that the percentage of NK cells expressing T-Bet differed in the three groups (p = 0.002), with fewer lymphocytes expressing T-Bet in the septic group than in the infection group (p = 0.002) (Figure 4-7C). The NK cells stimulated with PMA/I also showed that the percentage of NK cells expressing IFN-γ was different in the three groups (p = 0.003), with fewer NK cells of patients with sepsis expressing IFN-γ than the controls (p = 0.002) (Figure 4-7D). Thus, the septic patients exhibited a deficient NK T_H1 response.

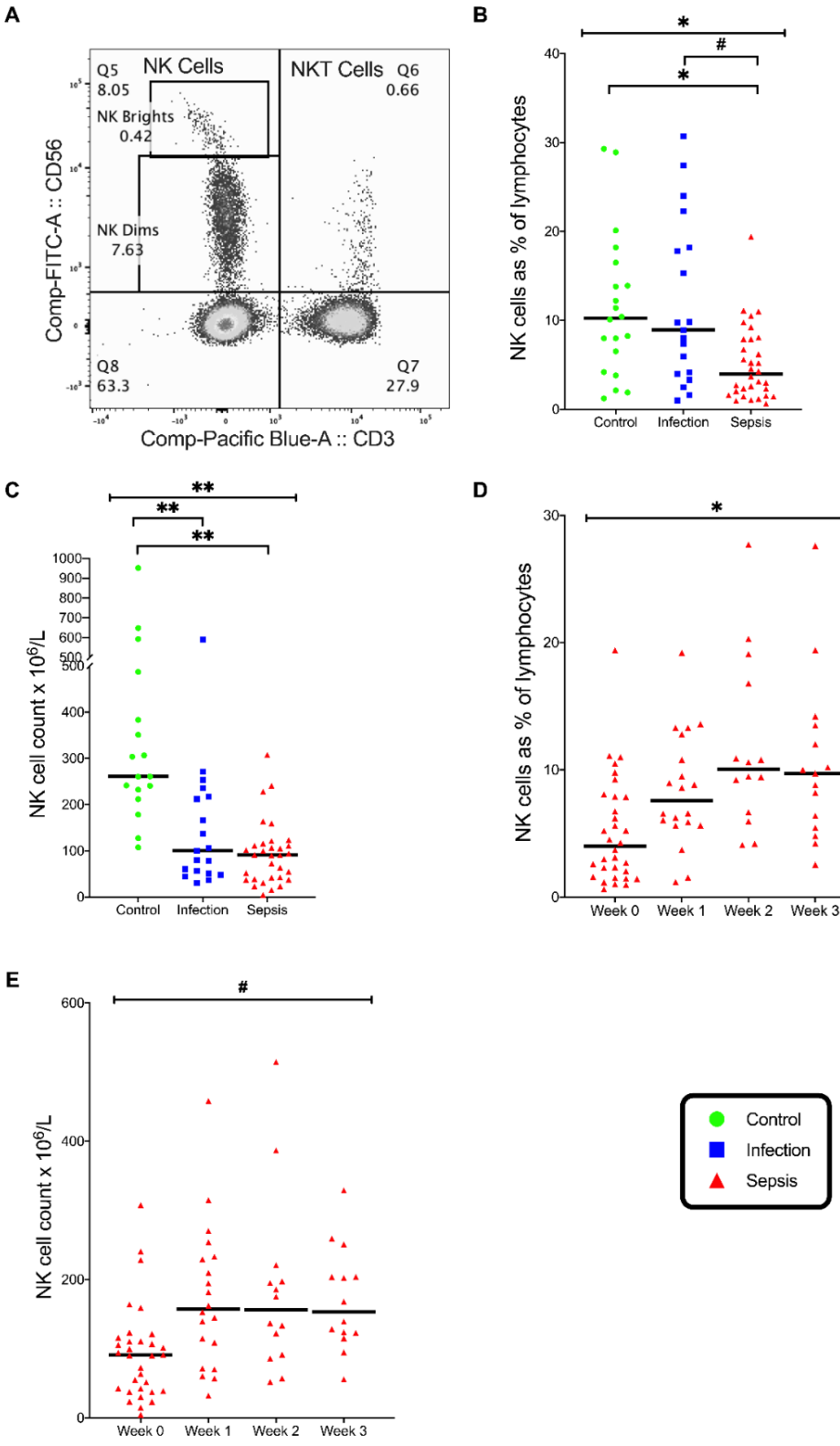

Figure 4-6 Natural Killer Cells

*(**A**) Flow cytometry plot on gated lymphocytes showing NK cells ($CD3^{-}CD56^{+}$) and $CD56^{+}$ T Cells ($CD3^{+}CD56^{+}$). (**B**) NK cells as a percentage of lymphocytes. (**C**) NK cell count in the three patient groups on admission. (**D**) Frequency of NK cells as a percentage of lymphocytes in septic patients over time. (**E**) NK cell count in septic patients over time. Control group (n = 20), infection group (n = 19), and septic group (n = 32). Graphs are plotted with bars representing the median. # $p < 0.05$; * $p \leq 0.01$; ** $p \leq 0.001$; *** $p \leq 0.0001$.*

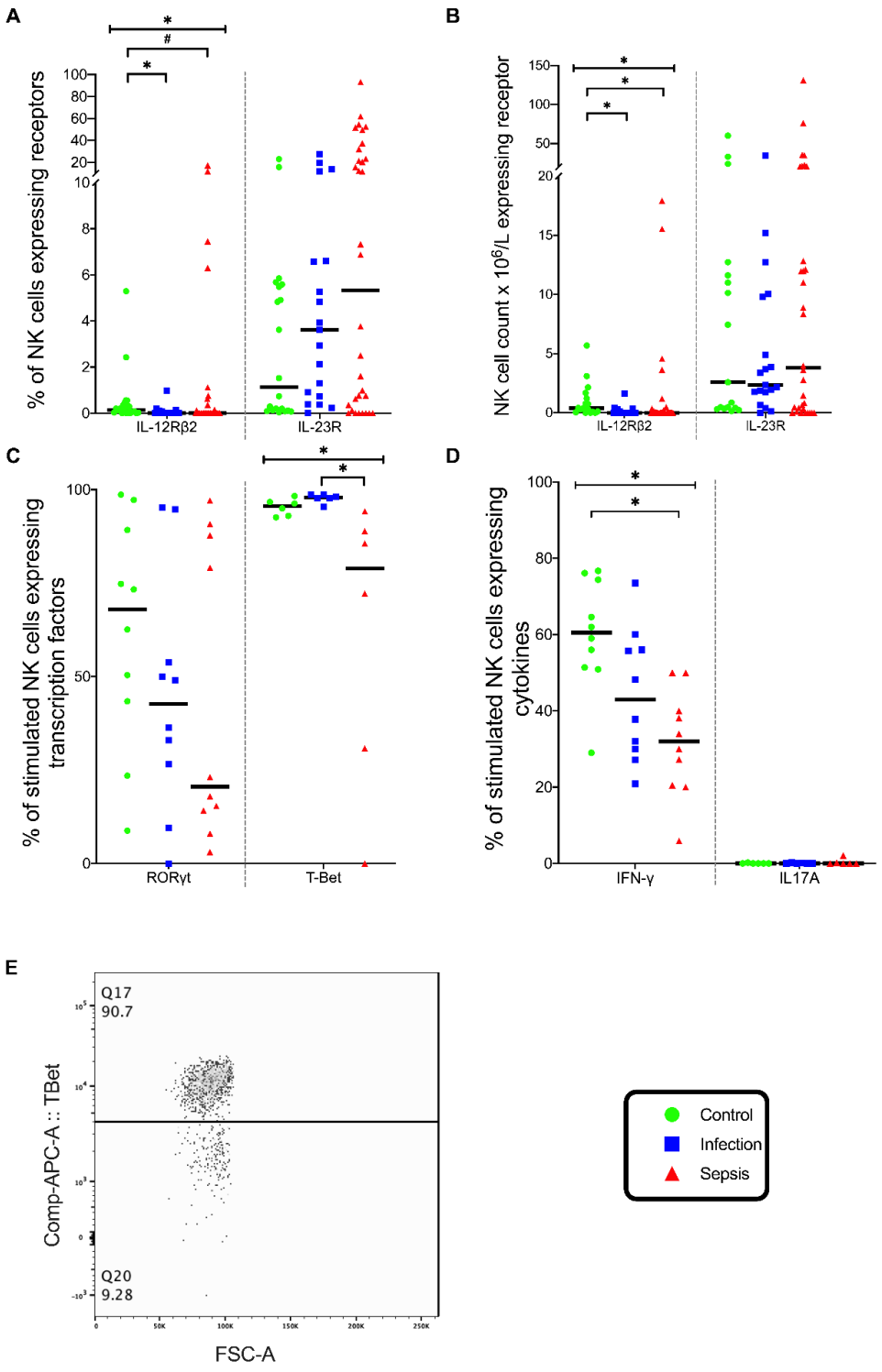

Figure 4-7 NK Cell Phenotype and stimulation

*(**A**) Frequency of NK cells expressing IL-12 receptor (IL-12Rβ2) and IL-23 receptor (IL-23R). (**B**) Absolute numbers of NK cells expressing IL-12Rβ2 and IL-23R. (**A**, **B**) Control group (n = 20), infection group (n = 19), and septic group (n = 32). (**C**) Frequency of NK cells expressing the transcription factors RORγt and T-Bet when stimulated with phorbol myristate acetate and ionomycin (PMA/I). (**D**) Frequency of NK cells expressing cytokines IL-17A and IFN-γ when stimulated with PMA/I. C-D, control group (n = 10), infection group (n = 10), and septic group (n = 10). (**E**) Flow cytometry plot on gated NK cells showing T-Bet expression. All the graphs are plotted with bars representing the median. # = $p < 0.05$; * = $p \leq 0.01$.*

4.6.5 Natural Killer T (NKT) Cells

The percentages of NKT cells differed across the three patient groups (p = 0.003), with more NKT cells in the infection group than in the septic group (p = 0.002) (Figure 4-8A). The percentage of naive NKT cells differed across patient groups ($p < 0.001$), with fewer naïve NKT cells in the septic group (p = 0.01) and the infection group ($p < 0.001$) than in the control group (Figure 4-8E).

The NKT cell counts differed across the three groups (p = 0.008), with lower NKT cell numbers in the septic group than in the control group (p = 0.04) and the infection group (p = 0.02) (Figure 4-8B). The NKT cell counts did not change over time in the septic patients (Figure 4-8C).

The naive NKT cell counts differed across the three groups ($p < 0.001$), with fewer naive NKT cells in the septic group than in the control group ($p < 0.001$) and fewer in the infection group than in the control group (p = 0.001) (Figure 4-8F). There were scant NKT central memory cells in the three groups. The NKT effector memory cell counts were different in the three groups (p = 0.02), with more NKT effector memory cells in the infection group than in the septic group (p = 0.035) (Figure 4-8F). The NKT terminally differentiated cells differed in the three groups (p = 0.002), with fewer NKT terminally differentiated cells in the septic group than in the control group (p = 0.03) and the infection group (p = 0.003) (Figure 4-8F). These lower naive NKT cell counts and increased NKT effector memory cells reflect NKT cell activation in sepsis and infection.

The NKT IL-23 receptor (IL-23R) expression was similar across the three patient groups (Figure 4-8D). However, the expression of IL-12 receptor (IL-12Rβ2) differed significantly across the three groups (p = 0.004), with the IL-12Rβ2 expression lower in the infection group than in the control group (p = 0.003) (Figure 4-8D). So few NKT cells expressed either IL-12Rβ2 or IL-23R that the comparisons of the cell counts between groups were deemed irrelevant.

Without stimulation, the percentage of NKT cells expressing T-Bet differed in the three groups (p = 0.01), with fewer NKT cells expressing T-Bet in the septic group than in the infection group (p = 0.01) (Figure 4-9A). In stimulated NKT cells, the expression of T-Bet was different in the three groups (p = 0.005), with fewer NKT cells expressing T-Bet in the septic group than those in the infection group (p = 0.004) (Figure 4-9A). In the stimulated NKT cells, the expression of RORγt was different in the three groups (p = 0.003), with fewer NKT cells expressing RORγt in the septic group than in the control group (p = 0.003) (Figure 4-9B).

In the stimulated NKT cells, the expression of IFN-γ was different in the three groups (p = 0.002), with fewer NKT cells expressing IFN-γ in the septic group than in the control group (p = 0.002) and the infection group (p = 0.036) (Figure 4-9C). When stimulated, the NKT cells expressed similar IL-17A in the three groups. (Figure 4-9D). Thus, the septic patients exhibited deficiencies in both NKT T_H1 and T_H17 responses.

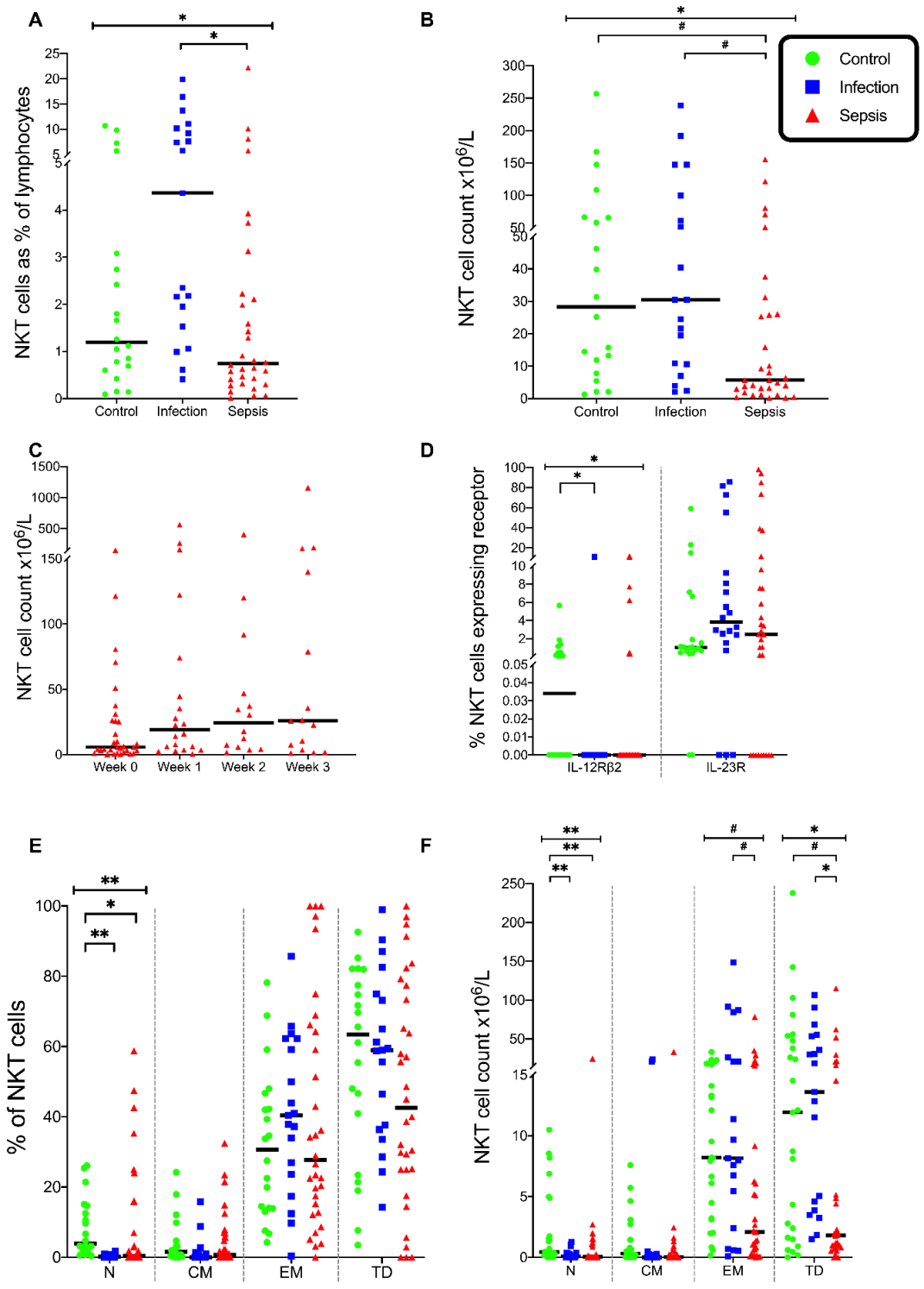

Figure 4-8 NKT cell phenotype

*(**A**) Frequencies of natural killer T (NKT) cells as a percentage of lymphocytes. (**B**) Absolute numbers of NKT cells in the three patient groups. (**C**) Total number of NKT cells in septic patients over time. (**D**) Frequency of NKT cells that express the IL-12 receptor (IL-12Rβ2) and IL-23 receptor (IL-23R). (**E**) Differentiation status of NKT cells in circulating blood. Naive, central memory, effector memory, and terminally differentiated (N, CM, EM, TD, respectively) differentiation states shown. (**F**) NKT cell count by differentiation status. Control group (n = 20), infection group (n = 19), and septic group (n = 32). Graphs are plotted with bars representing the median. * = p ≤ 0.01; ** = p ≤ 0.001.*

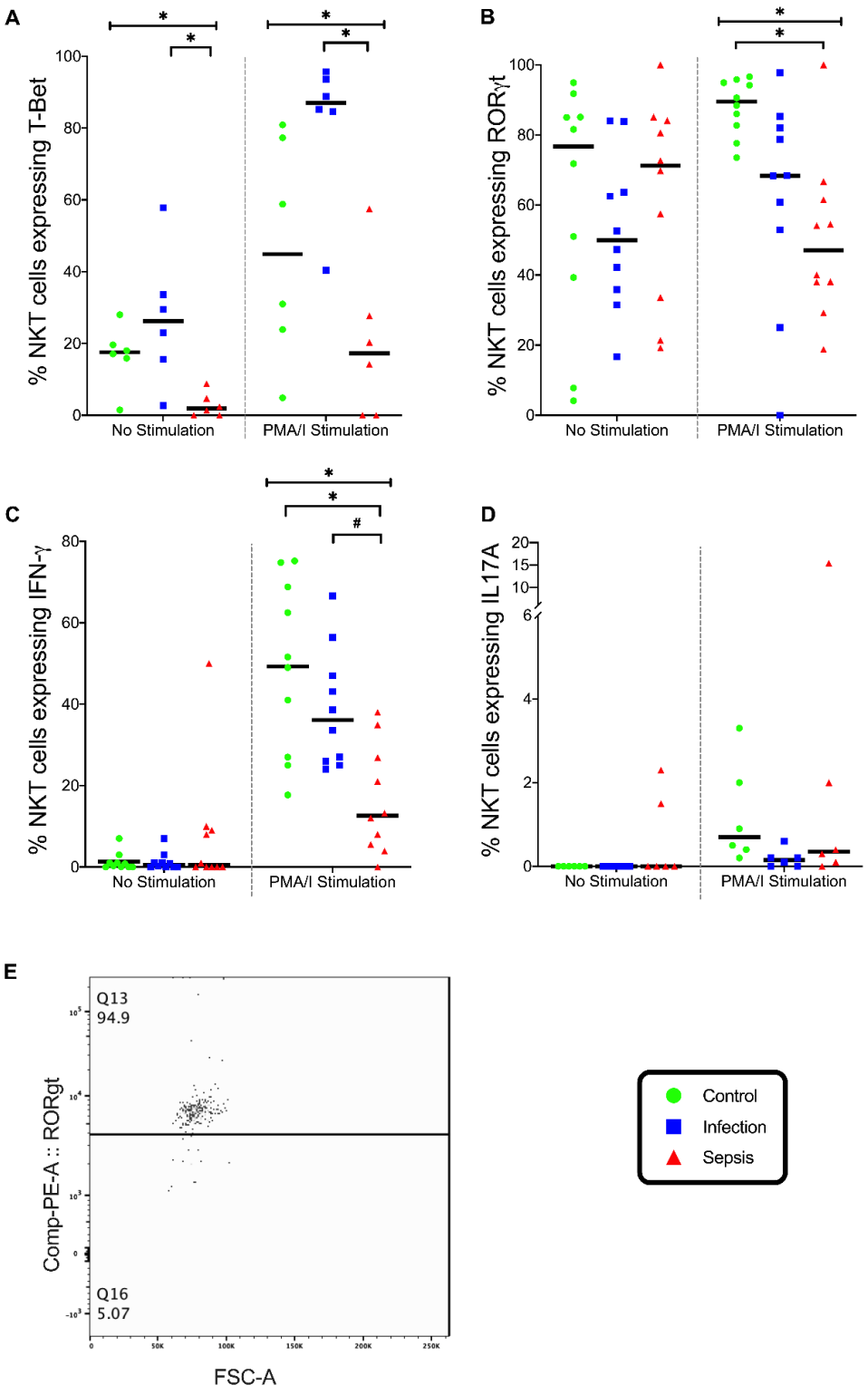

Figure 4-9 NKT Cell Stimulation

*(**A**) Frequencies of unstimulated natural killer T (NKT) cells and stimulated NKT cells with phorbol myristate acetate and ionomycin (PMA/I) expressing T-Bet. (**B**) Frequency of unstimulated NKT cells and stimulated NKT cells with PMA/I expressing RORγt. (**C**) Frequency of unstimulated NKT cells and stimulated NKT cells with PMA/I expressing IFN-γ. (**D**) Frequency of unstimulated NKT cells and stimulated NKT cells with PMA/I expressing IL-17A. (**E**) Flow cytometry plot on gated NKT cells expressing RORγt. Control group (n = 10), infection group (n = 10), and septic group (n = 10). Graphs are plotted with bars representing the median. # = $p < 0.05$; * = $p \leq 0.01$.*

4.6.6 Gamma-Delta (γδ) T Lymphocytes

The percentages of Vδ1 T cells were similar in the three groups (Figure 4-10C). However, the percentage of naive Vδ1 T cells were significantly different in the three groups (p = 0.001), with fewer naive Vδ1 T cells in the infection group than in the control group (p = 0.001) and fewer in the septic group than in the control group (p = 0.01) (Figure 4-10E). The absolute counts of Vδ1 T cells were similar in the three groups (Figure 4-10D).

The absolute counts of naive Vδ1 T cells were different in the three groups (p = 0.002); the naive Vδ1 T cell counts were lower in the septic group (p = 0.002) and the infection group (p = 0.016) than in the control group (Figure 4-10F). This data indicates Vδ1 T cell activation in both sepsis and infection, as was evident with the other non-MHC-restricted lymphocytes.

Few Vδ1 T cells expressed IL-12Rβ2 (Figure 4-10B). The expression of IL-23R was more frequent than that of IL-12Rβ2 and was significantly different across the three groups (p < 0.001) (Figure 4-10B). The Vδ1 IL-23R$^+$ expression was different in the three groups (p = 0.001), with fewer Vδ1 T cells expressing IL-23R in the infection group than in the control group (p = 0.001) (Figure 4-10B).

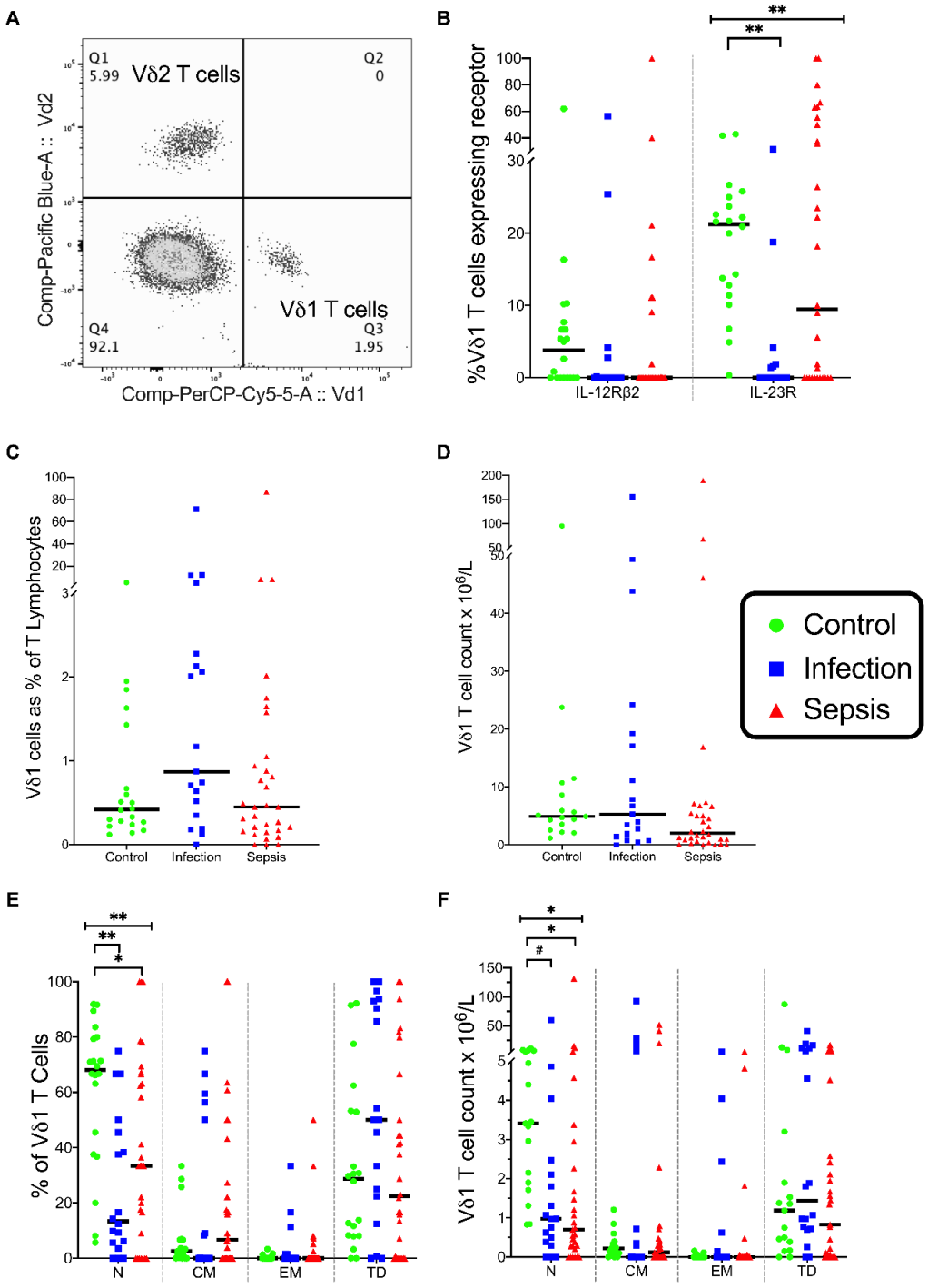

Figure 4-10 Vδ1 cell phenotype

*(**A**), Flow cytometry plot on gated T cells showing Vδ1 and Vδ2 cells. (**B**) Frequency of Vδ1 T cells expressing IL-12 receptor (IL-12Rβ2) and IL-23 receptor (IL-23R). (**C**) Frequency of T cells that express Vδ1 TCRs. (**D**) Vδ1 T cell count in the three patient groups. (**E**) Differentiation status of Vδ1 T cells. Naive, central memory, effector memory, and terminally differentiated (N, CM, EM, TD, respectively) differentiation states shown. (**F**) Differentiation status of Vδ1 T cells expressed as the total cell count. Control group (n = 20), infection group (n = 19), and septic group (n = 32). Graphs are plotted with bars representing the median. # = $p < 0.05$; * = $p \leq 0.01$; ** = $p \leq 0.001$.*

The Vδ1 T cell expression of T-Bet and RORγt was similar in the three patient groups (Figure 4-11A, B). In Vδ1 T cells stimulated with PMA/I, the IFN-γ expression differed between the patient groups (p = 0.02), with a greater IFN-γ expression in the infection group than in the septic group (p = 0.028) (Figure 4-11C).

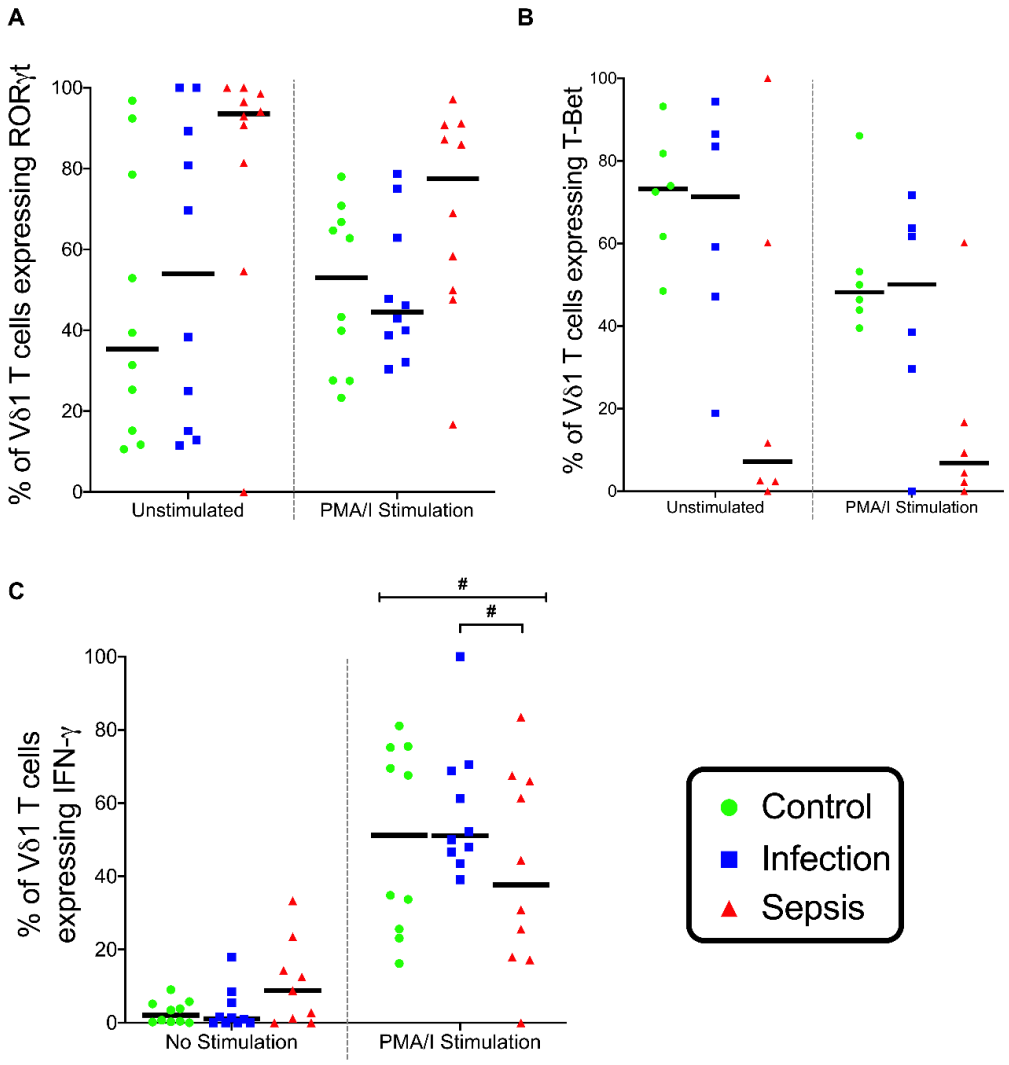

Figure 4-11 Vδ1 cell stimulation

*(**A**) Frequency of unstimulated and stimulated Vδ1 cells expressing RORγt. (**B**) Frequency of unstimulated and stimulated Vδ1 cells expressing T-Bet. (**C**) Frequency of unstimulated and stimulated Vδ1 cells expressing IFN-γ. All were stimulated with phorbol myristate acetate and ionomycin (PMA/I). Control group (n = 10), infection group (n = 10), and septic group (n = 10). Graphs are plotted with bars representing the median. # = p < 0.05.*

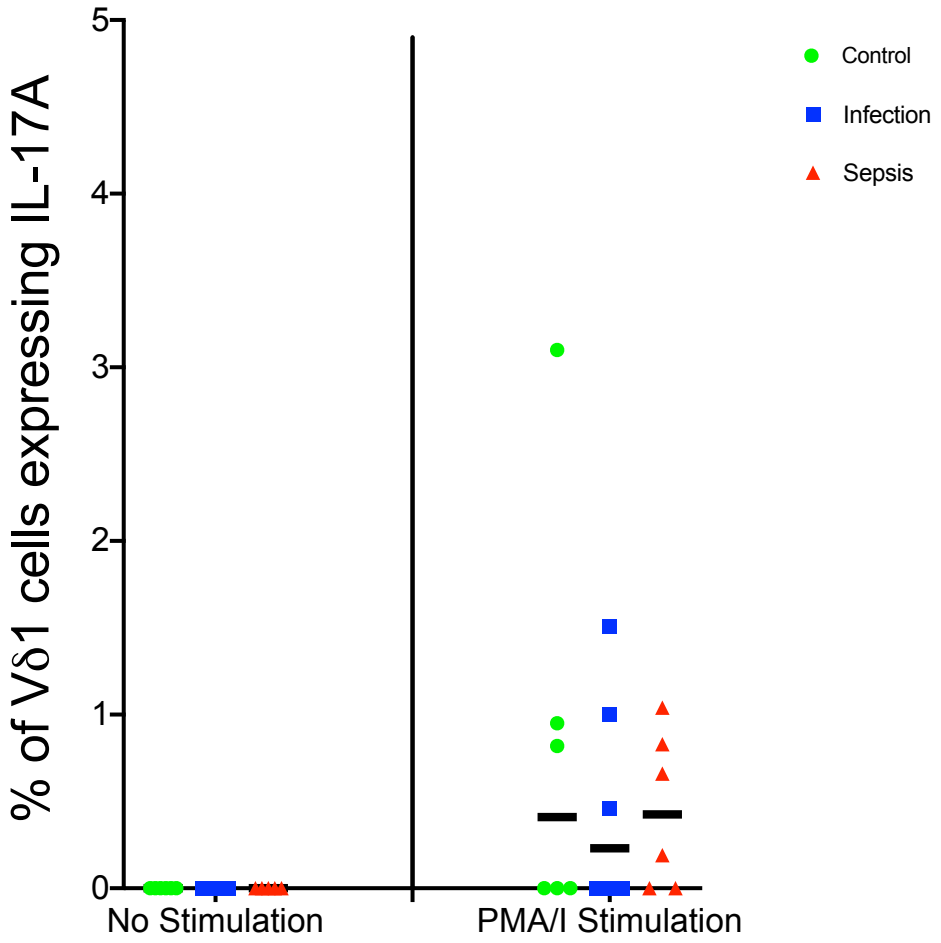

Figure 4-12 Vδ1 cell stimulation – IL-17A (not included in original paper)

Frequency of unstimulated and stimulated Vδ1 cells expressing IL-17A. Stimulated with phorbol myristate acetate and ionomycin (PMA/I). Control group (n = 10), infection group (n = 10), and septic group (n = 10). No statistically significance found across the groups.

The percentage of Vδ2 T cells was significantly different in the three groups ($p = 0.035$), with fewer Vδ2 T cells in the septic group than in the control group ($p = 0.034$) (Figure 4-12A). The percentage of naive Vδ2 T cells was significantly different in the three groups ($p < 0.001$), with a lower percentage naive Vδ2 T cells in the infection group ($p = 0.01$) and the septic group ($p < 0.001$) than in the control group (Figure 4-12C). This again indicates the activation of Vδ2 T cells with both infection and sepsis.

The absolute number of Vδ2 T cells was different in the three groups ($p < 0.001$); the number of Vδ2 T cells was lower in the septic group ($p < 0.001$) and the infection group ($p = 0.05$) than in the control group (Figure 4-12B). The number of naive Vδ2 T cells was different in the three groups ($p < 0.001$), with fewer naive Vδ2 T cells in the septic group ($p < 0.001$) and the infection group ($p = 0.001$) than in the control group (Figure 4-12D). Similarly, the number of central memory Vδ2 T cells was different in the three groups ($p = 0.004$), with fewer central memory Vδ2 T cells in the septic group ($p = 0.004$) and the infection group ($p = 0.03$) than in the control group (Figure 4-12D). The number of terminally differentiated Vδ2 T cells was different in the three groups ($p = 0.008$), with fewer terminally differentiated Vδ2 T cells in the septic group ($p = 0.03$) and the infection group ($p = 0.014$) than in the control group (Figure 4-12D). Again, this indicates Vδ2 T cell activation in both infection and sepsis, even though the number of Vδ2 T cells appears to be depleted, which may reflect lymphocyte redistribution away from the intravascular space. Very few Vδ2 T cells expressed either IL-12Rβ2 or IL-23R in the three patient groups.

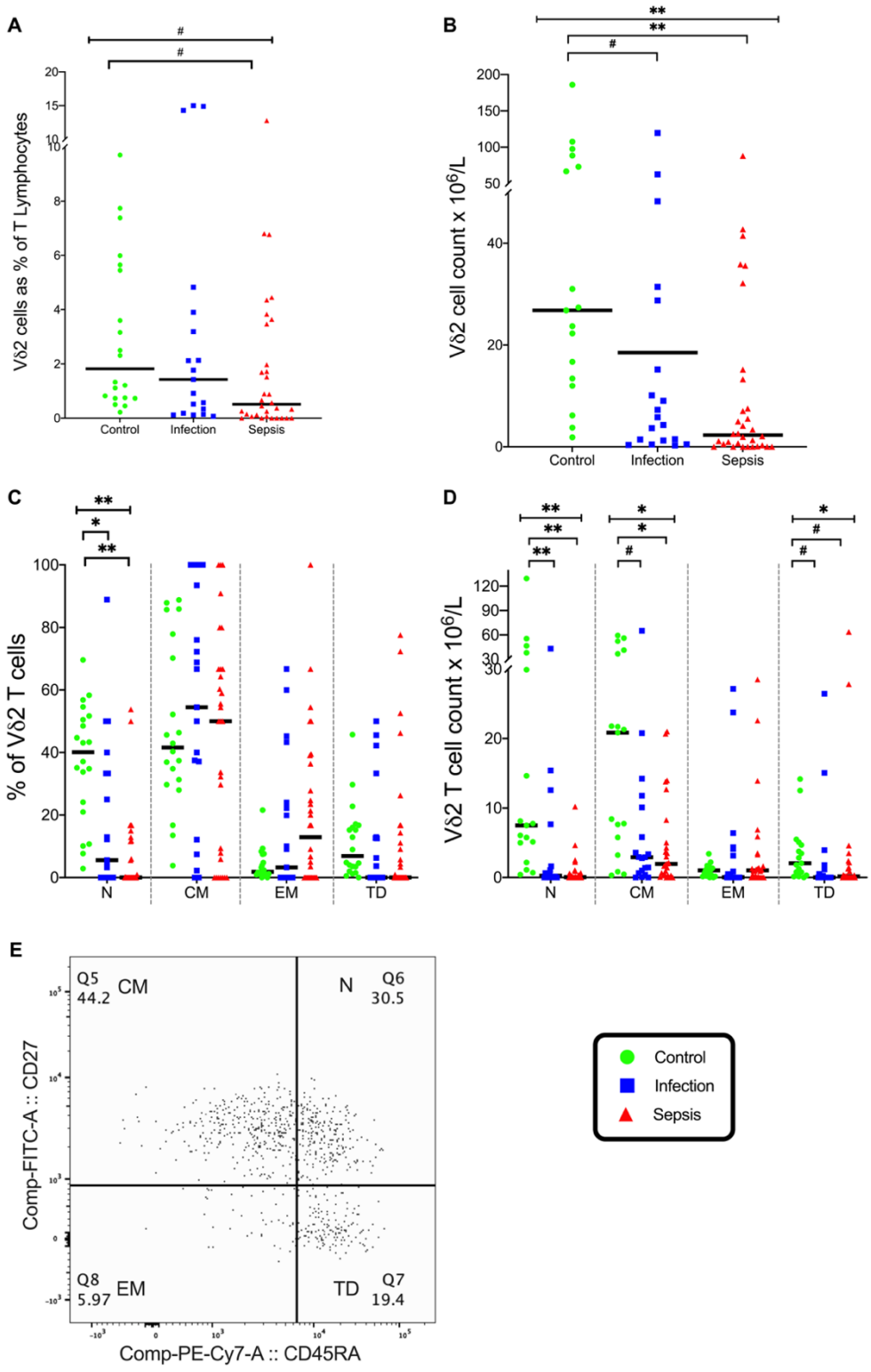

Figure 4-13 Vδ2 Cell Phenotype

*(**A**) Frequency of T lymphocytes that are Vδ2 Cells. (**B**) Vδ2 T cell count in the three patient groups. (**C**) Differentiation status of Vδ2 T cells. Naive, central memory, effector memory, and terminally differentiated (N, CM, EM, TD, respectively) differentiation states shown. (**D**) Differentiation status of Vδ2 T cells expressed as the total cell count. (**E**) Flow cytometry plot on gated Vδ2 T cells showing differentiation status. Control group (n = 20), infection group (n = 19), and septic group (n = 32). Graphs are plotted with bars representing the median. # = $p < 0.05$; * = $p \leq 0.01$; ** = $p \leq 0.001$.*

In stimulated Vδ2 T cells, the T-Bet expression differed across the three patient groups (p = 0.031), with a lower T-Bet expression in patients with sepsis than in the controls (p = 0.029) (Figure 4-13A). In unstimulated Vδ2 T cells, the RORγt expression differed across the three patient groups (p = 0.027), with a lower RORγt expression in patients with sepsis than in the controls (p = 0.054) (Figure 4-13B). In Vδ2 T cell stimulation with PMA/I, there was no difference in the IFN-γ expression between patient groups (Figure 4-13C). The expression of IL-17A was not detectable in the stimulation experiment of Vδ2 T cells.

Within patients with sepsis, there was no link between mortality and γδ T cell, NK cell, or NKT cell percentages or receptor expressions.

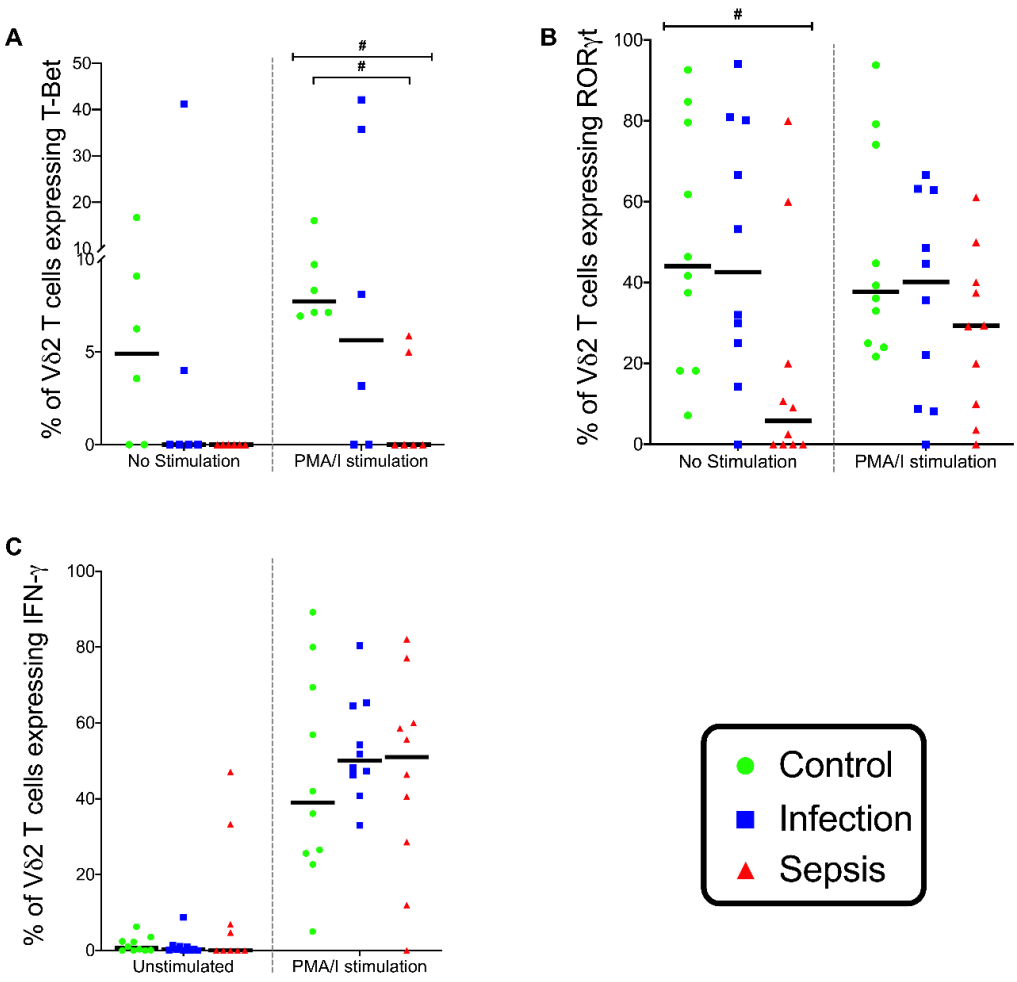

Figure 4-14 Vδ2 Cell Stimulation

*(**A**) Frequency of unstimulated and stimulated Vδ2 T cells expressing T-Bet. (**B**) Frequency of unstimulated and stimulated Vδ2 T cells expressing RORγt. (**C**) Frequency of unstimulated and stimulated Vδ2 T cells expressing interferon gamma (IFN-γ). Stimulated with phorbol myristate acetate and ionomycin (PMA/I). Control group (n = 10), infection group (n = 10), and septic group (n = 10). Graphs are plotted with bars representing the median. # = $p < 0.05$.*

4.7 Discussion

This study clearly links a mosaic of innate lymphocyte phenotypes with the clinical presentation of infection and sepsis in patients. Human sepsis studies of host immunity are problematic, as there is difficulty distinguishing between causal and coincidental molecular events. The very nature of human sepsis, with an unpredictable and often fulminant onset, precludes any attempt to establish the baseline immune functionality, which might predict subsequent sepsis onset or severity. To circumvent this problem, we included a third patient group in the study design. This third group of patients with infection, but without sepsis, may provide a more appropriate benchmark immune response from patients who tolerate infection with relative impunity rather than using healthy controls.

Prior studies have examined the association between innate lymphocytes and sepsis [93]. Grimaldi reported a decrease in MAIT cell count in patients with severe sepsis, but did not comment on the T_H1/ T_H17 phenotype or distinguish between the MAIT cell responses to infection and sepsis. In contrast, the present study noted a decrease in circulating MAIT cells with infection in the absence of sepsis, which suggests that the numeric depletion of MAIT cells is a common feature of all infections and may reflect redistribution rather than depletion. Although there was evidence of MAIT cell activation in both sepsis and infection, sepsis was linked with an altered MAIT phenotype, as T_H17 MAIT cells were reduced in sepsis, suggesting that the MAIT cell phenotype may be as important as absolute cell numbers in sepsis pathophysiology. As

previously described by Venet, γδ T cells were decreased in patients with septic shock [103].

This association is biologically plausible, as immunity is promoted by T_H17 cytokines against extracellular bacteria and fungi. They induce neutrophil recruitment to the site of inflammation by promoting antimicrobial peptide release and maintaining the intestinal barrier function [65, 66]. In addition, the archetypic T_H1 cytokine IFN-γ appears to be pivotally involved in generating an appropriate polymorphonuclear leukocyte bactericidal response and enhances antigen presentation by monocytes and macrophages, which in turn will give rise to an adaptive immune response [104].

It is notable that, despite the widespread expression of phenotype-specific transcription factors, few if any circulating lymphocytes expressed IL-12Rβ2 or IL-23R, with a considerably greater expression of IL-23R than IL-12Rβ2. Thus, circulating lymphocytes in patients with infection and sepsis may not as yet be fully activated, and hence assaying IL-17 cytokines from serum in patients with sepsis and infection may prove unrewarding [105]. However, the reduced expression of the T_H17-specific transcription factor RORγt in lymphocyte populations of septic patients provides more robust evidence for the link between human sepsis and deficient T_H17 responses in the majority of innate lymphocytes. Similarly, the enhanced expression of T-Bet by the innate lymphocytes of patients with infection but not sepsis provides inferential evidence that human sepsis is linked with a failure to elaborate a robust T_H1 response in specific innate lymphocytes. While Carvelli recently reported a decrease in T_H17 innate lymphoid cells in patients with sepsis, Carvelli did not

include patients with infection, and could not link patient outcome to a specific cellular phenotype [106].

$CD3^+$ $CD161^+$ lymphocytes are a composite population of diverse IL-17-producing lymphocytes [102, 107]. These $CD3^+$ $CD161^+$ lymphocytes are potent mediators of inflammation, and have been linked with chronic inflammatory diseases such as rheumatoid arthritis and correlate with disease severity in rheumatoid arthritis [108, 109]. However, in the present study the downregulation of RORγt among $CD3^+$ $CD161^+$ lymphocytes in patients with sepsis links to a decrease in T_H17 responsiveness and the occurrence of sepsis.

In this study, the proportion of the circulating lymphocyte pool expressing $CD3^+$ $CD161^+$ was lower in the septic patients who died. This lymphocyte subpopulation includes a diverse range of innate and adaptive T_H17 cells which may be crucially important in providing some measure of immunity in patients who have an existing adaptive immune paresis [61, 88]. This finding suggests that immunity in sepsis is composed of the net effects of several cell populations that together provide innate and adaptive T_H17 responses. However, the study was not designed or powered to investigate the mechanism of this association; specifically, whether there was any link between the occurrence of nosocomial infection and the depletion of $CD3^+$ $CD161^+$ lymphocytes in patients with sepsis.

Innate and adaptive lymphocyte activation was different in patients with sepsis. Many $CD3^+$ $CD161^+$ lymphocytes are MHC-restricted adaptive immune lymphocytes which were activated in patients with infection but not with sepsis.

This contrasts with MAIT, γδ T, and NK cells, which are non-MHC-restricted and were activated in both sepsis and infection. This failure to activate adaptive lymphocytes in septic patients reflects the well-described inhibition of antigen presentation pathways in patients with sepsis and is concordant with recent reports of adaptive immune paresis in septic patients [88].

4.8 Conclusions

Innate lymphocyte depletion was evident in patients with infection and sepsis, suggesting that lymphocyte depletion is not a hallmark of sepsis. Innate lymphocyte phenotypic differences appear to be of greater importance in the pathophysiology of sepsis than the absolute lymphocyte counts. Specifically, sepsis appears to be linked with the downregulation of the innate lymphocyte T_H17 phenotype and a failure to upregulate the T_H1 phenotype that was evident in patients with infection. In patients with sepsis, in the absence of an adaptive immune response, innate and innate-like T cells appear to assume a crucial role in providing host immunity and may determine the outcome.

A larger clinical study will be required to investigate the utility of specific lymphocyte activation indices as biomarkers for clinical outcome, such as mortality, in infected and septic patients. Lastly, this study demonstrates the benefit of including patients with infection in human studies of sepsis.

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Chapter 5

5 Summary and Discussion

5.1 Summary

In patients with sepsis, compared with patients who have infection, there is an obvious failure of MHC restricted $CD4^+$ and $CD8^+$ T lymphocyte activation, which affects T_H17 rather than T_H1 responsiveness. In contrast innate lymphocytes, that are not MHC dependant are activated in sepsis, but have attenuated T_H1 responsiveness.

Studies of sepsis in humans should include a comparator group of patients with infection who do not develop sepsis.

5.2 Demographic and Clinical Data

5.2.1 Control Group

In the control groups for both the immunophenotype and stimulation studies the subjects were recruited from a routine general practice phlebotomy clinic where they were screened by inclusion and exclusion criteria. None of the control group had recent infections.

5.2.2 Infection without Sepsis Group

In the infection without sepsis group the patients were admitted to hospital due to infection and recruited within 72 hours of a positive culture. These patients were treated with antibiotics. In the immunophenotyping none of this group died during their inpatient stay. In the stimulation group one patient died but this was more than 2 months after being admitted and the patient did not die due to infection. These patients were unwell as evidenced by their SOFA and APACHE II scores. Almost one third and one fifth of these patients had an acute kidney injury in the immunophenotyping study and the stimulation study respectively. None of this group required dialysis. One patient in both studies in this group received steroids after admission for treatment of their chronic obstructive lung disease. None of this group developed secondary infections.

5.2.3 Sepsis Group

In the sepsis group the patients were all recruited within 72 hours from admission to the intensive care unit for the immunophenotyping study and within 5 days in the stimulation study. These were all very unwell patients with high SOFA and APACHE II scores. 40% of the immunophenotyping group died in hospital and 30% of the stimulation group died in hospital which would be consistent with the known mortality for sepsis in developed countries. Almost

100% of these patients had vasoplegic shock requiring inotropes to maintain a mean arterial blood pressure greater than 65mmHg. These patients spent a median of a week on inotropes. Almost 90% of these patients required invasive mechanical ventilation. In the immunophenotyping group; 2 patients required non-invasive ventilation and 2 patients required supplemental oxygen. In the stimulation study; 1 patient was treated with non-invasive ventilation however the rest were invasively ventilated. In the immunophenotyping study 5 of the invasively ventilated patients had severe acute respiratory distress syndrome (ARDS), 13 patients had moderate ARDS, and 8 had mild ARDS. Acute respiratory distress syndrome were diagnosed as per the Berlin Criteria [110]. In the stimulation study 2 had severe ARDS, 5 had moderate ARDS, and 3 had mild ARDS. 35% (11 patients) of the immunophenotyping study required muscle relaxant infusions (atracurium infusions are used in our institution) and 6 patients required the use of inhaled nitric oxide as part of the management of hypoxia. In the sepsis group 50% (5 patients) of the patients required muscle relaxant infusion and 1 patient required inhaled nitric oxide as part of the management of hypoxia. In the immunophenotyping study 81% of the patients had an acute kidney injury, with 50% of all the patients in the group requiring continuous renal replacement therapy (CRRT). This would be similar to a German prevalence study showing that approximately 42% of patients with septic shock require renal replacement therapy [111]. 100% of the stimulation group had an acute kidney injury and 80% of these patients required CRRT. None of these patients were on steroids prior to admission to the intensive care unit but 18% of the immunophenotyping group and 50% of the stimulation group required

stress dose steroid as part of the management of vasoplegic shock. In our institution this would usually be hydrocortisone 50mg 6 hourly or 8 hourly and initiation is at the discretion of the treating clinician, typically when noradrenaline requirements are greater than 0.5 µg/kg/minute or when a second vasoconstricting agent is required. 50% of the patients in both studies developed secondary infections which were most often ventilator associated pneumonias. All patients were treated with antibacterial medications. In addition to antibacterial medications, 14 of 32 patients in the immunophenotyping group were treated with antifungals after antimicrobial therapy was rationalised whether empirically or targeted therapy, 3 of 10 patients in the stimulation group were treated with antifungals. In the immunophenotyping study 2 of the patients had candidaemias, 1 had aspergillus in sputum, 3 had candida in peritoneal fluid, 1 had candida in pleural fluid, and 6 had candida in either sputum or urine or both. In the stimulation study 1 patient had candida in peritoneal fluid, and 3 had candida in urine or sputum.

In summary the infection without sepsis group were unwell requiring admission to hospital for treatment of their infection with antibiotics. None of these patients succumbed to their infection and none developed secondary infections. The septic group were very unwell in multiorgan failure with the majority in septic shock and invasively ventilated.

5.3 Limitations of the Studies

The studies were performed on peripheral blood and thus examine circulating cells but it does not examine the cells at sites of infection. However comparing patients with infection without sepsis and patients with sepsis the differences seen would imply that there are differences in the peripheral circulating lymphocytes and thus this would most likely represent differences at sites of infection also.

In the stimulation experiment functional anti-CD3 antibodies and functional anti-CD28 antibodies were used to try to stimulate T cells. There were very little differences between those stimulated cells and the controls (unstimulated cells) when the flow cytometry results were analysed. I believe this was most likely due to the CD3 still being bound by the functional antibodies thus blocking the binding site for the fluorochrome-bound CD3 antibodies.

In the stimulation experiment lipopolysaccharide caused some stimulation but not as much as expected. I believe that this may have been due to the incubation time period of 5 hours which may have been too short, particularly when there may be an issue with antigen presenting cells in septic patients. In future experiments I would stimulate for longer in LPS as there are a range of protocols varying from 1 day to 4 days.

Phorbol 12-myristate 13-acetate (PMA) in combination with ionomycin offers excellent stimulation without the need for certain cell receptors and thus this stimulation was effective.

Panel 5 in the immunophenotyping study was designed to study Vδ3 cells and iNKT cells. Unfortunately the anti-Vδ3 fluorescence antibody did not result in any positive staining in any groups. The iNKT fluorescence bound antibody did stain for 6B11. The percentages were so few that it was not possible to analyse them in any meaningful way. iNKT cells accounted for a mean of 0.03%, 0.03%, and 0.1% of $CD3^+$ lymphocytes for control, infection, and sepsis groups respectively and a median of 0.01%, 0%, and 0.2% of $CD3^+$ lymphocytes for control, infection, and sepsis groups respectively. To analyse iNKT cells in these groups a larger quantity of blood would need to be drawn from the patients and iNKT separated from PBMCs using a magnetic bead separation technique.

5.4 Adapative T_H1 and T_H17 Immunity

5.4.1 Monocytes

This study shows that HLA-DR^+ Monocytes are downregulated in sepsis when compared to controls and infection. This is consistent with the literature [58], and it suggests that antigen presentation may be impaired in sepsis. This study could only demonstrate an extremely small amount of production of IL-12 by these cells which was similar across all patient groups. IL-23 was detected in unstimulated monocytes which was reduced in sepsis when compared to infection and control, which would inhibit a robust T_H17 response [85].

5.4.2 T_H1 $CD4^+$ and $CD8^+$ T Lymphocytes

This study did not find much expression of IL-12 receptor on $CD4^+$ T cells. This with the absence of T-Bet in $CD4^+$ T cells suggests that these cells are not T_H1 cells. The IL-12 receptor was found in small quantities on $CD8^+$ T cells. Most $CD8^+$ T cells expressed both T-Bet and RORγt together or just RORγt but very few expressed T-Bet alone. However of those few that express T-Bet alone there are significantly fewer in the sepsis group when compared to control. This would suggest that the majority of cells that are stimulated by IL-12 express both T-Bet and RORγt. $CD3^+$ CM, EM and TD cells displayed reduced T-Bet expression comparing sepsis to infection however as naïve $CD3^+$ T cells have similar T-Bet

expression across all groups, the decrement in T-Bet expression in differentiated cells suggests that there is an anomaly in T cell activation .

Upon stimulation with PMA and ionomycin, $CD4^+$ and $CD8^+$ T cells produced IFN-γ equally in all groups. Higher frequencies of $CD8^+$ T cells produced IFN-γ compared to $CD4^+$ T cells. Consequently there may not be any underlying defect in the cellular potential to elaborate T_H1 type response.

5.4.3 T_H17 $CD4^+$ and $CD8^+$ T Cells

IL-23 receptor was found on both $CD4^+$ and $CD8^+$ T cells, and to an order of magnitude greater than the IL-12 receptor. $CD4^+$ T cells tended to exclusively express RORγt, but RORγt expression was significantly down regulated in sepsis when compared to infection and control. RORγt was similarly expressed on $CD8^+$ T cells in control, infection, and sepsis.

IL-23 receptor expression on $CD3^+$ cells was increased in infection and sepsis when compared to control. IL-23 receptor was not found to be different on naïve and terminally differentiated $CD3^+$ cells however IL-23r was increased on central memory T cells in sepsis and increased on effector memory T cells in both sepsis and infection when compared to control. Expression of IL-23r on EM T cells was similar when comparing infection to sepsis. $CD3^+$ cells have reduced expression of RORγt in sepsis when compared to infection and control. Naïve $CD3^+$ cells have the same expression of RORγt however in $CD3^+$ CM and EM cells

RORγt expression is downregulated in sepsis compared to control, and in $CD3^+$ cells RORγt is downregulated in CM in sepsis when compared to infection.

Upon stimulation $CD4^+$ T cells produced small quantities of IL-17A whereas $CD8^+$ T cells did not produce IL-17A.

5.4.3.1 Discussion

IL-23 receptor is upregulated in infection and sepsis suggesting T_H17 activity is a normal response to infection. However RORγt is downregulated in sepsis on $CD3^+$ cells and $CD4^+$ T cells suggesting that T_H17 is somewhat suppressed in sepsis compared to infection. $CD8^+$ T cells have similar RORγt expression and do not seem to produce any IL-17A in response to stimulation. This would suggest that in sepsis there is less T_H17 activity compared to infection in T cells and particularly in $CD4^+$ T cells.

5.4.4 $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ Cell Differentiation

In sepsis and infection the $CD3^+$ cell count is reduced. The percentage of $CD3^+$ cells that are naïve are much lower in infection when compared to control and sepsis, with a much higher percentage of $CD3^+$ cells being EM in infection when compared to control and sepsis. More of the $CD3^+$ cells are TD when

comparing infection to sepsis. The percentage of $CD3^+$ cells that are $CD4^+$ is lower in infection than in control and sepsis whereas the percentage of $CD3^+$ cells that are $CD8^+$ are similar across all three groups. Naïve $CD8^+$ T cells were lower in the infection group compared to the sepsis group and control group. $CD8^+$ T cells tend to be differentiated into EM and in particular unlike $CD4^+$ T cells into TD cells, in all three groups.

There are far fewer Naïve $CD4^+$ T cells in the infection group when compared to control and sepsis with a much greater rise in EM cells in the infection group when compared to control and sepsis groups. Roughly 60% of $CD4^+$ T cells are EM cells compared to 20% of the sepsis group's. Over a two week period the proportion of EM $CD4^+$ T cells in the sepsis group gradually increases to similar levels as the infection group had had on day 1.

5.4.4.1 Discussion

T cell count and $CD4^+$ T cells are reduced in both infection and sepsis groups compared to control. However in the infection group there are less naïve T cells and more effector memory T cells than in the sepsis group. Naïve $CD8^+$ T cells are reduced in infection compared to sepsis and control. This suggests that there is a problem with T cell activation and differentiation in sepsis. In the septic group the EM T cells did rise over the two weeks, in those patients who were still alive, which suggests that the differentiation does occur but considerably

later than in patients who do not develop sepsis. This suggest that sepsis may be associated with a immunosuppressed state which is further supported by the finding in this study that T_{reg} cells were upregulated in sepsis.

5.5 Innate T_H1 and T_H17 Immunity

5.5.1 Natural Killer Cells

The frequency and number of natural killer (NK) cells in the sepsis group were reduced when compared to the control and infection groups. The frequency of NK cells increased over the three weeks following admission.

5.5.1.1 T_H1 NK Cells

There were scant NK cells expressing IL-12 receptor. Of the few that were expressed both sepsis and infection had reduced IL-12 receptor expression when compared to control. The overwhelming majority of NK cells expressed T-Bet when stimulated but this was reduced in the sepsis group compared to the infection group. When NK cells were stimulated there was significantly less IFN-γ produced by the NK cells in the septic group than in the control group.

5.5.1.2 T_H17 NK Cells

Similar numbers of NK cells expressed IL-23 receptor in all three groups. There was no significant difference in expression of RORγt with stimulation in all three groups, however there was minimal to no IL-17A detected. This result was expected, since NK cells typically do not produce T_H17 cytokines.

5.5.1.3 Discussion

The above findings suggest that NK Cells have a reduced T_H1 response in sepsis as indicated by the reduced T-Bet and reduced IFN-γ production in this group. There was no evidence of an altered NK T_H17 response in this study although production of IL-17A was not seen by these cells. Thus sepsis may be linked with anomalous NK T_H1 responsiveness, which is distinct from that seen with adaptive lymphocytes.

5.5.2 $CD56^+$ T Cells

For the purpose of this study natural killer T (NKT) cells were defined as $CD56^+$ T cells as discussed previously. The frequency of NKT cells was higher in infection than in sepsis and the NKT cell count in the infection group was similar to that of control. The NKT cell count was significantly lower in the sepsis group when compared to the control and infection group and the NKT cell count remained low over the following 3 weeks in the sepsis group.

5.5.2.1 $CD56^+$ T Cell Differentiation

The frequency and cell count of naïve NKT cells were reduced in sepsis and infection when compared to control. The frequency across the groups of CM, EM and TD NKT cells were similar, with the majority being of the TD phenotype followed by the EM phenotype and only few being of the CM phenotype. However, when looking at the cell counts there were significantly fewer EM NKT cells in the sepsis group when compared to the infection group, and significantly fewer TD NKT cells in the sepsis groups when compared to the infection and control groups. Thus, these non-MHC restricted innate lymphocytes are activated in patients with sepsis and infection.

5.5.2.2 T_H1 $CD56^+$ T Cells

There were minimal NKT cells expressing IL-12 receptor but of those that did, there were fewer expressing it in the infection group than the control. Without stimulation NKT T-Bet expression was significantly less in the septic group compared to the infection group. Upon stimulation T-Bet was dramatically upregulated in the infection group and not in the sepsis group with significantly less expression in the septic group compared to the infection group. Upon stimulation NKT cells in the sepsis group expressed significantly less IFN-γ when compared to the control and infection group.

5.5.2.3 T_H17 $CD56^+$ T Cells

Few NKT cells expressed IL-23 receptor and expression was similar in all groups. Upon stimulation RORγt expression in the sepsis group was significantly less compared to the control group. Upon stimulation scant IL-17A was detected and it was similar across all groups.

5.5.2.4 Discussion

NKT cells were defined as $CD56^+$ T cells in this thesis. iNKT cells were investigated separately which unfortunately were too scarce to analyse. NKT cell count seemed to be reduced in the septic group. There was evidence that differentiation was occurring in the septic and infection group but there were significantly fewer EM and TD NKT cells in the septic group. In the sepsis group T-Bet expression was low and we were unable to upregulate its expression with stimulation in this group. There was significantly less IFN-γ produced in the sepsis group. This suggests that there is a reduced T_H1 response to infection in patients who develop sepsis. There was reduced RORγt expression in the sepsis group which could also suggest a reduced T_H17 response in the septic group. However, the predominant effect appeared to be that in humans with infection, NKT cells may elaborate a T_H1 response which is defective in patients with sepsis.

5.5.3 MAIT Cells

Frequency of MAIT cells in the T cell population was less in the infection group when compared to the control group, and MAIT cell count was reduced in infection and sepsis groups when compared to control. MAIT cell count remained low over the following 3 weeks in the sepsis group.

5.5.3.1 MAIT Differentiation

The frequency of naive MAIT cells and CM MAIT cells were less in the infection and sepsis groups compared to control howevere the EM and TD MAIT cell frequencies were similar across all groups. The majority of MAIT cells were of the EM phenotype.

5.5.3.2 T_H1 MAIT Cells

There were very few MAIT cells expressing IL-12 receptor similarly in all groups. Without stimulation there was increased T-Bet expression in the MAIT cells of the infection group when compared to the sepsis group. With stimulation this became even more apparent with less T-Bet expression in the sepsis group compared to the infection group. When looking at the differentiated cells the TD MAIT cells expressed significantly more T-Bet in the infection group when compared to the sepsis group. Upon stimulation MAIT cells in each group expressed IFN-γ similarly.

5.5.3.3 T_H17 MAIT Cells

MAIT cells express IL-23 receptor in similar frequencies in all groups but the count of MAIT cells expressing IL-23 receptor differed with sepsis expressing signifantly less when compared to control. This low count of MAIT cells expressing IL-23 receptor remained similar over the following 3 weeks. When MAIT cells were stimulated, significantly less MAIT cells in the septic group expressed RORγt when compared to control. Examining the RORγt expression in differentiated MAIT cells revealed significantly less RORγt expression in the TD MAIT cells in the septic group compared to the control group. There was very little IL-17A detected upon stimulation of MAIT cells however it was similar in all groups.

5.5.3.4 Discussion

MAIT cell counts were reduced in both infection and sepsis and remained low in the sepsis group. There were less naive MAIT cells in both infection and sepsis suggesting there was differentiation in both groups occuring. MAIT cells in the infection group expressed more T-Bet than in the septic group suggesting that there is a reduced T_H1 response in patients who develop sepsis. There was less RORγt expressed in the MAIT cells when stimulated, particularly the TD MAIT cells suggesting reduced T_H17 response in patients with sepsis. This may be due

to an intrinsic issue as without stimulation RORγt expression was similar across the groups. However patients with sepsis may exhibit attenuated T_H1 and T_H17 responses. This can in part be accounted for by the heterogenous group of cells, both innate and adaptive immune lymphocytes that express the archetypic MAIT receptor CD161 (see discussion in Chapter 4).

5.5.4 Vδ1 Gamma-Delta T Lymphocytes

Vδ1 T cells had similar counts and frequencies across all three groups.

5.5.4.1 Vδ1 T Cell Differentiation

The frequency and cell count of naive Vδ1 T cells is reduced in both infection and sepsis groups when compared to control. There was no significant difference in CM, EM, and TD Vδ1 T cells across the groups.

5.5.4.2 T_H1 Vδ1 T Cells

Few Vδ1 T cells expressed IL-12 receptor and there were no differences across the groups. T-Bet expression by Vδ1 T cells was similar across all gorups.

Upon stimulation the Vδ1 T cells expressed less IFN-γ in the sepsis group when compared to the infection group.

5.5.4.3 T_H17 Vδ1 T cells

Vδ1 T cells expressed less IL-23 receptor in the infection group compared to control. RORγt expression by Vδ1 T cells was similar across the groups. IL-17A was expressed at similar low levels by Vδ1 T cells in all groups upon stimulation (Figure 4-12).

5.5.4.4 Discussion

There appears to be phenotypic differentiation in both infection and sepsis groups. T-Bet was similar across the groups but there was a reduced capacity to produce IFN-γ in the sepsis group. This suggests some reduction in capacity of the Vδ1 T cells to mount a T_H1 response in patients with sepsis. There did not appear to be a difference in the T_H17 response of Vδ1 T cells.

5.5.5 Vδ2 Gamma-Delta T Lymphocytes

Vδ2 T cells are the most abundant γδ T cells in circulating human blood. The frequency of Vδ2 T cells is reduced in sepsis when compared to control. The Vδ2 T cell counts are reduced in sepsis and infection when compared to control and appear to be much lower in sepsis than in infection.

These cells appear to be appropriately differentiating in infection and sepsis as suggested by the lower naïve cell count. Vδ2 T cells did not express much T-Bet, Median expression of 5% in controls and 0% in infection and sepsis. However, with stimulation the infection group expressed a median of roughly 5% whereas the sepsis group remained at a median of 0%. However approximately 40-50% of Vδ2 T cells produced IFN-γ on stimulation.

The percentage of naïve Vδ2 T cells is reduced in both infection and sepsis groups when compared to control. There was no significant difference between groups in CM, EM, and TD Vδ2 T cells. The naïve, CM, and TD Vδ2 T cell counts are reduced in infection and sepsis groups when compared to controls. The EM Vδ2 T cell counts are similar across all groups.

5.5.5.1 T_H1 Vδ2 T Cells

There were scant Vδ2 T cells expressing IL-12 receptors across all groups. T-Bet expression was low in Vδ2 T cells and with stimulation it was significantly lower in the sepsis group compared to the control group. With stimulation expression of IFN-γ was the same across all groups.

5.5.5.2 T_H17 Vδ2 T Cells

There were scant Vδ2 T cells expressing IL-23 receptors across all groups. RORγt was reduced in the sepsis group without stimulation but with stimulation it was upregulated and there were no differences in RORγt expression by Vδ2 T cells between the groups with stimulation. There was no IL-17A production detected with stimulation in Vδ2 T cells.

5.5.5.3 Discussion

There appears to be a reduced capacity of a T_H1 response in the sepsis group when the Vδ2 T cells are stimulated. RORγt expression in the Vδ2 T cells was upregulated with PMA/I stimulation in the sepsis group to similar levels as

the infection group however it was low in the sepsis group without stimulation suggesting a potential reduction in T_H17 Vδ2 T cell response to infection in the septic group.

5.6 Further Supplemental Discussion to Papers

This thesis demonstrated a reduction in T_H1 response in sepsis when compared to infection without sepsis in the global population of $CD3^+$ lymphocytes, NK cells, NKT cells, MAIT cells, Vδ1 and Vδ2 T cells. It also demonstrated a reduction in T_H17 response in sepsis when compared to infection without sepsis globally in $CD3^+$ lymphocytes, but more specifically in $CD4^+$ T cells, NKT cells, MAIT cells, and Vδ2 T cells.

The T_H1 and T_H17 responses in these cell lines was more robust in patients who had infection but did not develop sepsis, all of whom survived their infection. Thus, this thesis suggests that T_H17 and T_H1 responses may be protective from developing sepsis rather than acting as mediators of life-threatening inflammation. This is in keeping with other studies such as Ronit *et al* who reported a decrease in T_H17 lymphocytes, with endotoxin induced systemic inflammation [72]. But these findings are contrary to other suggestions from Mikacenic *et al.* and Liu *et al* who suggests that plasma IL-17 levels are directly correlated with worse prognosis in sepsis [51, 112]. Crucially though these studies did not investigate IL-17 responses in patients with infection without sepsis. While it may be found that IL-17 rises in sepsis it does not demonstrate causation of sepsis, as this may be appropriate or it may be inappropriately low in the context of infection. Human sepsis studies of host immunity are problematic, as there is difficulty distinguishing between causal and coincidental molecular events. This study highlights the importance of

including a normal or robust response to infection when examining immune responses to broaden our knowledge of immune responses to sepsis.

The absence of IL-12 receptor expression in both $CD4^+$ and $CD8^+$ T lymphocytes, which is consistent with existing human data suggests that immunity in elderly humans with sepsis is predominantly mediated by a T_H17 rather than a T_H1 response [70].

At presentation of sepsis there was reduced T_H17 $CD4^+$ T cell and T_H1 $CD8^+$ T cell differentiation. This study suggests a specific failure of the $CD4^+$ effector memory lymphocyte mediated T_H17 response. This is plausible as IL-17 enhances polymorphonuclear chemotaxis and activation, and is crucially important in mediating mucosal immunity, specifically for systemic *Candida* infections, which account for 20% of all infections in critically ill patients [80-82]. And indeed fungal secondary infections were seen in the septic group of patients in this study.

Naïve innate T cells in sepsis and infection groups were fewer than in control suggesting that the innate T cells were differentiating into effector phenotypes. This is to be expected for such non-MHC restricted innate lymphocytes in the presence of active infection. The activation of these non-MHC restricted lymphocytes in septic patients contrasts starkly with the activation failure of adaptive T lymphocytes in patients with sepsis and emphasises the central role of antigen presentation and adaptive immunity in human response to extra cellular bacterial infection.

This study and thesis demonstrate the importance and value of comparing immune responses in sepsis to those of immune responses to infection without sepsis. Future studies should include this format. Murine immune responses to sepsis have been studied in the past but unfortunately these models rarely translate into those of the human immune responses. The human immune response to infection and sepsis is extremely complex with multiple immune cells interacting in concert. Some immune cells appear to have plasticity, for example those of macrophages [113], and thus an immune response may have cell types switching from one phenotype to another, thus it is important to follow the immunophenotyping for more than one time point as was done in this work. Ideally the immune system should be analysed before infection as well however it is currently not possible to predict which patients will develop sepsis in response to infection.

A larger clinical study will be required to investigate the utility of specific lymphocyte activation indices as biomarkers for clinical outcome, such as mortality, in infected and septic patients. Future studies will harness more information with the advent of more sophisticated multi-colour flow cytometers which can analyse more than 8 colours per event which this study was limited to.

This study raises some interesting questions regarding the immune response to sepsis and infection in humans that warrant investigation. The first is why the adaptive T cells are not differentiating from naïve to effector states in the septic group, as this might suggest potential immunomodulation therapies in sepsis, to stimulate T cell differentiation. Causes of reduced T_H1 and in

particular T_H17 responses with potential avenues for immunomodulation therapies should also be explored. Specifically, the roles of the extended IL-1 and IL-6 cytokine families in T cell differentiation in human sepsis are worth investigating.

6 References

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM *et al*: **The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)**. *JAMA* 2016, **315**(8):801-810.
2. Vincent JL, Jones G, David S, Olariu E, Cadwell KK: **Frequency and mortality of septic shock in Europe and North America: a systematic review and meta-analysis**. *Crit Care* 2019, **23**(1):196.
3. Seymour CW, Rea TD, Kahn JM, Walkey AJ, Yealy DM, Angus DC: **Severe sepsis in pre-hospital emergency care: analysis of incidence, care, and outcome**. *Am J Respir Crit Care Med* 2012, **186**(12):1264-1271.
4. Vincent JL, Marshall JC, Namendys-Silva SA, François B, Martin-Loeches I, Lipman J, Reinhart K, Antonelli M, Pickkers P, Njimi H *et al*: **Assessment of the worldwide burden of critical illness: the intensive care over nations (ICON) audit**. *Lancet Respir Med* 2014, **2**(5):380-386.
5. Fleischmann C, Scherag A, Adhikari NK, Hartog CS, Tsaganos T, Schlattmann P, Angus DC, Reinhart K: **Assessment of Global Incidence and Mortality of Hospital-treated Sepsis. Current Estimates and Limitations**. *Am J Respir Crit Care Med* 2016, **193**(3):259-272.
6. Kaukonen KM, Bailey M, Suzuki S, Pilcher D, Bellomo R: **Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000-2012**. *Jama* 2014, **311**(13):1308-1316.
7. Ireland HSE: **National Sepsis Report 2018**. In.; 2018.
8. Rowe T, Araujo KL, Van Ness PH, Pisani MA, Juthani-Mehta M: **Outcomes of Older Adults With Sepsis at Admission to an Intensive Care Unit**. *Open Forum Infect Dis* 2016, **3**(1):ofw010.
9. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR: **Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care**. *Crit Care Med* 2001, **29**(7):1303-1310.
10. United Nations DoEaSA, Population Division (2017). : **World Population Ageing 2017**. 2017((ST/ESA/SER.A/408).).
11. Ferrer R, Artigas A, Suarez D, Palencia E, Levy MM, Arenzana A, Pérez XL, Sirvent JM: **Effectiveness of treatments for severe sepsis: a prospective, multicenter, observational study**. *Am J Respir Crit Care Med* 2009, **180**(9):861-866.
12. Liu VX, Fielding-Singh V, Greene JD, Baker JM, Iwashyna TJ, Bhattacharya J, Escobar GJ: **The Timing of Early Antibiotics and Hospital Mortality in Sepsis**. *Am J Respir Crit Care Med* 2017, **196**(7):856-863.
13. Kumar A, Roberts D, Wood KE, Light B, Parrillo JE, Sharma S, Suppes R, Feinstein D, Zanotti S, Taiberg L *et al*: **Duration of hypotension before initiation of effective antimicrobial therapy is the critical**

determinant of survival in human septic shock. *Crit Care Med* 2006, **34**(6):1589-1596.
14. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, Steingrub JS, Garber GE, Helterbrand JD, Ely EW *et al*: **Efficacy and safety of recombinant human activated protein C for severe sepsis**. *N Engl J Med* 2001, **344**(10):699-709.
15. Rivers E, Nguyen B, Havstad S, Ressler J, Muzzin A, Knoblich B, Peterson E, Tomlanovich M: **Early goal-directed therapy in the treatment of severe sepsis and septic shock**. *N Engl J Med* 2001, **345**(19):1368-1377.
16. Annane D, Sébille V, Charpentier C, Bollaert PE, François B, Korach JM, Capellier G, Cohen Y, Azoulay E, Troché G *et al*: **Effect of treatment with low doses of hydrocortisone and fludrocortisone on mortality in patients with septic shock**. *Jama* 2002, **288**(7):862-871.
17. Ranieri VM, Thompson BT, Barie PS, Dhainaut JF, Douglas IS, Finfer S, Gårdlund B, Marshall JC, Rhodes A, Artigas A *et al*: **Drotrecogin alfa (activated) in adults with septic shock**. *N Engl J Med* 2012, **366**(22):2055-2064.
18. Mouncey PR, Osborn TM, Power GS, Harrison DA, Sadique MZ, Grieve RD, Jahan R, Tan JC, Harvey SE, Bell D *et al*: **Protocolised Management In Sepsis (ProMISe): a multicentre randomised controlled trial of the clinical effectiveness and cost-effectiveness of early, goal-directed, protocolised resuscitation for emerging septic shock**. *Health Technol Assess* 2015, **19**(97):i-xxv, 1-150.
19. Sprung CL, Annane D, Keh D, Moreno R, Singer M, Freivogel K, Weiss YG, Benbenishty J, Kalenka A, Forst H *et al*: **Hydrocortisone therapy for patients with septic shock**. *N Engl J Med* 2008, **358**(2):111-124.
20. Peake SL, Delaney A, Bailey M, Bellomo R, Cameron PA, Cooper DJ, Higgins AM, Holdgate A, Howe BD, Webb SA *et al*: **Goal-directed resuscitation for patients with early septic shock**. *N Engl J Med* 2014, **371**(16):1496-1506.
21. Annane D, Vignon P, Renault A, Bollaert PE, Charpentier C, Martin C, Troché G, Ricard JD, Nitenberg G, Papazian L *et al*: **Norepinephrine plus dobutamine versus epinephrine alone for management of septic shock: a randomised trial**. *Lancet* 2007, **370**(9588):676-684.
22. Russell JA, Walley KR, Singer J, Gordon AC, Hébert PC, Cooper DJ, Holmes CL, Mehta S, Granton JT, Storms MM *et al*: **Vasopressin versus norepinephrine infusion in patients with septic shock**. *N Engl J Med* 2008, **358**(9):877-887.
23. Gordon AC, Mason AJ, Thirunavukkarasu N, Perkins GD, Cecconi M, Cepkova M, Pogson DG, Aya HD, Anjum A, Frazier GJ *et al*: **Effect of Early Vasopressin vs Norepinephrine on Kidney Failure in Patients With Septic Shock: The VANISH Randomized Clinical Trial**. *Jama* 2016, **316**(5):509-518.
24. Warren BL, Eid A, Singer P, Pillay SS, Carl P, Novak I, Chalupa P, Atherstone A, Pénzes I, Kübler A *et al*: **Caring for the critically ill patient. High-dose antithrombin III in severe sepsis: a randomized controlled trial**. *Jama* 2001, **286**(15):1869-1878.
25. Annane D, Cariou A, Maxime V, Azoulay E, D'Honneur G, Timsit JF, Cohen Y, Wolf M, Fartoukh M, Adrie C *et al*: **Corticosteroid treatment**

and intensive insulin therapy for septic shock in adults: a randomized controlled trial. *Jama* 2010, **303**(4):341-348.
26. Brunkhorst FM, Engel C, Bloos F, Meier-Hellmann A, Ragaller M, Weiler N, Moerer O, Gruendling M, Oppert M, Grond S *et al*: **Intensive insulin therapy and pentastarch resuscitation in severe sepsis**. *N Engl J Med* 2008, **358**(2):125-139.
27. Abraham E, Reinhart K, Opal S, Demeyer I, Doig C, Rodriguez AL, Beale R, Svoboda P, Laterre PF, Simon S *et al*: **Efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) in severe sepsis: a randomized controlled trial**. *Jama* 2003, **290**(2):238-247.
28. Perner A, Haase N, Guttormsen AB, Tenhunen J, Klemenzson G, Åneman A, Madsen KR, Møller MH, Elkjær JM, Poulsen LM *et al*: **Hydroxyethyl starch 130/0.42 versus Ringer's acetate in severe sepsis**. *N Engl J Med* 2012, **367**(2):124-134.
29. Venkatesh B, Finfer S, Cohen J, Rajbhandari D, Arabi Y, Bellomo R, Billot L, Correa M, Glass P, Harward M *et al*: **Adjunctive Glucocorticoid Therapy in Patients with Septic Shock**. *N Engl J Med* 2018, **378**(9):797-808.
30. O'Dwyer MJ, Dempsey F, Crowley V, Kelleher DP, McManus R, Ryan T: **Septic shock is correlated with asymmetrical dimethyl arginine levels, which may be influenced by a polymorphism in the dimethylarginine dimethylaminohydrolase II gene: a prospective observational study**. *Crit Care* 2006, **10**(5):R139.
31. Winkler MS, Kluge S, Holzmann M, Moritz E, Robbe L, Bauer A, Zahrte C, Priefler M, Schwedhelm E, Böger RH *et al*: **Markers of nitric oxide are associated with sepsis severity: an observational study**. *Crit Care* 2017, **21**(1):189.
32. Bogdan C: **Nitric oxide synthase in innate and adaptive immunity: an update**. *Trends Immunol* 2015, **36**(3):161-178.
33. Winkler MS, Nierhaus A, Rösler G, Lezius S, Harlandt O, Schwedhelm E, Böger RH, Kluge S: **Symmetrical (SDMA) and asymmetrical dimethylarginine (ADMA) in sepsis: high plasma levels as combined risk markers for sepsis survival**. *Crit Care* 2018, **22**(1):216.
34. Joffre J, Hellman J, Ince C, Ait-Oufella H: **Endothelial Responses in Sepsis**. *Am J Respir Crit Care Med* 2020, **202**(3):361-370.
35. Ziegler T, Horstkotte J, Schwab C, Pfetsch V, Weinmann K, Dietzel S, Rohwedder I, Hinkel R, Gross L, Lee S *et al*: **Angiopoietin 2 mediates microvascular and hemodynamic alterations in sepsis**. *J Clin Invest* 2013, **123**(8):3436-3445.
36. Bettelli E, Korn T, Oukka M, Kuchroo VK: **Induction and effector functions of T(H)17 cells**. *Nature* 2008, **453**(7198):1051-1057.
37. Vignali DA, Collison LW, Workman CJ: **How regulatory T cells work**. *Nat Rev Immunol* 2008, **8**(7):523-532.
38. LaMarche NM, Kohlgruber AC, Brenner MB: **Innate T Cells Govern Adipose Tissue Biology**. *J Immunol* 2018, **201**(7):1827-1834.
39. Godfrey DI, Koay HF, McCluskey J, Gherardin NA: **The biology and functional importance of MAIT cells**. *Nat Immunol* 2019, **20**(9):1110-1128.
40. Chien YH, Meyer C, Bonneville M: **γδ T cells: first line of defense and beyond**. *Annu Rev Immunol* 2014, **32**:121-155.

41. Bonneville M, O'Brien RL, Born WK: **Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity**. *Nat Rev Immunol* 2010, **10**(7):467-478.
42. Mangan BA, Dunne MR, O'Reilly VP, Dunne PJ, Exley MA, O'Shea D, Scotet E, Hogan AE, Doherty DG: **Cutting edge: CD1d restriction and Th1/Th2/Th17 cytokine secretion by human Vδ3 T cells**. *J Immunol* 2013, **191**(1):30-34.
43. Mao Y, Yin S, Zhang J, Hu Y, Huang B, Cui L, Kang N, He W: **A new effect of IL-4 on human γδ T cells: promoting regulatory Vδ1 T cells via IL-10 production and inhibiting function of Vδ2 T cells**. *Cell Mol Immunol* 2016, **13**(2):217-228.
44. Spooner CE, Markowitz NP, Saravolatz LD: **The role of tumor necrosis factor in sepsis**. *Clin Immunol Immunopathol* 1992, **62**(1 Pt 2):S11-17.
45. Lv S, Han M, Yi R, Kwon S, Dai C, Wang R: **Anti-TNF-α therapy for patients with sepsis: a systematic meta-analysis**. *Int J Clin Pract* 2014, **68**(4):520-528.
46. Flohé SB, Agrawal H, Flohé S, Rani M, Bangen JM, Schade FU: **Diversity of interferon gamma and granulocyte-macrophage colony-stimulating factor in restoring immune dysfunction of dendritic cells and macrophages during polymicrobial sepsis**. *Mol Med* 2008, **14**(5-6):247-256.
47. Aikawa N: **[Cytokine storm in the pathogenesis of multiple organ dysfunction syndrome associated with surgical insults]**. *Nihon Geka Gakkai Zasshi* 1996, **97**(9):771-777.
48. Cohen J: **The immunopathogenesis of sepsis**. *Nature* 2002, **420**(6917):885-891.
49. Ge Y, Huang M, Yao YM: **Biology of Interleukin-17 and Its Pathophysiological Significance in Sepsis**. *Front Immunol* 2020, **11**:1558.
50. Cauvi DM, Williams MR, Bermudez JA, Armijo G, De Maio A: **Elevated expression of IL-23/IL-17 pathway-related mediators correlates with exacerbation of pulmonary inflammation during polymicrobial sepsis**. *Shock* 2014, **42**(3):246-255.
51. Mikacenic C, Hansen EE, Radella F, Gharib SA, Stapleton RD, Wurfel MM: **Interleukin-17A Is Associated With Alveolar Inflammation and Poor Outcomes in Acute Respiratory Distress Syndrome**. *Crit Care Med* 2016, **44**(3):496-502.
52. Fleischmann C, Thomas-Rueddel DO, Hartmann M, Hartog CS, Welte T, Heublein S, Dennler U, Reinhart K: **Hospital Incidence and Mortality Rates of Sepsis**. *Dtsch Arztebl Int* 2016, **113**(10):159-166.
53. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, Richards DR, McDonald-Smith GP, Gao H, Hennessy L *et al*: **Genomic responses in mouse models poorly mimic human inflammatory diseases**. *Proc Natl Acad Sci U S A* 2013, **110**(9):3507-3512.
54. Hotchkiss RS, Monneret G, Payen D: **Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach**. *Lancet Infect Dis* 2013, **13**(3):260-268.
55. Venet F, Filipe-Santos O, Lepape A, Malcus C, Poitevin-Later F, Grives A, Plantier N, Pasqual N, Monneret G: **Decreased T-cell repertoire diversity in sepsis: a preliminary study**. *Crit Care Med* 2013, **41**(1):111-119.

56. Lukaszewicz AC, Grienay M, Resche-Rigon M, Pirracchio R, Faivre V, Boval B, Payen D: **Monocytic HLA-DR expression in intensive care patients: interest for prognosis and secondary infection prediction**. *Crit Care Med* 2009, **37**(10):2746-2752.
57. Hotchkiss RS, Osmon SB, Chang KC, Wagner TH, Coopersmith CM, Karl IE: **Accelerated lymphocyte death in sepsis occurs by both the death receptor and mitochondrial pathways**. *J Immunol* 2005, **174**(8):5110-5118.
58. Monneret G, Lepape A, Voirin N, Bohe J, Venet F, Debard AL, Thizy H, Bienvenu J, Gueyffier F, Vanhems P: **Persisting low monocyte human leukocyte antigen-DR expression predicts mortality in septic shock**. *Intensive Care Med* 2006, **32**(8):1175-1183.
59. Grimaldi D, Louis S, Pene F, Sirgo G, Rousseau C, Claessens YE, Vimeux L, Cariou A, Mira JP, Hosmalin A *et al*: **Profound and persistent decrease of circulating dendritic cells is associated with ICU-acquired infection in patients with septic shock**. *Intensive Care Med* 2011, **37**(9):1438-1446.
60. Forel JM, Chiche L, Thomas G, Mancini J, Farnarier C, Cognet C, Guervilly C, Daumas A, Vely F, Xeridat F *et al*: **Phenotype and functions of natural killer cells in critically-ill septic patients**. *PLoS One* 2012, **7**(12):e50446.
61. Hotchkiss RS, Monneret G, Payen D: **Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy**. *Nat Rev Immunol* 2013, **13**(12):862-874.
62. Inoue S, Suzuki-Utsunomiya K, Okada Y, Taira T, Iida Y, Miura N, Tsuji T, Yamagiwa T, Morita S, Chiba T *et al*: **Reduction of immunocompetent T cells followed by prolonged lymphopenia in severe sepsis in the elderly**. *Crit Care Med* 2013, **41**(3):810-819.
63. Venet F, Pachot A, Debard AL, Bohé J, Bienvenu J, Lepape A, Monneret G: **Increased percentage of CD4+CD25+ regulatory T cells during septic shock is due to the decrease of CD4+CD25- lymphocytes**. *Crit Care Med* 2004, **32**(11):2329-2331.
64. Faivre V, Lukaszewicz AC, Alves A, Charron D, Payen D, Haziot A: **Human monocytes differentiate into dendritic cells subsets that induce anergic and regulatory T cells in sepsis**. *PLoS One* 2012, **7**(10):e47209.
65. Zhu J, Yamane H, Paul WE: **Differentiation of effector CD4 T cell populations (*)**. *Annu Rev Immunol* 2010, **28**:445-489.
66. Damsker JM, Hansen AM, Caspi RR: **Th1 and Th17 cells: adversaries and collaborators**. *Ann N Y Acad Sci* 2010, **1183**:211-221.
67. Naylor K, Li G, Vallejo AN, Lee WW, Koetz K, Bryl E, Witkowski J, Fulbright J, Weyand CM, Goronzy JJ: **The influence of age on T cell generation and TCR diversity**. *J Immunol* 2005, **174**(11):7446-7452.
68. Group NCA: **National Consent Policy**. In. Edited by National Consent Advisory Group HsE, Ireland. Dublin, Ireland.; 2017.
69. Zhang M, Gong J, Presky DH, Xue W, Barnes PF: **Expression of the IL-12 receptor beta 1 and beta 2 subunits in human tuberculosis**. *J Immunol* 1999, **162**(4):2441-2447.
70. Chognard G, Bellemare L, Pelletier AN, Dominguez-Punaro MC, Beauchamp C, Guyon MJ, Charron G, Morin N, Sivanesan D, Kuchroo V *et al*: **The dichotomous pattern of IL-12r and IL-23R expression**

elucidates the role of IL-12 and IL-23 in inflammation. *PLoS One* 2014, **9**(2):e89092.
71. Venet F, Foray AP, Villars-Méchin A, Malcus C, Poitevin-Later F, Lepape A, Monneret G: **IL-7 restores lymphocyte functions in septic patients**. *J Immunol* 2012, **189**(10):5073-5081.
72. Ronit A, Plovsing RR, Gaardbo JC, Berg RM, Hartling HJ, Ullum H, Andersen Å B, Madsen HO, Møller K, Nielsen SD: **Inflammation-Induced Changes in Circulating T-Cell Subsets and Cytokine Production During Human Endotoxemia**. *J Intensive Care Med* 2017, **32**(1):77-85.
73. Yan Z, Xiaoyu Z, Zhixin S, Di Q, Xinyu D, Jing X, Jing H, Wang D, Xi Z, Chunrong Z *et al*: **Rapamycin attenuates acute lung injury induced by LPS through inhibition of Th17 cell proliferation in mice**. *Sci Rep* 2016, **6**:20156.
74. Brunialti MK, Santos MC, Rigato O, Machado FR, Silva E, Salomao R: **Increased percentages of T helper cells producing IL-17 and monocytes expressing markers of alternative activation in patients with sepsis**. *PLoS One* 2012, **7**(5):e37393.
75. Maravitsa P, Adamopoulou M, Pistiki A, Netea MG, Louis K, Giamarellos-Bourboulis EJ: **Systemic over-release of interleukin-17 in acute kidney injury after septic shock: Clinical and experimental evidence**. *Immunol Lett* 2016, **178**:68-76.
76. Wynn JL, Wilson CS, Hawiger J, Scumpia PO, Marshall AF, Liu JH, Zharkikh I, Wong HR, Lahni P, Benjamin JT *et al*: **Targeting IL-17A attenuates neonatal sepsis mortality induced by IL-18**. *Proc Natl Acad Sci U S A* 2016, **113**(19):E2627-2635.
77. Paraschos MD, Patrani M, Pistiki A, Katsenos C, Tsaganos T, Netea MG, Giamarellos-Bourboulis EJ, Mandragos K: **Defective cytokine production early after multiple traumas: Modulation in severe sepsis**. *Cytokine* 2015, **76**(2):222-226.
78. Breslow JM, Meissler JJ, Jr., Hartzell RR, Spence PB, Truant A, Gaughan J, Eisenstein TK: **Innate immune responses to systemic Acinetobacter baumannii infection in mice: neutrophils, but not interleukin-17, mediate host resistance**. *Infect Immun* 2011, **79**(8):3317-3327.
79. Nordfjäll K, Larefalk A, Lindgren P, Holmberg D, Roos G: **Telomere length and heredity: Indications of paternal inheritance**. *Proc Natl Acad Sci U S A* 2005, **102**(45):16374-16378.
80. Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, Migaud M, Israel L, Chrabieh M, Audry M *et al*: **Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity**. *Science* 2011, **332**(6025):65-68.
81. Milner JD, Brenchley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, Kanno Y, Spalding C, Elloumi HZ, Paulson ML *et al*: **Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome**. *Nature* 2008, **452**(7188):773-776.
82. Liu B, Tan W, Barsoum A, Gu X, Chen K, Huang W, Ramsay A, Kolls JK, Schwarzenberger P: **IL-17 is a potent synergistic factor with GM-CSF in mice in stimulating myelopoiesis, dendritic cell expansion, proliferation, and functional enhancement**. *Exp Hematol* 2010, **38**(10):877-884.e871.

83. Shen H, Zhang W, Abraham C, Cho JH: **Age and CD161 expression contribute to inter-individual variation in interleukin-23 response in CD8+ memory human T cells**. *PLoS One* 2013, **8**(3):e57746.
84. Schmitt V, Rink L, Uciechowski P: **The Th17/Treg balance is disturbed during aging**. *Exp Gerontol* 2013, **48**(12):1379-1386.
85. Langrish CL, McKenzie BS, Wilson NJ, de Waal Malefyt R, Kastelein RA, Cua DJ: **IL-12 and IL-23: master regulators of innate and adaptive immunity**. *Immunol Rev* 2004, **202**:96-105.
86. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ: **IL-23 drives a pathogenic T cell population that induces autoimmune inflammation**. *J Exp Med* 2005, **201**(2):233-240.
87. Cosmi L, De Palma R, Santarlasci V, Maggi L, Capone M, Frosali F, Rodolico G, Querci V, Abbate G, Angeli R *et al*: **Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor**. *J Exp Med* 2008, **205**(8):1903-1916.
88. Coakley JD, Breen EP, Moreno-Olivera A, Al-Harbi AI, Melo AM, O'Connell B, McManus R, Doherty DG, Ryan T: **Dysregulated T helper type 1 (Th1) and Th17 responses in elderly hospitalised patients with infection and sepsis**. *PLoS One* 2019, **14**(10):e0224276.
89. Treiner E, Duban L, Bahram S, Radosavljevic M, Wanner V, Tilloy F, Affaticati P, Gilfillan S, Lantz O: **Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1**. *Nature* 2003, **422**(6928):164-169.
90. Dusseaux M, Martin E, Serriari N, Peguillet I, Premel V, Louis D, Milder M, Le Bourhis L, Soudais C, Treiner E *et al*: **Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells**. *Blood* 2011, **117**(4):1250-1259.
91. Ben Youssef G, Tourret M, Salou M, Ghazarian L, Houdouin V, Mondot S, Mburu Y, Lambert M, Azarnoush S, Diana JS *et al*: **Ontogeny of human mucosal-associated invariant T cells and related T cell subsets**. *J Exp Med* 2018, **215**(2):459-479.
92. Smith DJ, Hill GR, Bell SC, Reid DW: **Reduced mucosal associated invariant T-cells are associated with increased disease severity and Pseudomonas aeruginosa infection in cystic fibrosis**. *PLoS One* 2014, **9**(10):e109891.
93. Grimaldi D, Le Bourhis L, Sauneuf B, Dechartres A, Rousseau C, Ouaaz F, Milder M, Louis D, Chiche JD, Mira JP *et al*: **Specific MAIT cell behaviour among innate-like T lymphocytes in critically ill patients with severe infections**. *Intensive Care Med* 2014, **40**(2):192-201.
94. Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, Kronenberg M: **CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution**. *J Exp Med* 1998, **188**(8):1521-1528.
95. Bendelac A, Savage PB, Teyton L: **The biology of NKT cells**. *Annu Rev Immunol* 2007, **25**:297-336.
96. Passos ST, Silver JS, O'Hara AC, Sehy D, Stumhofer JS, Hunter CA: **IL-6 promotes NK cell production of IL-17 during toxoplasmosis**. *J Immunol* 2010, **184**(4):1776-1783.
97. Young JS, Monaghan SF, Chung CS, Cioffi WG, Ayala A, Heffernan DS: **Divergent invariant natural killer T-cell response to sepsis of**

abdominal vs. non-abdominal origin in human beings. *Surg Infect (Larchmt)* 2015, **16**(1):29-35.
98. Heffernan DS, Monaghan SF, Chung CS, Cioffi WG, Gravenstein S, Ayala A: **A divergent response of innate regulatory T-cells to sepsis in humans: circulating invariant natural killer T-cells are preserved**. *Hum Immunol* 2014, **75**(3):277-282.
99. Bai L, Picard D, Anderson B, Chaudhary V, Luoma A, Jabri B, Adams EJ, Savage PB, Bendelac A: **The majority of CD1d-sulfatide-specific T cells in human blood use a semiinvariant Vdelta1 TCR**. *Eur J Immunol* 2012, **42**(9):2505-2510.
100. Mangan BA, Dunne MR, O'Reilly VP, Dunne PJ, Exley MA, O'Shea D, Scotet E, Hogan AE, Doherty DG: **Cutting edge: CD1d restriction and Th1/Th2/Th17 cytokine secretion by human Vdelta3 T cells**. *J Immunol* 2013, **191**(1):30-34.
101. Tyler CJ, Doherty DG, Moser B, Eberl M: **Human Vgamma9/Vdelta2 T cells: Innate adaptors of the immune system**. *Cell Immunol* 2015, **296**(1):10-21.
102. Maggi L, Santarlasci V, Capone M, Peired A, Frosali F, Crome SQ, Querci V, Fambrini M, Liotta F, Levings MK *et al*: **CD161 is a marker of all human IL-17-producing T-cell subsets and is induced by RORC**. *Eur J Immunol* 2010, **40**(8):2174-2181.
103. Venet F, Bohe J, Debard AL, Bienvenu J, Lepape A, Monneret G: **Both percentage of gammadelta T lymphocytes and CD3 expression are reduced during septic shock**. *Crit Care Med* 2005, **33**(12):2836-2840.
104. Boehm U, Klamp T, Groot M, Howard JC: **Cellular responses to interferon-gamma**. *Annu Rev Immunol* 1997, **15**:749-795.
105. White M, Lawless MW, O'Dwyer MJ, Grealy R, Connell BO, Stordeur P, Kelleher D, McManus R, Ryan T: **Transforming growth factor beta-1 and interleukin-17 gene transcription in peripheral blood mononuclear cells and the human response to infection**. *Cytokine* 2010.
106. Carvelli J, Piperoglou C, Bourenne J, Farnarier C, Banzet N, Demerle C, Gainnier M, Vely F: **Imbalance of Circulating Innate Lymphoid Cell Subpopulations in Patients With Septic Shock**. *Front Immunol* 2019, **10**:2179.
107. van der Geest KSM, Kroesen BJ, Horst G, Abdulahad WH, Brouwer E, Boots AMH: **Impact of Aging on the Frequency, Phenotype, and Function of CD161-Expressing T Cells**. *Front Immunol* 2018, **9**:752.
108. Miao J, Zhang K, Qiu F, Li T, Lv M, Guo N, Han Q, Zhu P: **Percentages of CD4+CD161+ and CD4-CD8-CD161+ T cells in the synovial fluid are correlated with disease activity in rheumatoid arthritis**. *Mediators Inflamm* 2015, **2015**:563713.
109. Miao J, Zhang K, Lv M, Li Q, Zheng Z, Han Q, Guo N, Fan C, Zhu P: **Circulating Th17 and Th1 cells expressing CD161 are associated with disease activity in rheumatoid arthritis**. *Scand J Rheumatol* 2014, **43**(3):194-201.
110. Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS: **Acute respiratory distress syndrome: the Berlin Definition**. *Jama* 2012, **307**(23):2526-2533.
111. Oppert M, Engel C, Brunkhorst FM, Bogatsch H, Reinhart K, Frei U, Eckardt KU, Loeffler M, John S: **Acute renal failure in patients with**

severe sepsis and septic shock--a significant independent risk factor for mortality: results from the German Prevalence Study. *Nephrol Dial Transplant* 2008, **23**(3):904-909.

112. Liu Y, Wang X, Yu L: **Th17, rather than Th1 cell proportion, is closely correlated with elevated disease severity, higher inflammation level, and worse prognosis in sepsis patients**. *J Clin Lab Anal* 2021, **35**(5):e23753.
113. Liu YC, Zou XB, Chai YF, Yao YM: **Macrophage polarization in inflammatory diseases**. *Int J Biol Sci* 2014, **10**(5):520-529.