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Unconventional T-cell driven inflammatory responses during acute peritonitis: implications for diagnosis and therapy of peritoneal dialysis patients

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Abstract

Scientific background. Infection remains a major cause of morbidity and technique failure in PD patients. The mechanisms that underpin the clinical severity of peritonitis episodes and their link to outcomes remain poorly defined. γδ T cells together with MAIT cells play a crucial role in orchestrating acute immune responses by the recognition of metabolites (HMB-PP and vitamin B2 derivatives) present in many pathogenic bacteria. My work aimed to understand the molecular and cellular mechanisms underlying the local recognition of bacterial pathogens by peritoneal unconventional T cells, which could be exploited for targeted therapies and novel point of care diagnostic test.

Approach. The local and systemic frequency of unconventional T was analysed before and during acute microbial infections, in a well-defined cohort of individuals with end-stage kidney disease receiving peritoneal dialysis (PD). In addition, the responsiveness of peritoneal unconventional T cells to HMB-PP and/or vitamin B2 producing bacteria was assessed *ex vivo*.

Results. This study demonstrated that: *(i)* peritoneal Vγ9/Vδ2 T cells and MAIT cells are elevated in patients with infections caused by HMB-PP and/or vitamin B2 positive bacteria (*e.g. E. coli*) but not in infections caused by HMB-PP and vitamin B2 negative species (*e.g. Streptococcus*); *(ii)* peritoneal Vγ9/Vδ2 T cells and MAIT cells are dominant producers of the pro-inflammatory cytokines TNF-α and IFN-γ in response to HMB-PP and/or vitamin B2 positive bacteria; and *(iii)* in turn, TNF-α and IFN-γ are potent stimulators of peritoneal mesothelial cells and fibroblasts. Outcome analyses showed that infections caused by bacteria that are able to activate Vγ9/Vδ2 T-cells and/or MAIT cells were associated with higher risks of technique failure such as mortality and catheter removal.

Conclusions. My studies provide a molecular basis for the existence of pathogen-specific immune fingerprints that have diagnostic and prognostic value, identify key pathways by which unconventional T-cells can amplify early inflammatory responses, and highlight potential therapeutic targets that may be exploited to improve outcomes.

Table of Contents

List of Figures

List of tables

List of abbreviaitons

ANOVA	Analysis of variance
ANZDATA	Australia and New Zealand Dialysis Transplant Registry
APC	Antigen presenting cell
APD	Automated peritoneal dialysis
BSA	Bovine serum albumin
CAPD	Continuous ambulatory peritoneal dialysis
CD	Cluster of differentiation
CD277	Butyrophilin subfamily member A1
CKD	Chronic kidney disease
CMV	Cytomegalovirus
CoM	Conditioned Medium
CXCL 8	CXC chemokine ligand 8 (also known as IL-8)
DC	Dendritic cell
DMAPP	Di-methyl-allyl pyrophosphate
DMEM	Dulbecco's Modified Eagle Medium
DMRL	6,7-dimethyl-8-D-ribityllumazine
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
EPCR	Endothelial protein C receptor
ESRD	End-stage renal disease
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
HD	Haemodialysis
HLA	Human leukocyte antigen
HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HPFB	Human Peritoneal Fibroblast
HPMC	Human peritoneal mesothelial cells
ICAM	Intercellular adhesion molecule
ICOS	TCR-inducible costimulatory receptor
IFN-γ	Interferon gamma
IL	Interleukin
IPP	Isopentenyl pyrophosphate
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LB	Luria Broth
LFA 1	Lymphocyte function-associated antigen 1
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MAIT	Mucosal-associated invariant T cells
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1

M-CSF	Macrophage colony stimulating factor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MMT	Mesothelial to mesenchymal transition
ND	Not Done
NEAA	Non-essential aminoacid
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NKG2D	NK group 2, member D receptor
NKT	Natural Killer T cells
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PD	Peritoneal dialysis
PDE	Peritoneal Dyalisis Effluent
Pe	Phycoerythrin
PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptor
RBC	Red blood cells
RM	Repeated Measure
RNA	Ribonucleic acid
RPMI	RPMI-1640 media
SD	Standard deviation
SEM	Standard error of the mean
sIL-6R	Soluble interleukin-6 receptor
TCR	T cell receptor
TGFβ	Transforming growth factor-beta
TH	T helper cells
TLR	Toll-like receptor
Tnaive	Naive T cell
TNF-α	Tumour necrosis factor-alpha
Treg	Regulatory T cells
UTI	Urinary tract infection

Chapter 1. Introduction

1.1 Overview of the immune response

The immune system defines the sets of cells and molecules that protect the body against foreign agents, known collectively as antigens. This system is made up of two components that complement each other: the innate and the adaptive immune responses (Mackay et al., 2000a).

The innate immune response is a non-specific line of defence, which is invoked at equal magnitude every time that a pathogen is encountered. This response includes phagocytic cells such as neutrophils and monocytes and inflammatory cells such as basophils and mast cells. Together, these cells protect the body from microbes present at the site of infections. This process is mediated by the release of complement proteins and cytokines (e.g. interferons and tumour necrosis factor (TNF)-α). Indeed, innate immune cells are able to recognise pathogen-associated molecular patterns (PAMPs). In this way innate immune cells can distinguish foreign microbes from self-components (Medzhitov, 2007). Cell recruitment to the site of infection and subsequent activation is the major cause of the phenomenon called "inflammation". Overall, this process is beneficial, eliminating pathogens and promoting tissue healing. However, dysregulation of the innate immune and inflammatory responses may lead to tissue damage and death (Medzhitov, 2007, 2008)

The adaptive immune response is triggered when a pathogen overcomes the first line of defence of the innate immune response. It occurs throughout the lifetime of an individual allowing adaptation to infections and preparing the immune system for a potential future challenge by the same pathogen. This response is mediated mainly by antigen-specific B cells and T cells, which are activated by antigen-presenting cells (APC) such as dendritic cells (DC) and macrophages. Once activated, B cells mainly secrete antigen specific immunoglobulins (antibodies) that help eliminate extracellular microorganisms. Activated T cells are able to kill infected cells directly or through the activation of macrophages (Mackay et al., 2000a).

1.1.1 T cell development

Committed T cells are generated in the bone marrow and migrate to the thymus to complete their development. Pre-mature thymocytes lack expression of the T cell receptor (TCR) and of the co-receptors CD4 and CD8. They are referred to as double negative (DN) thymocytes. The TCR consists of two chains, which are formed by a process of gene rearrangement. During this process there is first the generation of the TCR β, γ and δ chains and then the TCRα. In this way thymocytes with a successful pre-TCR proliferate and switch from DN to a CD4 and CD8 double positive (DP) phenotype. DP TCR αβ thymocytes interact with cortical epithelial cells that express major histocompatibility complex (MHC) class I or II associated with self-peptides. The DP thymocytes, which recognise self-peptides resulting in an intermediate level of TCR signalling, develop into single positive thymocytes. TCRs that binds MHC-class I complexes become $CD8^+$ T cells, whereas TCRs that bind MHC-class II complexes become $CD4^+$ T cells. T cells are then ready to be exported to the peripheral circulation as naïve T cells (Attaf et al., 2015; Mackay et al., 2000b; Yates, 2014)

1.1.2 Unconventional T cells

The rearrangement of the genes encoding for TCRα and TCRβ can generate a vast TCR diversity to recognise an extensive range of antigens. Usually peptides derived from extracellular antigens are processed and presented in association with a highly polymorphic MHC complex. αβ T cells that are activated by this type of antigen presentation are referred to as "conventional" and are different from "unconventional" T cell. Indeed, this last population of cells *i*) does not recognise classical peptide antigens, *ii*) becomes activated by presenting molecules encoded by non-polymorphic genes localised outside the MHC locus such as CD1a, CD1b, CD1c, CD1d and MR1 (Van Rhijn and Moody, 2015), *iii*) tends to localize in non-lymphoid tissues, and *iv*) is abundant in peripheral blood for a rapid antigen response and proliferation (Godfrey et al., 2015).

αβ T cells belonging to the unconventional T cells group include mucosal associated invariant T (MAIT) cells, invariant natural killer T (iNKT) cells and germline-encoded mycolyl-reactive (GEM) T cells. Another group of unconventional T cells are γδ T cells. This last group together with αβ T cells and B cells represent the third lymphocytes lineage discovered in jawed vertebrates (Liuzzi et al., 2015; Vantourout and Hayday, 2013).

The importance of unconventional T cells lies in their ability to bridge the innate and the adaptive immune responses through the activation of inflammatory cells and the generation of antigen presenting cells (Tyler et al., 2015; Ussher et al., 2014a). In particular, Vγ9/Vδ2 T cells and MAIT cells are able to discriminate among a wide range of pathogens by the recognition of metabolic molecules (Liuzzi et al., 2015), which are essential for certain microorganisms and are absent in human cells. These antimicrobial responses are acquired at the foetal development stage, when these unconventional T cells already manifest effector function prior to the encounter with foreign pathogens (Dimova et al., 2015; Leeansyah et al., 2014).

1.1.3 Human γδ T cells

γδ T cells develop in the thymus before αβ T cells and acquire antimicrobial responses during foetal development (Pablo Pereira and Susumu Tonegawa, 1993). In humans, they typically constitute 1-5% of all lymphocytes in blood. However, they are able to localize in tissue (skin, intestine and reproductive tract) and make up to 50 % of T cells during microbial infections (Vantourout and Hayday, 2013). γδ T cells in the blood express a Vδ2 chain mostly paired to a Vγ9 chain, whereas the majority of γδ T cells in epithelial tissue carry a Vδ1 chain (LeFranc et al., 1986; Morita et al., 2007).

γδ T cells do not respond to peptide antigens presented by MHC class I and II that normally stimulate the majority of conventional αβ T cells. Extensive studies have been performed on the activation of $V\delta2^{+}$ T cells in the presence of so-called phosphoantigens (pAg). Distinctly, it has been suggested that $V\delta1^{+}$ T cells are enriched in tissue mucosa and recognise self and foreign lipids presented by CD1, and the stress related MHC-like molecule MICA and MICB (Godfrey et al., 2015; Groh et al., 1998; Uldrich et al., 2013). In addition, Willcox *et al*. showed that $V\delta2^{neg}$ T cells can bind the endothelial protein C receptor (EPCR), a ligand present in cytomegalovirus (CMV) infected cells with homology to CD1d. Moreover, the study showed that this binding was independent of the presence of lipids (Willcox et al., 2012).

1.1.3.1 Vγ9/Vδ2 T cells and pyrophosphate antigens

Vγ9/Vδ2 T cells represent the major γδ T cell subset present in peripheral blood, constituting up to 5% of the lymphocytes, but able to reach up to 50% and more of all peripheral T cells in infected patients (Morita et al., 2007). They respond to a class of low molecular weight molecules called "phosphoantigens" such as isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These molecules are generated in eukaryotes and some microbes via the mevalonate pathway through the action of the upstream enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase and downstream enzymes such as isopentenyl-PP (IPP) isomerase and farnesyl pyrophosphate synthase (Tanaka et al., 1995). Normally the levels of IPP present in microbes are not sufficient to induce an effective γδ T cell activation. Instead, it was found that metabolites derived from the non-mevalonate (MEP) pathway (Figure 1.1, Table 1.3) were able to induce a full activation of γδ T cells. Bacteria producing IPP via the classic mevalonate pathway failed to do so (Jomaa et al., 1999). These findings suggested that other compounds in addition to IPP were responsible for this activation. The discovery of the compound that was able to induce such a strong activation of γδ T cells came from studies where enzymes in the MEP pathway of the *E. coli* strains were disrupted or deleted from the genome. This study led to the discovery of a novel metabolite, (*E*)-4-hydroxy-3-methyl-2-butenyl pyrophosphate (HMB-PP), which is the direct precursor of IPP and 10,000 times more potent in stimulating Vγ9/Vδ2 T cells than IPP itself (Eberl et al., 2003; Hintz et al., 2001).

It has been shown that dysregulation of the mevalonate pathway in tumour cells leads to an accumulation of IPP followed by activation of Vγ9/Vδ2 T cells. This mechanism is caused by alteration of the mevalonate pathway with increased expression of the enzyme HMG-CoA reductase (Gober et al., 2003; Harwood et al., 1991). Manipulation of the mevalonate pathway by aminobisphosphonates such as zoledronate and related drugs can be used experimentally to sensitise target cells to γδ T cell-mediated killing for novel cancer immunotherapies (Dieli et al., 2007; Meraviglia et al., 2010; Wilhelm et al., 2003)

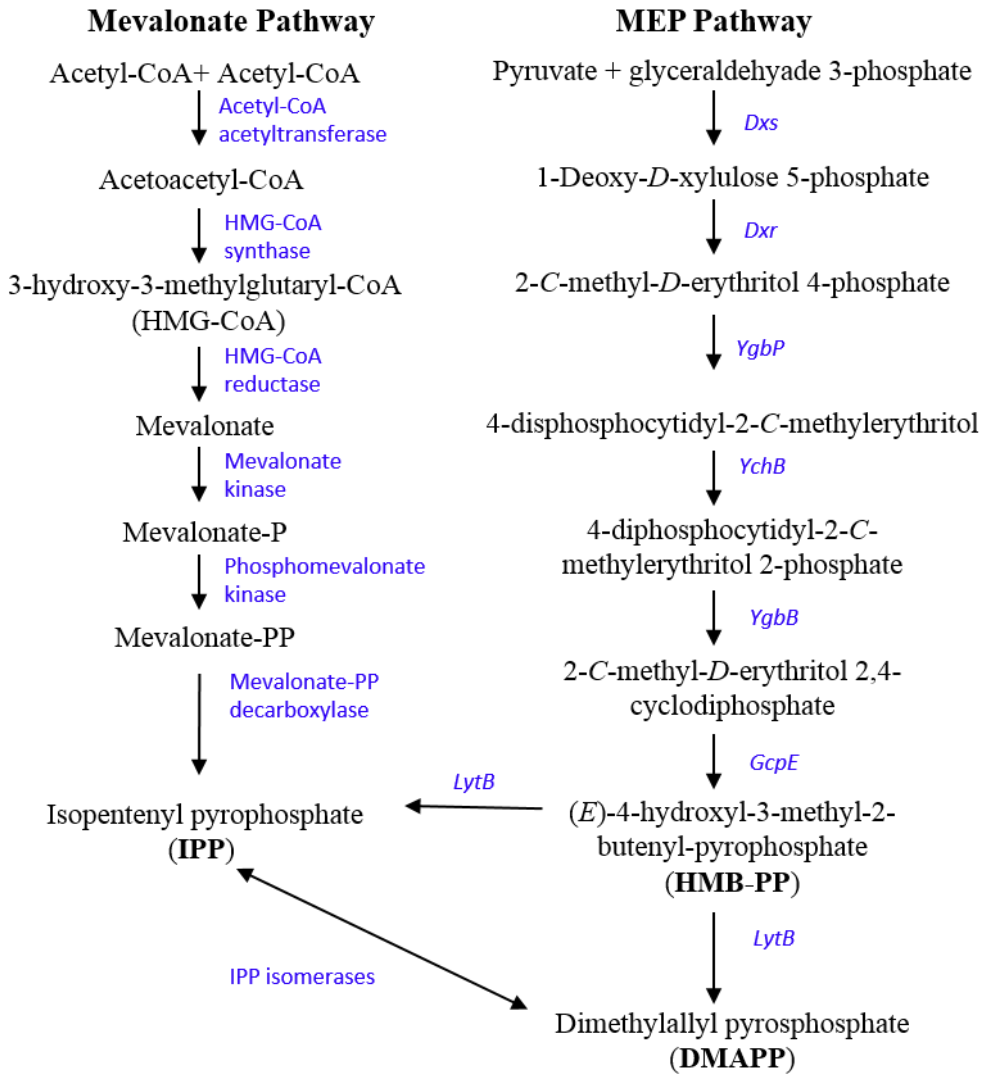

Figure 1.1 Mevalonate and MEP pathway for isoprenoid biosynthesis.
The MEP pathway is found in most Eubacteria (with the exception of Gram$^+$ cocci) as well as apicomplexan protozoa and chloroplasts of algae and higher plants. The mevalonate pathway is found in Archaebacteria, Eubacteria such as Gram$^+$ cocci, eukaryotes and the cytoplasm of plants. Adapted from Morita et al. 2007.

1.1.3.2 Vγ9/Vδ2 T cell activation: HMB-PP and microbial infection

HMB-PP is an intermediate of the MEP pathway, which is found in Eubacteria such as mycobacteria and Gram negative bacteria such as *Pseudomonas, Salmonella, Vibrio, E. coli, Neisseria* and *Brucella*. These bacteria are responsible for diseases such as tuberculosis, diphtheria, typhoid, plague and cholera (Gill et al. 2006; Eberl et al. 2003), (Table 1.1). *Listeria monocytogenes* is unique in being the only pathogenic species known to possess both the mevalonate and MEP pathways (Begley et al. 2004b). Staphylococci and streptococci do not use the MEP pathway (Morita et al. 2007).

The MEP pathway is also present in plant chloroplasts, which share common ancestry with an organelle present in parasitic protozoa responsible for the spread of malaria (Morita et al., 2007). Indeed, Vγ9/Vδ2 T cells are able to proliferate and exert effector functions during infections caused by *Plasmodium falciparum* (Costa et al., 2011). This parasite is able to invade red blood cells and expand in the microcirculation. The control of this parasite is fundamental to avoid the progression of the disease. Once activated γδ T cells contribute to the parasite growth inhibition. This mechanism is mediated mainly by cytotoxic molecule like granulsyn but not perforin (Costa et al., 2011; Farouk et al., 2004). γδ T cells activation is triggered either during the intraerythrocytic parasitic stage or by the extracellular merozoite stage. Indeed, γδ T cells once activates are able to expand to up to 40% of all T cells and inhibit the replication of *Plasmodium* by a TCR-dependent mechanism (Bordessoule, Gaulard, and Mason 1990; Elloso et al. 1994).

One of the most detrimental pathogens possessing the MEP pathway is *Mycobacterium tuberculosis* (Table 1.1). In a macaque model of *M. tuberculosis* infections, Vγ9/Vδ2 T cells were found distributed in lungs, bronchial lymph nodes and spleens (Huang et al., 2008a). It may be that peripheral Vγ9/Vδ2 T cells reach the site of infection through trans-endothelial migration conferring protection against this bacterium (Huang et al., 2008a). Moreover, it has been shown that Vγ9/Vδ2 T cells were able to arise a memory-type immune response when *Mycobacterium bovis* BCG-vaccinated monkeys were infected with *M. tuberculosis* by aerosol (Chen et al., 2013; Shen et al., 2002). At the same time the lack of Vγ9/Vδ2 T cells can be associated with low protection against the infections. Indeed, the deficiency of Vγ9/Vδ2 T cells may explain why HIV patients show a poor protection against *M. tuberculosis* infections (Chen et al., 2013; Martini et al., 2002).

It has been shown that γδ T cells respond to neutrophils after bacterial phagocytosis. This activation is dependent on the ability of bacteria to produce HMB-PP and independent of the presence of other pathogen associated molecular patterns such as LPS (Davey et al., 2011a). The addition of alkaline phosphatase to these co-cultures can abrogate this response, confirming that HMB-PP is the main soluble activator released by neutrophils upon phagocytosis of bacteria that is able to stimulate Vγ9/Vδ2 T cells (Davey et al., 2011a). The mechanism by which HMB-PP is recognized by Vγ9/Vδ2 T cells is still unclear. However, a functional Vγ9/Vδ2 TCR and cell-cell contact are mandatory for full γδ T cell activation (Eberl et al., 2009; GREEN et al., 2004).

Of note, excessive γδ T cell stimulation can have detrimental consequences. In bacterial sepsis γδ T cell are activated in less than 24 hours and their frequency correlates with severity of the inflammatory response and clinical outcome (Davey et al., 2014; Matsushima et al., 2004). Moreover, a dysregulation of the crosstalk between monocytes and Vγ9/Vδ2 T cells at the site of infection can be the cause of excessive production of inflammatory mediators and increase of morbidity and mortality in HMB-PP$^+$ infection (Eberl et al., 2009; Lin et al., 2013).

Table 1.1. Microbial pathogen HMB-PP production.

HMB-PP$^+$		
Gram$^-$ bacteria	**Gram$^+$ bacteria**	**Other bacteria**
Acinetobacter baumannii	*Bacillus anthracis*	*Ehrlichia chaffeensis*
Enterobacter aerogenes	*Clostridium difficile*	*Mycoplasma penetrans* [b]
Escherichia coli	*Corynebacterium diphtheriae*	*Treponema pallidum*
Francisella tularensis	*Listeria monocytogenes* [a]	
Haemophilus influenzae	*Mycobacterium tuberculosis*	
Helicobacter pylori	*Propionibacterium acnes*	
Klebsiella pneumoniae		
Neisseria meningitidis		
Pseudomonas aeruginosa		**Protozoan parasites**
Salmonella enterica		*Plasmodium falciparum*
Shigella dysenteriae		*Toxoplasma gondii*
Vibrio cholerae		
Yersinia pestis		
HMB-PP$^-$		
Gram$^-$ bacteria	**Gram$^+$ bacteria**	**Other bacteria**
Legionella pneumophila	*Enterococcus faecalis*	*Borrelia burgdorferi*
	Staphylococcus aureus	*Mycoplasma genitalium* [b]
	Streptococcus pneumoniae	*Rickettsia prowazekii*
	(*Listeria innocua*) [a]	

[a] The HMB-PP species *L. innocua* is non-pathogenic but listed here because of its close phylogenetic relation to the HMB-PP$^+$ pathogen *L. monocytognes* (Begley et al., 2004).
[b] Most mycoplasma species have no isoprenoid biosynthesis of their own. *M. penetrans* is the only human pathogenic mycoplasma known so far to produce HMB-PP (Eberl et al., 2004). Taken from Eberl and Moser 2009.

1.1.3.3 Presentation of phosphoantigens to Vγ9/Vδ2 T cells by BTN3

Although the discovery of γδ T cells dates back three decades and the discovery of phosphoantigens two decades, it remains unclear how γδ T cells are able to sense pAg. However, it is clear that cell-cell contact is necessary for full activation of Vγ9/Vδ2 T cells and that a cell surface receptor is implicated in this process. It is known that B7 related receptors are implicated in the induction of TCR-activated proliferation and differentiation of naïve T cells. This family includes members such as the *Skint* and butyrophilin (BTN) subfamilies. *Skint1* is implicated in thymic selection, maturation, and skin-tissue homing of murine Vγ5/Vδ1 dendritic epidermal T cells (DETCs) (Barbee et al., 2011; Harly et al., 2014; Rhodes et al., 2016) This molecule is composed of two butyrophilin-related Ig domain

and three transmembrane regions. The human proteins which are most similar to it are the BTNs. These are type I membrane proteins with two immunoglobulins domain, IgV and IgC2 and a cytosolic domain B30.2 (PRYSPRY) (Karunakaran et al., 2014). This domain is shared with the one present in TRIM molecules, where it acts as PRR binding associated with infections. Although a crucial role of BTN3 for human Vγ9/Vδ2 T cells response has been confirmed, there are contradictory studies about this interaction (Rhodes et al., 2016; Vavassori et al., 2013).

Independent studies described an interaction of pAg molecules with the BTN3A1 B30.2 domain, confirming the presence of a binding pocket where mutation of charged residues can render T cells unresponsive to BTN3A1 (Hsiao et al., 2014; Sandstrom et al., 2014). The intracellular HMB-PP sensing might be favoured by an interaction between the cytoskeletal adaptor protein periplakin and the leucine motif located next to the cytoplasmic B30.2. This interaction is followed by conformational changes in the B30.2 domain and transmission of conformational changes to the cell surface (Rhodes et al., 2015) (Figure 1.2). A second model supports pAg binding to the external IgV domain of BTN3A1 mimicking a MHC like presenting molecule. A third model suggests that BTN3A simply acts as adhesion factor promoting cell-cell interation without taking part in TCR ligand recognition (Rhodes et al., 2016; Vavassori et al., 2013) (Figure 1.2).

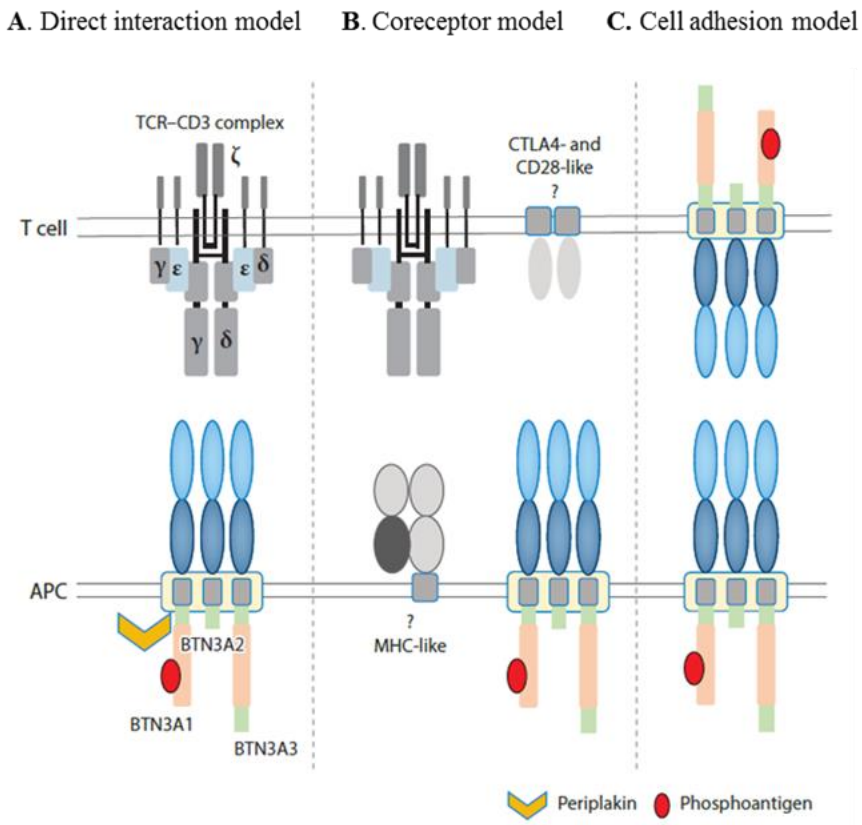

Figure 1.2. Currently proposed models for the presentation of phosphoantigens to Vγ9Vδ2 TCR by BTN3A molecules.
(A) Direct interaction model: BTN3A1 is the ligand for the Vγ9/Vδ2 TCR. (B) The coreceptor model: the γδ TCR interacts with a MHC-like molecule and BTN3A with a structure related to CTLA4 and CD28. (C) The cell adhesion model: BTN3A structure mediates homotypic interactions to promote cell contact among T cells and APCs. Taken from Rhodes, Reith, and Trowsdale 2016.

1.1.3.4 Vγ9/Vδ2 T cell effector functions

Once activated Vγ9/Vδ2 T cells are able to perform different effector functions such as DC maturation, priming of $CD4^+$ and $CD8^+$ T cells and supporting survival and maturation of monocytes and neutrophils (Davey et al., 2011a; Eberl et al., 2009; Moser and Eberl, 2007).

During the adaptive immune response DC, upon recognition of danger signal via PRR, are able to upregulate co-stimulatory molecules and migrate to the lymph node to present the up taken antigen to the αβ T cells. However, activated Vγ9/Vδ2 T cells, thanks to the release of IFN-γ and TNF-α, are able to induce upregulation of HLA-DR, CD86 and CD83 on immature DC (Tyler et al., 2015). In addition, the co-culture of these two types of cells lead to the upregulation of CCR7, a receptor essential for DC migration to the secondary lymphoid tissue for antigen presentation. In this regard, it has been shown that Vγ9/Vδ2 T cells-matured DC were able to prime $CD4^+$ T cells towards a Th1 phenotype. This response was due to an increase IFN-γ release by activated $V\delta2^+$ T cells (Shrestha et al., 2005). CCR7 can also be upregulated on activated γδ T cells, triggering their recruitment to the lymph nodes. Once in the lymph nodes, it is believed that γδ T cells can act as APCs by the upregulation of co-stimulatory molecule such as CD80, CD86 and CD40 and the adhesion receptors CD11a, CD18 and CD54. Moreover, γδ T cells are able to polarize $CD4^+$ T cells toward Th1 phenotype, through the production of IFN-γ and TNF-α (Brandes, 2005; Moser and Brandes, 2006).

Vγ9/Vδ2 T cell effector features are not possible without cell-cell contact, explaining why the presence of accessory monocytes is beneficial for Vγ9/Vδ2 T cell activation by HMB-PP. This was demonstrated by an *in vitro* study in our laboratory, where HMB-PP induced monocyte activation within 6-18 hours, at a physiologically relevant concentration of 0.1 nM HMB-PP in the presence of a ratio of Vγ9/Vδ2 T cells and monocytes as low as 1-50-1:500. (Eberl and Moser, 2009). Indeed, activated Vγ9/Vδ2 T cells can induce monocyte differentiation into APC. This can occur by downregulation of the marker CD14 and upregulation of CD40, CD86 and HLA-DR and DC related markers CD83 and CD209. This effect is also induced on monocytes present in the peritoneal cavity in the presence of HMB-PP. This scenario mimics what happens at early stages on inflammation when low number of bacteria produce HMB-PP and monocytes outnumber local γδ T cells (Eberl et al., 2009).

The expression of inflammatory chemokines receptors on Vγ9/Vδ2 T cells (e.g. CCR2, CCR5,CCR6 and CXCR3) confirm the capacity of these innate immune cells to migrate to

the site of infection (Brandes, 2003; Cipriani et al., 2000; Glatzel et al., 2002) (Figure 1.3). Once at the site of infection, Vγ9/Vδ2 T cells are also able to shape the neutrophil phenotype during cell activation. This action involves: *i*) activation of Vγ9/Vδ2 T cells by HMB-PP$^+$ microbes phagocytosed by neutrophils, *ii)* release of pro-inflammatory cytokines IFN-γ and TNF-α, *iii*) expression of APC-related markers such as CD40, CD54, CD64 and CD83 as well as MHC molecules on neutrophils, and *iv*) subsequent activation of αβ T cells (Davey et al., 2011a, 2014) (Figure 1.3).

Another important function played by Vγ9/Vδ2$^+$ T cells is the release of cytotoxic molecules or the induction of apoptosis in the presence of infected or tumour cells. Indeed, γδ T cells can kill infected molecule through the engagement of death-inducing receptors (FAS/FAS ligand) and the release of cytolytic granules containing perforins and graenzym and/or granulysin (Bonneville et al., 2010). Once released perforin formed pores on the target cells membrane facilitating the entry of graenzym A and B leading to the target cell lysis. Granulysin released by Vγ9/Vδ2$^+$ T cells has been associated with the protection against detrimental pathogens such as *M. tuberculosis* (Dieli et al., 2001) and the inhibition of the growth of the parasite responsible for malaria (*P. falciparum*) (Farouk et al., 2004). Granulysin acts increasing the influx of calcium from extracellular and intracellular stores contributing in this way to the cell mitochondrial damage (Krensky and Clayberger, 2009).

There are others Vγ9/Vδ2 T cells functions that will not be discussed here such as the importance of these cells in providing B cells help in the production of immunoglobulins (Caccamo et al., 2006; Tyler et al., 2015)

Altogether, these findings highlight the role of Vγ9/Vδ2$^+$ T cells in bridging the innate and adaptive immune response in the presence of HMB-PP$^+$ microbes by the immediate release of pro-inflammatory cytokines and expression of APC-related markers implicated in CD4$^+$ and CD8$^+$ T cells expansion.

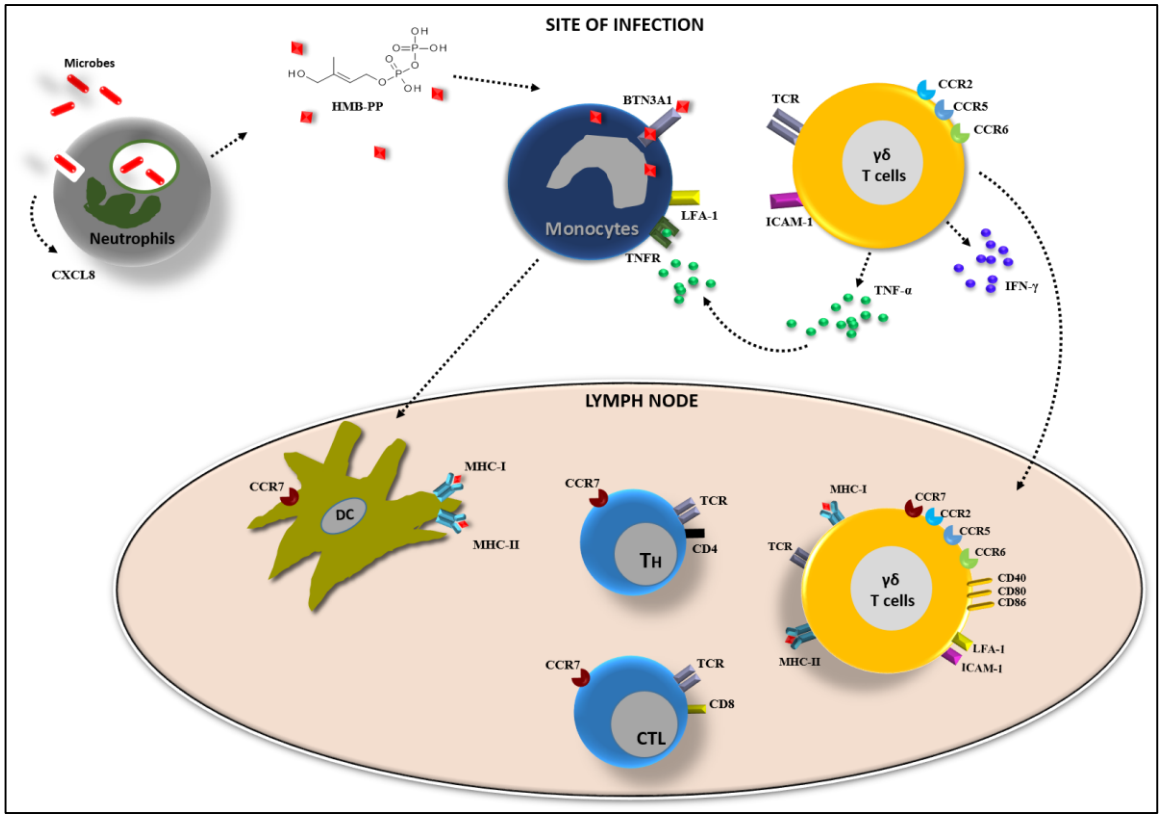

Figure 1.3. HMB-PP dependent interaction between γδ T cells, neutrophils and monocytes and migration to the lymph node in acute microbial infection.
The presence of inflammatory chemokines such as CXCL8 leads to recruitment of γδ T cells, monocytes and neutrophil to the site of infection. Neutrophils engulf invading microbes and release microbial metabolites such as HMB-PP into the microenvironment, which become visible to γδ T cells in the context of BTN3A1 and contact-dependent signals provided by monocytes or other accessory cells. Activated γδ T cells release pro-inflammatory cytokines such as TNF-α, which supports local γδ T cell expansion and acts as a monocyte and neutrophil survival signal. Crosstalk between monocytes and γδ T cells induces differentiation of γδ T cells into APCs and migration to the lymph node, where they initiate a microbe specific $CD4^+$ and $CD8^+$ T cell activation and expansion. Adapted from Moser and Eberl 2011 and Davey et al. 2011.

1.1.3.5 Human γδ T cells in metabolic disorders and inflammatory diseases

Apart from the beneficial role of Vγ9/Vδ2 T cells in antimicrobial defence, uncontrolled activation of these cells at the site of infection may result in a dysregulated inflammation process, with implications for inflammatory and metabolic diseases.

It has been shown that Vγ9/Vδ2 T cells tend to increase in the skin of patients with psoriasis and decrease in the peripheral blood (Laggner et al., 2011). This cell recruitment was associated with the expression of the chemokine receptor CCR6 on peripheral Vγ9/Vδ2 T cells. Vγ9/Vδ2 T cells in the psoriatic lesions where able to release an array of cytokines implicated in tissue inflammation such as IL-17A and TNF-α, and to induce activation of keratinocytes (Laggner et al., 2011). Similarly, another study highlighted the presence of Vγ9/Vδ2 T cells in the intestinal mucosa, which after activation were able to promote activation of αβ T cells via production of IFN-γ (McCarthy et al., 2013). Moreover, gut-homing Vγ9/Vδ2 T cells tend to increase in patients with Crohn's disease when compared with healthy controls. This may be caused by an increased gut permeability, changes in microbiota composition and translocation and activation of Vγ9/Vδ2 T cells by bacterial products (McCarthy et al. 2015).

Together with other unconventional T cells, γδ T cells are reduced in blood and duodenum of patients with coeliac disease. Among all γδ T cell subpopulations, these patients presented a predominant $V\delta1^+$ profile, highlighting the possible contribution of these cells to the gut repair via TGF-β production (Dunne et al., 2013).

1.1.4 Human MAIT cells

Another type of cells that bridge the innate and the adaptive immune system are MAIT cells. They represent up to 5% of peripheral T cells and mucosal tissue but reach up to 50% of T cell in the liver (Lopez-Sagaseta et al., 2013). Their TCR is characterized by the expression of a Vα7.2-Jα33/12/20 chain paired with a limited number of Vβ2 segments, and has the capacity to recognize ligands restricted to the MHC related-1 (MR1) molecule (Lopez-Sagaseta et al., 2013).

MAIT cells exit the thymus as "naïve" T cells and the intra thymic selection is restricted to the presence of hematopoietic MR1 positive cells excluding B cells. On the contrary, B cells and commensal flora are necessary for the selection of MAIT cells and expansion either in the peripheral blood or in the mucosal tissue (Gapin, 2009). This last study has been confirmed in patients with mutated Bruton tyrosine kinase, who have reduced levels of MAIT cells (Treiner et al., 2003)

1.1.4.1 The MR1 protein and its ligands

By conducting refolding assays followed by mass spectrometry, Kjer-Nielsen *et al.* discovered in 2012 that cell culture medium induced refolding of human MR1 *in vitro*, suggesting the presence of MR1 ligand(s) in the medium. The same results were found when MR1 was cultured with supernatant from *Salmonella typhimurium.* These findings led to the identification of two vitamin B compounds: vitamin B9 (folic acid) derivatives and vitamin B2 (riboflavin) intermediates (Kjer-Nielsen et al., 2012).

Products of folic acid such as 6-formylpterin (6-FP) were able to cause a rapid upregulation of MR1 but did not stimulate MAIT cells. On the contrary, MAIT cells were only activated in the presence of intermediates of the riboflavin pathway. This pathway includes ligands known as ribityllumazines including 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH), 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe) and, as the most potent activator, reduced 6-hydroxymethyl-8-D-ribityllumazine (rRL-6-CH_2OH). McWilliam *et al.* described that MAIT cell activation was caused by the presence of a ribityl tail in the ribityllumazine structures, which was absent in the formylpterins (McWilliam et al., 2015) (Figure 1.4 and Table 1.3).

Recently it was discovered that the most potent MAIT cell activators are in fact the pyrimidines 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-

oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU). These molecules are formed upon reaction of the precursor 5-amino-6-D-ribitylaminouracil (5-A-RU) with methylglyxal or glyoxal present in host cells and bacteria; once formed they perfectly accommodate in the MR1 binding cleft (Corbett et al., 2014; McWilliam et al., 2015) (Figure 1.5 and Table 1.3).

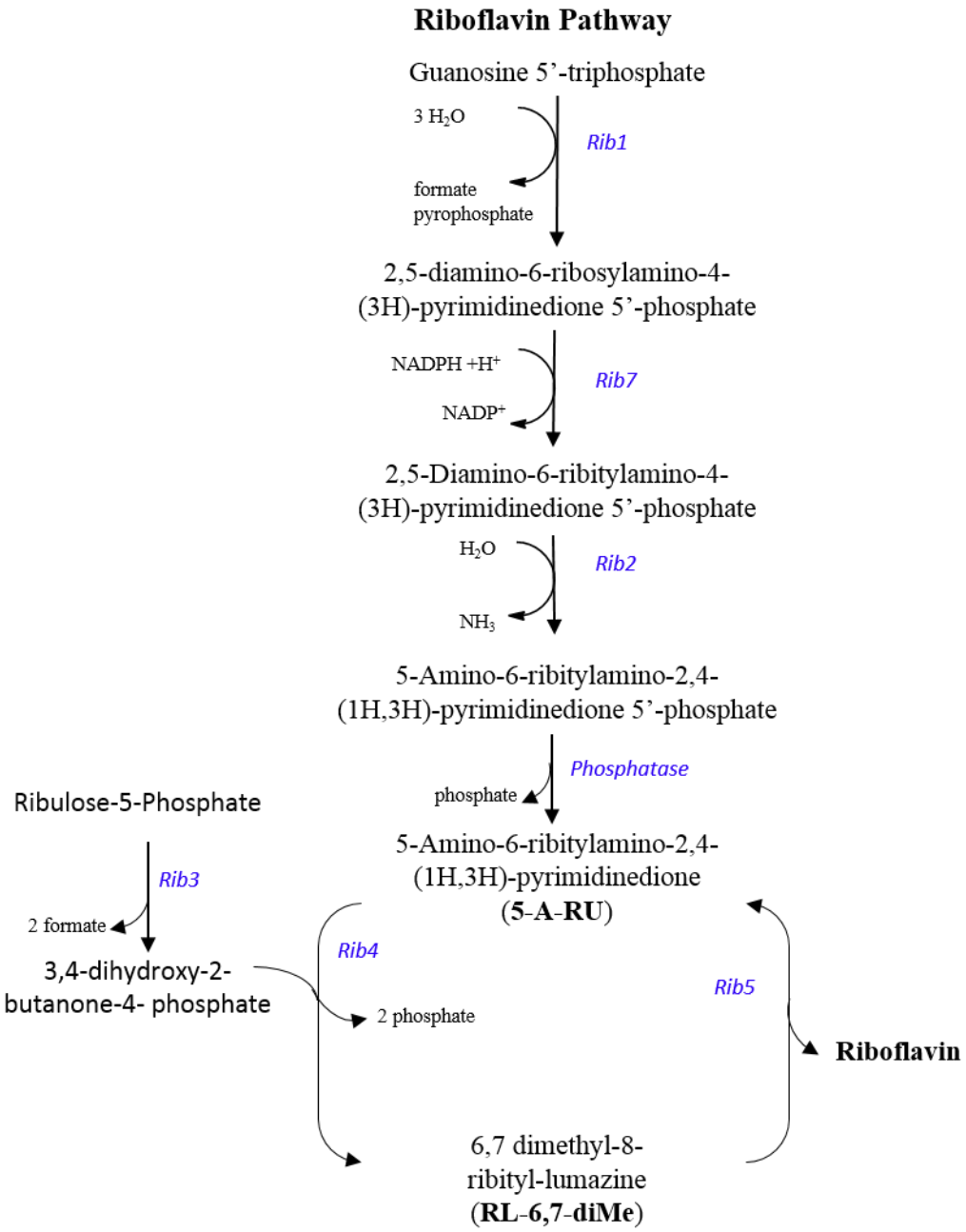

Figure 1.4 Schematic representation of the riboflavin biosynthesis pathway.
Gene names follows the nomenclature of the *Saccharomyces* Genome Database (SGD). RIB1: GTP cyclohydrolase II, RIB7: 2,5-Diamino-6- ribosylamino-4-(3H)-pyrimidinone-5'-phosphate reductase, RIB2: 2,5-Diamino-6-ribitylamino-4-(3H)-pyrimidinone-5'-phosphate deaminase, RIB3: 3,4-dihydroxy-2-butanone-4-phosphate synthase, RIB4: 6,7-dimethyl-8-ribityllumazine synthase, RIB5: riboflavin synthetase. Adapted from Marx *et al.* (Marx et al., 2008)**.**

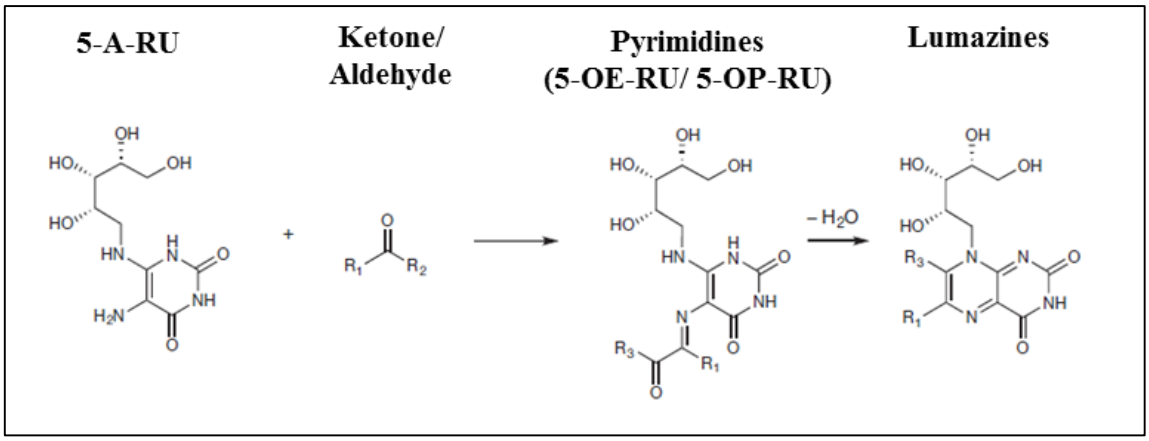

Figure 1.5. MR1 restricted antigens.
Reaction scheme for the condensation of 5-amino-ribityl uracil (5-A-RU) with small reactive aldehydes or ketones such as glyoxal or methylglyoxal to form pyrimidines and lumazines. Adapted from McWilliam *et al.* (McWilliam et al., 2015).

The MHC class I-related protein MR1 is able to process antigens by an endocytic pathway, which do not include the typical proteasome degradation and transporter associated with antigen processing (TAP). On the contrary, it requires the MHC class II chaperone invariant chain (Ii) and HLA-DM for endocytic trafficking (Huang et al., 2008b).

Recent analyses have shown that MAIT cells were able to recognise pathogens in *ex-vivo* experiments using distinct TCR repertoires both between and within individuals (Gold et al., 2014). This results suggested that MAIT cells may discriminate between pathogen derived-ligands in a clonotype-dependent manner (Gold et al., 2014).

1.1.4.2 MAIT cells: microbial reactivity

In 2010 Le Bourhis *et al.* observed that both human and mice MAIT cells were able to respond in a MR1 dependent manner to APC co-cultured in the presence of live bacteria such as Enterobacteriacae, *Staphylococcus* and *Mycobacterium* but not in the presence of *Enterococcus* and *Streptococcus* (Le Bourhis et al. 2010). In addition, this responsiveness was always directed against bacteria but not viruses (Gold et al. 2010). It was demonstrated that mice lacking of MR1 were more susceptible to *K. pneumoniae* infection, whereas wild-type mice were able to clear the infection in two days (Georgel et al. 2011). This activity was abrogated in the presence of anti-MR1 neutralising antibodies, and the activation was translated in release of IFN-γ. It was later confirmed that the activation of MAIT cells was

caused by the vitamin B2 pathway, which was present in the most detrimental pathogenic and commensal Gram negative (*E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and Gram positive (*Mycobacterium tuberculosis*, *Staphylococcus aureus* and *Clostridium difficile*) bacteria but not in *Streptococcus* and *Enterococcus* species (Table 1.2). This study emphasized the role of MAIT cells in pathogen defence (Cowley, 2014; Kjer-Nielsen et al., 2012).

Table 1.2. MAIT cell activating and non-activating microbial pathogens

Bacteria riboflavin+	**Bacteria riboflavin^{-}**
Escherichia coli	*Streptococcus spp.*
Pseudomonas aeruginosa	*Enterococcus faecalis*
Klebsiella pneumoniae	*Listeria monocytogenes*
Staphylococcus aureus	
Staphylococcus epidermidis	
Mycobacterium abscessus	
Mycobacterium tuberculosis	
Salmonella typhimurium	
Yeast riboflavin^{+}	
Candida albicans	
Candida glabrata	
Saccharomyces cerevisiae	
Adapted from Kjer- Nielsen et al. 2012.	

1.1.4.3 MAIT cells: antimicrobial functions

Recent research showed that Vα7.2^{+} T cells from neonate-derived thymuses had the capacity to react against *Mycobacterium tuberculosis* and release TNF-α *in vitro*, in the absence of prior antigenic exposure (Gold et al., 2013). Furthermore, cells derived from peripheral blood had already a memory phenotype, highlighting the capacity of MAIT cells to adapt in response to the microenvironmental signal shaping intrinsic effector functions.

MAIT cells typically express a naïve phenotype in cord blood (CD45RA^{+} CCR7^{+} CD62L^{+}) and an effector memory phenotype in adult (CD45RO^{+} CCR7^{-} CD62L^{-} CD27^{+} CD28^{+}). They are usually identified as Vα7.2^{+} and CD161^{++} T cells and are also positive for IL-18Rα and IL-12R receptors (Figure 1.6) (Dusseaux et al. 2011). CD161 is a C-type lectin-like membrane receptor which binds the ligand lectin-like transcript 1 (LLT1), with yet unclear function. A mRNA microarray study on CD161^{++} T cells described the association between the presence of this receptor and the expression of IL-12 and IL-18 receptors, highlighting the production of IFN-γ as one of the main feature of these cells (Fergusson et al., 2014).

MAIT cells are able to produce various cytokines including IL-2, IFN-γ, TNF-α and IL-17 (Figure 1.6). The latter is associated with expression of the Th17 transcriptional factor RORγt. However, the production of IL-17 is restricted to CD161^{++} MAIT cells, and stimulation of the TCR is necessary for a complete activation (Dusseaux et al., 2011). In addition, MAIT cells are also able to upregulate granzyme B, especially enhancing perforin expression, after interaction with infected epithelial cells (Le Bourhis et al., 2013; Jeffery et al., 2016; Kurioka et al., 2015).

Similar to Vγ9/Vδ2 T cells, MAIT cells express chemokine receptors implicated in tissue tropism such as CCR5, CCR6, CCR9 and CXCR6 confirming the capacity of these cells to migrate to the tissue during episodes of infections (Figure 1.6) (Dusseaux et al., 2011; Treiner et al., 2003). Indeed, neutrophils which engulf vitamin B2^{+} bacteria at the site of infection are able to induce MAIT cell activation (Davey et al., 2014). Once activated, MAIT cells, similarly to Vγ9/Vδ2 T cells, trigger survival and differentiation of neutrophil in APC highlighting the role of these cells in orchestrating inflammatory events at the site of infection.

The contribution of MAIT cells to inflammation is also due to the capacity of these cells to become activated in the presence of IL-12 and IL-8 in a MR1 independent fashion (Ussher

et al., 2014b). IL-12 and IL-18 are pro-inflammatory cytokines released by APC after microbial stimulation. Indeed, APCs infected with *E. faecalis*, a bacterial species lacking vitamin B2, are able to produce sufficient IL-12 and IL-18 to activate MAIT cells. The same type of responses is induced in the presence of TLR4 and TLR8 agonists (Ussher et al., 2014b). This means that MAIT cells can be activated in the presence of different PAMPs and can amplify inflammatory response even in the absence of antigen recognition.

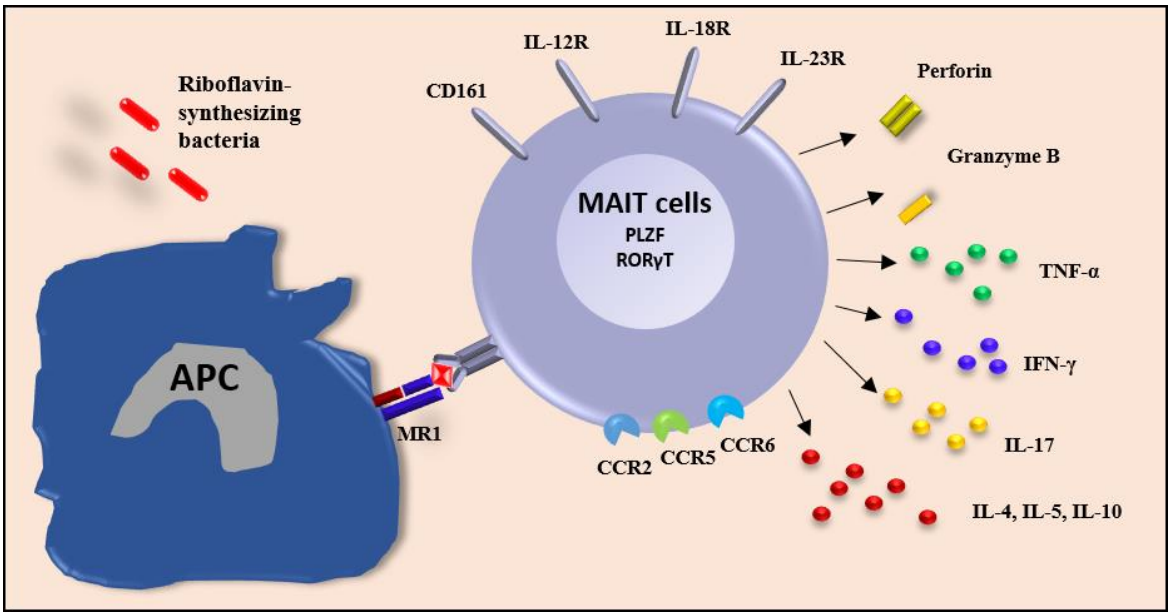

Figure 1.6. Overview of MAIT cell activation by riboflavin synthesizing bacteria.
Bacteria utilizing the riboflavin pathway enter in the APC cells. Riboflavin intermediates are loaded onto MR1 in the endoplasmic reticulum and transported to the cell surface by the Golgi apparatus. At the cell surface, MR1 and the bound ligand are presented to MAIT cells, which become activated and lyse infected cells by the release of perforin and granzyme B and pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-17. Adapted from Hinks 2016 (Hinks, 2016).

1.1.4.4 MAIT cells in infectious disease

Studies performed in the last few years have focused on the variation in frequencies of MAIT cells in the peripheral blood between healthy and infected patients. In particular during HIV infection, $CD8^{+}CD161^{++}$ MAIT cells were found to be dramatically reduced in blood of early and chronic HIV patients compared to healthy controls, and were not rescued after 2 years of antiretroviral therapy (Cosgrove et al., 2013). It was suggested that the decrease of this population was partially due to a downregulation of CD161. However, in 2014 Fernandez *et al.*, using a MR1 tetramer, showed that MAIT cells were absent from the $V\alpha7.2^{+}$ $CD161^{-}$ population (Fernandez et al., 2014). One of the major implications of HIV

infection is the depletion of intestinal $CD4^+$ T cells and the destruction of the intestinal immune system. It is possible that the loss of the gut integrity and translocation of microbial products into the blood stream may induce migration of MAIT cells to the gut followed by depletion associated with apoptosis caused by persistent cell activation. All these mechanisms may explain the progressive loss of MAIT cells in the periphery and the high susceptibility to *M. tuberculosis* in patients with HIV infection (Cosgrove et al., 2013; Gold et al., 2015; Sonnenberg et al., 2005).

Alterations in MAIT cells subsets have also been described in patients with severe sepsis. In this regard, Grimaldi *et al*. observed that peripheral MAIT cells were decreased in early septic patients with severe chronic infection and in critically ill patients (Grimaldi et al., 2014). Of note, MAIT frequencies were less decreased in patients with infections caused by streptococci, suggesting a ligand-specific recruitment to the site of infection in response to pathogens utilizing the riboflavin pathway (Szabo et al., 2015).

1.1.4.5 MAIT cells in metabolic disorder and inflammatory disease

MAIT cells appear numerically and functionally impaired in in metabolic disorder and inflammatory disease. Type 2 diabetes (T2D) and obesity are associated with low grade inflammation and alterations in the gut microbiota. Indeed, it was observed that patients with these disorders had lower levels of MAIT cells in the periphery compared to the healthy control (Apostolopoulos et al., 2016; Magalhaes et al., 2015). In obese patients, MAIT cell numbers were higher in the adipose tissue than in blood. Moreover, in both patients group MAIT displayed a Th17-like inflammatory profile. This suggests that different metabolic and inflammatory mediators released in obesity and T2D patients may affect MAIT cell activation. Similarly to this study Harms *et al.* showed the presence of terminally differentiated $CD27^-$ MAIT cells in a young cohort of type 1 diabetic patients (T1D). It was suggested that this phenotype is induced by an increase level of IL-18 in the same cohort of patients (Harms et al., 2015).

In an inflammatory disorder such as asthma MAIT cell levels were found to be reduced in blood and bronchial biopsies. Indeed, the deficiency of these cells was associated with an increased risk of infection with riboflavin-synthesizing pathogen such as *Mycobacterium tuberculosis* (Hinks, 2016). Another study described the changes in frequency of MAIT

cells in inflammatory bowel disease. In this study MAIT cells were found to be decreased in blood and increased in the inflamed tissue and were associated with a Th17 profile (Serriari et al., 2014). Similarly, MAIT cells were found in large proportions in patients with psoriatic skin (Johnston and Gudjonsson, 2014; Teunissen et al., 2014), in the synovial fluid of rheumatoid arthritis patients (Cho et al., 2014) and were reduced in peripheral blood of patient with cystic fibrosis (Smith et al., 2014) and systemic lupus (Cho et al., 2014).

1.1.5 Other pathogen-specific unconventional T cells: NK T cells and GEM T cells.

NKT cells are unconventional T αβ cells with a semi-invariant TCR restricted by CD1d. Two types of NKT cells have been described. Invariant iVα14^{+}NKT cells, usually called type I NKT cells, express the Vα14-Jα18 chain in mice and Vα24-Jα18 in humans. Type II NKT cells express a diverse non-Vα14^{+} TCRs (Rossjohn et al., 2012). This second population was discovered observing that NK cells function was detectable in mice lacking type I NKT cells but not in mice lacking of CD1d molecule (Godfrey and Kronenberg, 2004). NKT cells represent approximately 0.1% of T cells in human blood and are strongly activated in the presence of the marine sponge-derived glycolipid α-galactosylceramide (α-GalCer), which is generally used for immunotherapeutic applications. Type II NKT cells do not recognize α-GalCer but recognize the myelin-derived glycolipid sulfatide (Godfrey et al., 2015) (Table 1.3).

NKT cells respond promptly upon TCR engagement, releasing Th1 and Th2 type cytokines. During the late phase of activation, they tend to produce more IFN-γ and TNF-α (Gumperz et al., 2002). Like MAIT cells, NKT cell numbers are reduced in inflamed tissues in diseases such as rheumatoid arthritis, lupus and inflammatory bowel disease (Balato et al., 2009). They are also implicated in microbial defence and inhibition of autoimmune disease (Gumperz and Brenner, 2001; Taniguchi et al., 2003).

A third population of unconventional T cells that expresses a highly restricted TCR α-chain are germline-encoded, mycolyl-reactive (GEM) T cells. They are CD4^{+} and express the same Vα7.2 segment as MAIT cells but rearranged to the α chain joining region 9 (TRAJ9), which differs from the MAIT J_{α}. Notably, GEM T cells react against lipids presented by CD1b molecules. This interaction leads to the release of antimicrobial cytokines such as TNF-α and IFN-γ (Kronenberg and Zajonc, 2013; Van Rhijn and Moody, 2015) (Table 1.3).

Mycolic acid (MA) is the main component of the *M. tuberculosis* cell wall used by this pathogen to resist to the immune cells attack. A study done on MA reactive T cells demonstrated that TB patients, but not healthy donors, were more responsive to this antigen. The response was measured as release of IFN-γ (Montamat-Sicotte et al., 2011) and was inhibited after blocking of CD1b with monoclonal antibody. The authors also observed that IFN-γ–secreting MA-specific T cells were undetectable in the blood of some patients but were present in the lung of TB patient. This observation suggests a preferential recruitment of these cells to the site of infection.

To study lipid-reactive T cells in the context of TB, Van Rhijn *et al.* generated a CD1b tetramer loaded with glucose monomycolate (GMM), which is produced *in vivo* during TB infection and activates CD1b reactive T cells. The clone sorted from the CD1b tetramer positive cells comprised 0.01% of all circulating T cells. This clone was able to produce a large amounts of IFN-γ and IL-2 after treatment with GMM but less so after MA treatment. This result suggested that GMM is the prevalent antigen triggering activation of GEM T cells (Van Rhijn et al. 2013). Although at low frequency, compared with others unconventional T cells, CD1b tetramers also identified GEM T cells in blood of healthy donors. These cells had low CD69 expression and lacked of the transcriptional factor PLZF responsible for the effector phenotype, suggesting that GEM T cells are more dependent on infection to drive expansion. A second population of GMM-specific T cells with lower avidity for CD1b tetramer has been identified recently. This population expresses a TCR with a preference of the TRAV17 and TRAV4-1 genes (Van Rhijn et al., 2014).

Altogether, these findings emphasize the importance of GEM T cells in the diagnosis of tuberculosis and, given the non-polymorphic nature of CD1 protein, GEM T cells could be an important target for vaccine development (Godfrey et al., 2015; Kronenberg and Zajonc, 2013; Liuzzi et al., 2015).

Table 1.3 Non polymorphic targets of human T cells

Target	TCR Type	Natural antigens	Synthetic agonists
CD1a	αβ	Many lipids	Fatty acid, squalene, wax esters, dideoxymycobactin
CD1b	αβ	Many lipids	Mycolic acid, sulfoglycolipid, phosphatidylinositol mannoside, glycerol monomycolate
	GEM T cells	Glucose monomycolate	Glucose monomycolate
	LDN5-like	Glucose monomycolate	Glucose monomycolate
CD1c	αβ	Many lipids	Phosphomycoketide, mannosyl-phosphomycoketide, methyl-lysophosphatidic acid
CD1c	γδ	Unknown	None
CD1d	αβ		
	Type I NKT	α-GalCer and other α-linked glycolipids	Many a-GalCer analogues
	Type II NKT	Many lipids	Lysophosphatidylcholine, PPBF
CD1d	γδ		
	$TRDV1^+$		
	$TRDV3^+$		
MR1	αβ	Riboflavin derivatives	5-OP-RU
	MAIT cells		5-OE-RU
			RL-6-Me7-OH
			RL-6,7-diMe
Butyrophilin	γδ		
	$TRDV2^+$ $TRGV9^+$	HMB-PP, IPP	Synthetic phosphoantigens

References: (Chien et al., 2014; Van Rhijn et al., 2015; Rossjohn et al., 2012; Sullivan et al., 2008; Vantourout and Hayday, 2013)

Adapted from Godfrey et al.

1.2 Peritoneal dialysis

1.2.1 General overview

Renal replacement therapies are employed in patients with end-stage renal disease (ESRD) to replace the normal filtration functions of the kidneys. The severity of kidney failure is divided into different stages (1 to 5) based on the glomerular filtration rate (GFR). Kidney failure is defined as GFR lower than 30 ml/min per 1.73 m^2 (stage 4). The most common reason for renal failure in the UK is diabetic renal disease followed by glomerulonephritis, pyelonephritis, hypertension and renal vascular disease (UK Renal Registry 17th Annual Report, 2015) (Table 1.4).

Table 1.4 Primary renal diagnosis RRT incidence rate (2013) per million population

Diagnosis	England	N Ireland	Scotland	Wales	UK
Diabetes	25.2	20.2	22.7	31.8	25.1
Glomerulonephritis	14.1	10.4	15.6	17.8	14.3
Pyelonephritis	6.6	13.1	8.4	5.5	6.9
Hypertension	8.3	5.5	3.0	3.6	7.5
Polycystic kidney	7.4	9.8	7.7	7.8	7.5
Renal vascular disease	4.9	7.1	6.2	10.7	5.3
Other	18.7	15.8	16.5	13.6	18.1
Uncertain aetiology	13.9	14.8	13.9	22.1	14.4
Data not available	12.3	1.6	0.2	1.9	10.4
All	**111**	**98**	**94**	**115**	**110**

Patients with kidney failure will receive a renal replacement treatment (RRT) such as renal transplantation, peritoneal dialysis (PD) and haemodialysis (HD). However, most of the time, the lack of available organ donors in addition to comorbidity conditions in the patient means that PD and HD are the only available treatments.

The 2013 UK renal registry contains records of patients who have been at least 90 days on RRT. Among them 66.9% were on HD, 19.0% on PD and only 8.3% on transplant (UK Renal Registry 17th Annual Report, 2015). Within the UK, Wales shows the highest incidence of RRT (Figure 1.7).

The patient choice between PD and HD depends on the patient lifestyle, geographic location (near or far from the dialysis centre), and patient and family employment. Patients on PD are in fact more likely to rate their medical care highly than patients on HD (Rubin et al., 2004). The reason is because PD offers a better quality of life, with higher freedom for the patients than HD, and the possibility of home therapy with close medical care. Indeed, PD is more cost effective and is characterised by better survival rates in the first two years of dialysis treatment compared to HD (Chaudhary et al., 2011). Furthermore, complications related to infection appear to be higher in HD than in PD (Chaudhary et al., 2011; Sinnakirouchenan and Holley, 2011).

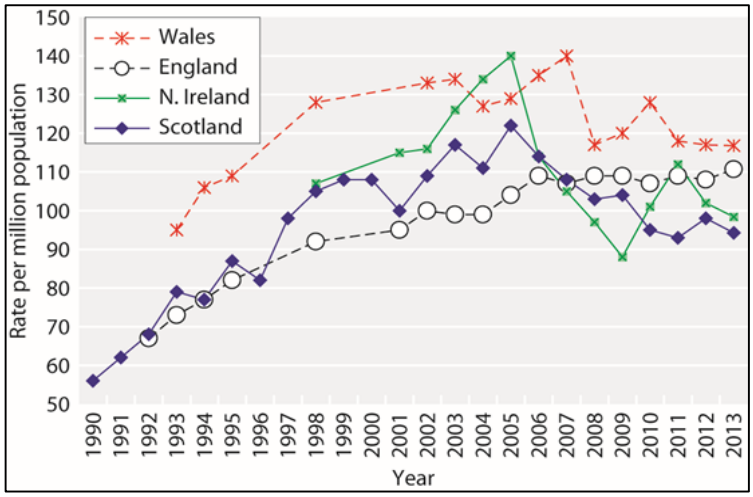

Figure 1.7. RRT incidence in the UK 1990-2013.
Taken from UK Renal Registry 17th annual report (UK Renal Registry 17th Annual Report, 2015)**.**

1.2.2 Peritoneal dialysis techniques

The preservation of three main components of PD therapy are fundamental for optimal PD treatment. These are: 1) PD catheter, 2) PD solutions and 3) the peritoneal membrane with blood vessels associated to it. During PD a dialysis solution (dialysate) is introduced into the patient's peritoneal cavity by a catheter inserted into the abdominal cavity. The peritoneal membrane is used as dialyser to remove waste products and fluids by a diffusion and ultrafiltration process. The dialysis solutions contains: *i*) electrolytes such as sodium, potassium, magnesium and calcium; *ii*) a buffer (e.g. lactate and /or bicarbonate) and *iii*) an osmotic agent that makes the solution hyperosmolar. Typically the latest can be a substance with high molecular weight such as icodextrin or with low molecular weight such as glucose and amino acids.

During the dwell time, the transport of solute across the peritoneum can be described by three processes, namely diffusion, ultrafiltration and convection processes. Diffusion allows the transport of solute as a consequence of the concentration gradient from the more concentrated area (capillary blood) to a less concentrated area (PD solution). This brings solutes such as creatinine and urea to flow from the blood to the PD fluid and osmotic agents such as glucose to flow in the opposite direction. This process depends on the peritoneal surface area and on the concentration gradient, which will affect the dwell time.

Different models have been proposed to describe the transport of solute from the capillary to the peritoneal cavity. The most recent model is the "three pore model" (Rippe et al., 2004), which assumes that the transport across the membrane is due to the presence of pores of different dimensions. Large pores (100-200 Å), clefts between endothelial cells, allow the passage of macromolecules, whereas small pores (40-60 Å), that may be present in the endothelium, allow the transport of small solute and water. The latter can also pass through ultra-small pores (aquaporins, 2.3-15 Å) (Rippe et al., 2004).

Ultrafiltration is accomplished by an osmotic pressure gradient between the capillary and the PD solutions. This is achieved making the dialysate hypertonic adding high concentration of glucose or icodextrin depending on the dwell time. During this process small solutes are also transported by bulk flow of water (convective motion) (Teitelbaum and Burkart, 2003).

1.2.3 Peritoneal dialysis modality

Depending on the peritoneal membrane transport status and performance, patients can be treated with two types of PD: continuous ambulatory peritoneal dialysis (CAPD) and automated peritoneal dialysis (APD). In CAPD, patients perform three manual short exchanges of dialysate during the day and two exchanges during the night, each with 2 to 2.5 litres of dialysate solution. In APD, the patients program a dialysis cycler to perform multiple automated exchanges overnight, whereas the last "fill" of fluid is kept in the abdomen during the day (Teitelbaum and Burkart, 2003).

1.2.4 Technique failure in PD

There are different reasons that force PD patients to discontinue the therapy and these are grouped into infection and non-infection related reasons.

1.2.4.1 Infectious complications

Although PD patients have a better survival prospective than patient on HD in the first 2 years of therapy (Chaudhary et al., 2011), technique failure caused by episodes of peritonitis is still high throughout the course of the therapy. Peritonitis is a common cause for catheter removal, transfer to HD, ultrafiltration failure and death in PD patients. Furthermore, there is a wide variation in peritonitis rates among treatment centres: from a very low rate of episodes per year in Taiwan (n=0.06) to a very high rate of episodes in Israel (n=1.66 per year) (Piraino et al., 2011). UK centres are somewhat in the middle (n=0.14 episodes to 1.0 episodes per year) (Davenport, 2009) but still far from the rate that should be achieved according to the guidelines by the International Society for Peritoneal Dialysis (ISPD) (< 0.24 per year). The differences among centres depend to a large extent on patient training and different protocols to prevent infections (Piraino et al., 2011).

1.2.4.1.1 Diagnosis

In most cases, the first signs of peritonitis reported by a patient are cloudy effluents and abdominal pain. If the count of white blood cells (WBC) present in the effluent is greater than 100/µl, with more than half of the cells being polymorphonuclear cells, an episode of peritonitis is clinically confirmed (Teitelbaum and Burkart, 2003). Afterwards, a standard culture technique is used to identify the responsible organism for an appropriate antibiotic selection.

Normally the culture results take up to 3 days, however if there are no results after 72 hours the culture is defined as negative (Fahim et al., 2010a). This can be caused by different reasons, such as *i*) inappropriate culture methods (e.g. suboptimal sample collection and processing); *ii*) recent antibiotic exposure; *iii*) presence of fastidious organisms such as mycobacteria; *iv*) chemical agents used during the treatment; or *v*) by effluent eosinophilia. ISPD recommends that culture-negative episodes should not exceed more than 20% of all episodes, but a rate higher than this percentage has been reported among 12% of the units in Australia (Fahim et al., 2010a).

1.2.4.1.2 Microbiology

Single Gram$^+$ bacteria are responsible for almost 50% of PD associated infections. These are followed by single Gram$^-$ infection (up to 25% of all cases), fungal and polymicrobial infections. Among Gram$^+$ bacteria, infections caused by the skin colonizer coagulase negative *Staphylococcus* (CoNS, 30% of all peritonitis episodes) are the most frequent ones, mainly caused by touch contamination at the time of catheter insertion. This is a clear indication of a problem with the training methods adopted by the PD nurses and consequently by the patients (Ghali et al., 2011). Touch contamination together with catheter infection can also lead to *Staphylococcus aureus* infection, whereas *Streptococcus* and *Enterococcus* infections are typically associated with contamination from the gastrointestinal tract and tunnel infection. A less common Gram$^+$ infection is the one caused by *Corynebacterium*, which like CoNS, is difficult to identify as pathogen given its contribution to the normal skin microbiota (Li et al., 2010; Piraino et al., 2011).

The most frequent Gram$^-$ bacterium in PD associated peritonitis is *Pseudomonas aeruginosa*, which like *S. aureus* is linked to high rates of catheter removal, high probability of hospitalizations and transfer to HD (Siva et al., 2009). This is followed by infections

caused by non-*Pseudomonas* Gram$^-$ bacteria such as *E. coli, Klebsiella* and *Proteus*. Gram$^-$ bacteria may arise from bowel sources such as constipation, colitis or transmural migration, and are able to form biofilms around the catheter. The same biofilm composition can allow bacteria to acquire resistance to antibiotics (Dasgupta and Larabie, 2001; Jarvis et al., 2010; Szeto et al., 2006). The organisms found in polymicrobial infections are mainly Gram$^+$ CoNS and *S. aureus* and Gram$^-$ *E. coli* and *Pseudomonas* (Ghali et al., 2011).

1.2.4.1.3 Treatment of peritonitis

When symptoms of peritonitis are confirmed, empiric antimicrobial therapies to cover both Gram$^+$ and Gram$^-$ infections are adopted. Gram$^+$ bacteria are targeted with vancomycin and cephalosporins, whereas Gram$^-$ bacteria are targeted with aminoglycosides or third generation cephalosporins. The choice of the right antibiotic depends on the patient and unit history for the organisms considered. Usually antibiotics are administered intraperitoneally to the dwell bag for at least 6 hours and the treatment should be continued for 2 weeks (Li et al., 2010). This can be revaluated as soon as the culture results are obtained. Three weeks of antibiotics treatment are suggested for infections caused by *S. aureus*, Gram$^-$ and enterococcal infections (Li et al., 2010). The London UK audit registered a high cure rate for CoNS (77%) and culture negative results (77%) followed by *S. aureus* infections (47%). On the contrary, cure rates for Gram$^-$ infections are as low as 20-60% (Davenport, 2009). This means that patients presenting with peritonitis caused by Gram bacteria are more likely to discontinue PD (Cho and Johnson, 2014; Jarvis et al., 2010).

Indeed, in many cases the traditional diagnostic methods are not specific or rapid enough to target the cause of infection. As a result, patients are exposed to improper and unnecessary antibiotic treatment contributing for example to the spread of multidrug resistance (Arias and Murray, 2009). Moreover, if the effluent fails to clear within 5 days from the commencement of antibiotic treatment the catheter should be removed to avoid peritoneal membrane failure; this type of peritonitis is called **refractory**. Catheter removal is also considered in case of **relapsing** peritonitis, which refers to a new episodes of peritonitis that occurs within 4 weeks of completion of therapy with a recent episode with the same organism. **Repeating** peritonitis is instead defined as an infection that occurs with the same organism but after more than 4 weeks from the completion of the therapy; while **recurrent** peritonitis is an episode that occurs within 4 weeks but with a different organism (Burke et al., 2011; Piraino et al., 2011).

Collectively, this means that there is an urgent need of novel methods for early diagnosis of peritonitis to prevent technique failure. It is crucial to treat episodes of peritonitis on time to avoid worse outcomes. In a recent study, Szeto et al. reported how to predict relapsing or repeat peritonitis by measuring DNA fragments in PD effluent of patients with peritonitis (Szeto et al., 2013). In addition, in our laboratory, Lin et al. described how to use local cellular and humoral responses (immune fingerprints) evoked by the specific interaction of the immune system with microbial pathogens to discriminate between $Gram^{-}$, $Gram^{+}$ and culture negative episodes of peritonitis (Lin et al., 2013).

1.2.4.2 Non-infectious complications

Besides microbial infections, there are different non-infectious complications that may lead to cessation of PD, all of which have in common functional changes in the peritoneum.

- **Peritoneal membranes changes**: long-term therapy exposition to a non-physiological PD fluid can be harmful to the peritoneal membrane. Glucose, used as osmotic agent, together with low pH and presence of lactate can lead to modifications of the peritoneal membrane with vascular proliferation and fibrosis.

- **Ultrafiltration failure**: it increases with time on PD; this is caused by an increased transport of small solutes followed by gradient dissipation during the dwell time. If an osmotic agent at slow absorption fails to solve this problem, patients are forced to move to HD.

- **Encapsulating peritoneal sclerosis (EPS)**: this is a complication followed by chronic abdominal inflammation resulting in bowel encapsulation. Long time on PD seems to be the main risk of factor for this complication (Lai and Leung, 2010)

- **Other reasons**: PD can be also be discontinued for mechanical (e.g. catheter obstruction) or metabolic abnormalities (e.g. hypoalbuminaemia, hyperglycaemia and weight gain) (Teitelbaum and Burkart, 2003).

1.2.5 Chronic inflammation in PD

1.2.5.1 Anatomy of the peritoneal cavity

The peritoneum is a large serous complex membrane consisting of parietal and visceral layers. The parietal peritoneum covers the inner surface of the abdominal and pelvic walls including the diaphragm, whereas the visceral peritoneum covers the gastrointestinal tract,

liver and spleen and forms the omentum and visceral mesentery (Blackburn and Stanton, 2014). The visceral peritoneum is a reflection of the parietal peritoneum on the viscera. This reflection allows the peritoneum to form potential spaces. The space between the parietal peritoneum and the visceral peritoneum forms the peritoneal cavity, where the PD fluid is introduced. However, during PD it is the parietal peritoneum that performs most of peritoneal transport, whereas only 30% of the visceral peritoneum is in contact with the fluid (Flessner, 1996).

The peritoneal membrane is a single layer of flat squamous epithelial cells called mesothelial cells, which are located on a thin basement membrane. Underneath this membrane there is a thick layer of connective tissue composed of fibres of collagen, scattered fibroblasts and blood vessels (Figure 1.8).

Between the parietal and visceral layer there is typically 50-100 ml of fluid, necessary for the lubrication of the peritoneal surfaces. This fluid contains water, electrolytes, solutes, proteins and cells. These last are mainly mesothelial cells followed by macrophages, mast cells and fibroblasts. Under normal conditions there is a small net filtration from the capillary blood to the peritoneal cavity. The mesothelium cells layer is interrupted by intercellular gaps called stomas, which are connected with mediastinal lymph nodes where foreign antigens can be ingested by macrophages and presented to T and B lymphocytes in the lymphatic nodes. The integrity of these components is fundamental for successful PD therapy (Teitelbaum and Burkart, 2003).

1.2.5.2 The role of leukocytes during PD associated inflammation

In a healthy adult, the peritoneal fluid contains up to 90% monocytes/macrophages, 5% lymphocytes, and 5% of neutrophils. This scenario changes extensively during episodes of peritonitis and differs between culture negative and culture positive episodes of peritonitis (Glik and Douvdevani, 2006; Lin et al., 2013).

In the first phase of the inflammation, bacterial products are recognized by resident peritoneal cells such as mesothelial cells, macrophages and DCs. After activation these cells release different vasoactive substances such as prostaglandins and histamines and chemokines such as CXCL8. This results in vasodilation and blood vessels permeability eliciting the infiltration of neutrophil (polymorphonuclear cells) at the site of infection (Figure 1.8). Indeed, at day 1 of the infection neutrophils can reach up to 95% of all

peritoneal cells (Glik and Douvdevani, 2006; Lai and Leung, 2010). Inflammatory mediators such as TNF-α, TGF-β, IL-1β, IL-6, IL-10, IL-22 and CXCL8 increase during episodes of acute peritonitis (Lin et al., 2013). Activated neutrophils in turn release granules with toxic content such as reactive oxygen and nitrogen species. They are also able to release soluble IL-6 receptor (sIL6-R), which together with IL-6 is responsible for the switch between polymorphonuclear and mononuclear cell recruitment to the site of infection. Indeed, IL-6 induce the release of CC chemokines such as CCL5, CCL2 and CXCL10 at the site of infection (Hurst et al., 2001; Jones, 2005) (Figure 1.8).

At the site of infection, DCs, capture the antigen and migrate to the lymph nodes. Once at the lymph nodes, DCs are able to induce a $CD4^+$ and $CD8^+$ specific T cells response, which then differentiate, proliferate and migrate to the site of infection (Figure 1.8). $CD4^+$ T cells are able to differentiate in different subsets (*e.g.* Th1, Th2, Th17, Treg and Th22 lineage) characterized by the release of different inflammatory mediators. A study on stable CAPD patients showed that these patients had high frequency of Th2 cells compared with control donors (Enríquez et al., 2002; Yokoyama et al., 2001). However, this pattern changes during episodes of peritonitis where there is an increase of Th1 cells and also Th17 cells (Wang and Lin, 2005). Moreover, it has been shown that the balance of the axis Th17-Treg is fundamental to avoid peritoneal damage. In effect, the presence of only TGF-β pushes the balance toward Treg activation by the induction of Foxp3 (Liappas et al., 2015). Instead, the simultaneous presence of inflammatory mediator such as IL-6 can tilt the balance toward activation of Th17 cells, which are the main cell type secreting IL-17 and contribute to the membrane damage (Liappas et al., 2015; Rodrigues-Díez et al., 2014). Moreover, in a mouse model with repeated episodes of infection, it has been demonstrated that IL-6 was responsible for the induction of peritoneal fibrosis enhancing IFN-γ secreting Th1 cells. This resulted in an increased production of matrix metalloproteinases in mesothelial cells, and the effect was reversed when STAT1 and IFN-γ signals were impaired (Fielding et al., 2014). On the contrary, other studies have shown that Tregs cells have a protective effect on the peritoneal membrane, when mice were treated with agonists able to activate the peroxisome proliferator-activated receptor-γ (PPAR-γ) (Sandoval et al., 2010).

Lin *et al*. showed that the peritoneal cell composition was markedly different between $Gram^-$ and $Gram^+$ infections. Peritoneal leukocytes in patients infected with $Gram^-$ bacteria were dominated by a large proportion of neutrophils and high levels of IL-β, IL-10 and TNF-α. While these patients had only a relatively low proportion of T cells, they showed an

enrichment of peritoneal γδ T cells (Lin et al., 2013). On the contrary, Gram$^+$ infections were dominated by elevated numbers of CD4$^+$ and CD8$^+$ T cells and peritoneal levels of CXCL10, IFN-γ and IL-22. This suggests that the specific immune response is shaped by the type of pathogen present at the site of the infection.

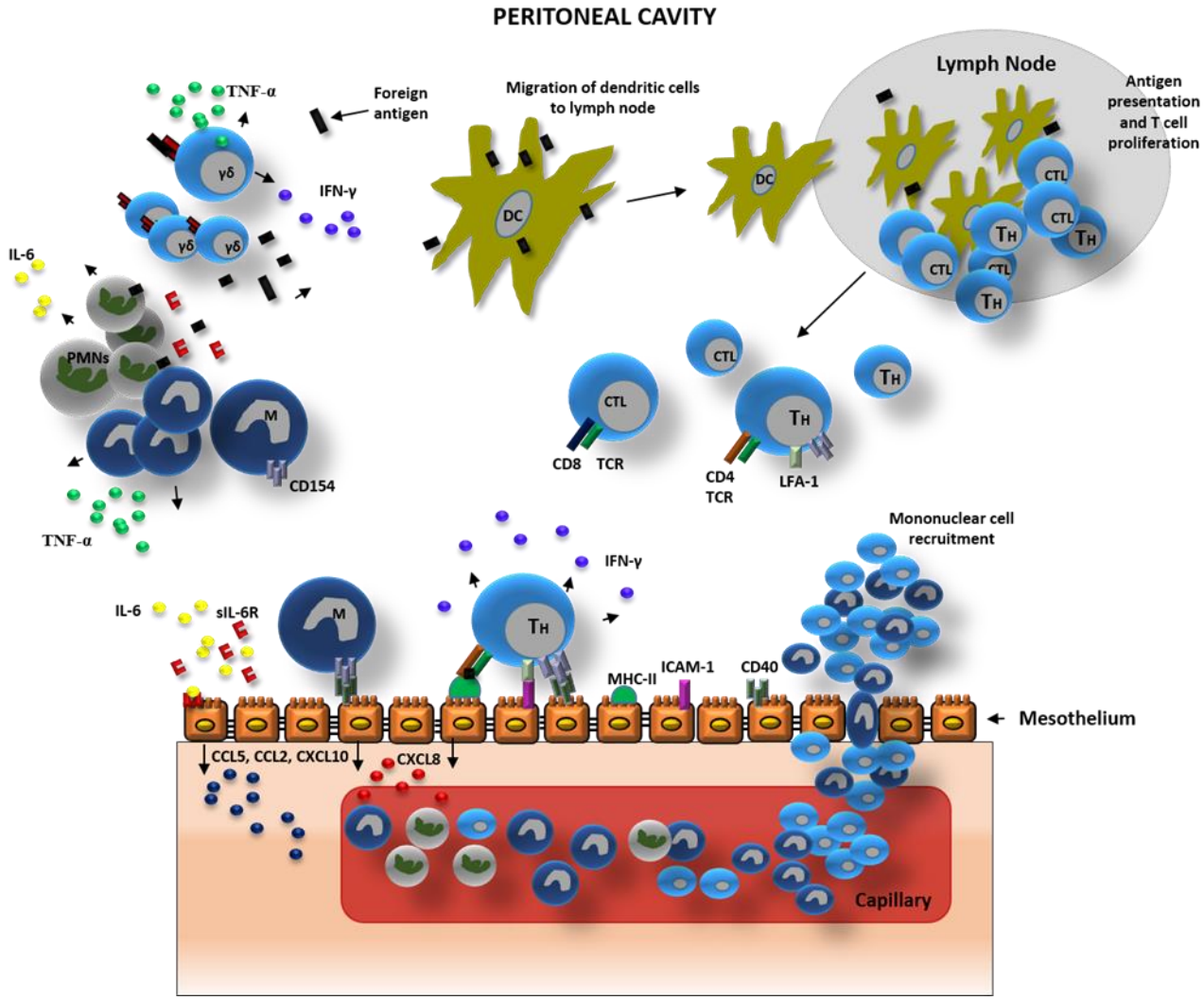

Figure 1.8. Leukocyte activation in the peritoneum.
The initial reaction against pathogens is induced by immune cells already present at the site of infection like peritoneal monocytes/macrophages (M) and recruited cells such as polymorphonucelar cells (PMNs) and γδ T cells. Activated PMNs release the soluble form of interleukin-6 receptor (sIL-6R), which together with IFN-γ secreted by activated γδ T cells and IL-6 released by macrophages, induce peritoneal mesothelial cell stimulation and activation. Once activated they release chemokines responsible of mononuclear cells migration to the site of infection. IFN-γ is also important for the expression of MHC class II on both macrophages and mesothelial cells. Dendritic cells (DCs) take up antigens and migrate to the lymph node for antigen presentation to T cells. Antigen-specific T helper (Th) cells and cytotoxic T lymphocytes (CTLs) proliferate and migrate from lymph nodes to the peritoneum. Th cells may also become activated locally by antigen presented MHC class II molecules expressed on mesothelial cells. Adapted from Glik and Douvdevani 2006 (Glik and Douvdevani, 2006).

1.2.5.3 Human peritoneal mesothelial cells

Human peritoneal mesothelial cells (HPMC) are the first cells to encounter the pathogen during episodes of infection. Normally, they play an essential role in fluid and solute transport through the peritoneal membrane. However, many studies in the last few years have focused on the role of these cells in peritoneal inflammation and tissue repair. Given the acquisition of tight cell-cell contact including tight junctions, gap junctions, adherent junctions and desmosomes, HPMC have a clearly defined apical basal polarity. The integrity of these adherent cell proteins is fundamental for the formation/integrity of a continuous mesothelial monolayer.

HPMC are endowed with microvilli and cilia essential for the movement of solutes across the peritoneal cavity and protect the peritoneal membrane from damage (Mutsaers, 2002). Given the location of these cells between the blood vessels and the peritoneal cavity, they are the first cells to be implicated in the peritoneal immune defence. During the first phase of the innate response, $Gram^+$ and $Gram^-$ bacteria can directly activate HPMCs via TLR2 and TLR5 receptors present on their cell surface (Colmont et al., 2011; Visser et al., 1996). The interaction with these receptors triggers the release of pro-inflammatory cytokines, such as IL-6, IL-1α and IL-1β as well as chemokines such as CXCL8, CXCL10, CCL2 and CCL5. Resident macrophages can also contribute to the HPMC activation by releasing TNF-α and IL-1β, which together with IFN-γ elicits a positive feedback on HPMC chemokines production (Betjes et al., 1993; Park et al., 2003; Topley et al., 1993a; Visser et al., 1998).

HPMC participate in the inflammation also by the expression of adhesion molecules such as ICAM-1, which are necessary for leukocyte migration across the mesothelium. Hausmann *et al*. showed that HPMC were not only able to upregulate the expression of MHC class II molecule in the presence of IFN-γ but were also able to present *S. aureus* α-toxins to $CD4^+$ T cells releasing the T cell growth factor IL-15 (Hausmann et al., 2000). The ligation and the presence of a functional CD40 receptor on mesothelial cells is fundamental for T cell activation (Basok et al., 2001). This results in a full activation of $CD4^+$ T cells not only by interaction with the TCR but also binding the leukocytes associated molecule LFA-1 and the adhesion molecule ICAM-1 on HPMC.

1.2.5.4 Human peritoneal fibroblasts

Human peritoneal fibroblasts (HPFB) are another component of the peritoneal cell compositions implicated in PD associated inflammation. They are scattered in the connective tissue and are mainly responsible for extracellular matrix and collagen production. HPFB are characterized by a spindle-shape conformation and by the expression of the fibroblast-specific protein-1 (FSP-1). In the presence of inflammatory mediators HPFB can adopt an activated phenotype characterized by the expression of α-SMA, acquiring an increased proliferation and motility. In this case they are called "myofibroblasts". In addition, Kawka *et al*. observed that HPFB can be divided into two subsets according to the expression of CD90 (Thy-1), which is associated with a myofibroblastic phenotype (Witowski et al., 2015). In non-PD patients HPFB express the marker CD34, normally associated with bone marrow stem cells. The function of these markers is still unknown but is reduced in patients on long term PD and during peritoneal fibrosis (Jiménez-Heffernan et al., 2004).

Like mesothelial cells, peritoneal fibroblasts actively participate in the peritoneal inflammation. It has been shown that they react to the presence of activated macrophages by releasing the chemokines CCL2 and CXCL8. This effect was found to be mainly induced by the presence of IL-1β (Witowski Janusz and Jörres Achim, 2006; Witowski et al., 2001). IL-1β is also able to induce the secretion of colony-stimulating factor (G-CSF) by HPFB, which permits neutrophil mobilization and survival (Witowski et al., 2009). Moreover, after treatment with IL-1β and TNF-α, peritoneal fibroblasts release CCL5. Similar to mesothelial cells, this chemokine is synergistically upregulated in the presence of IFN-γ followed by the expression of CD40 receptor (Brouty-Boyé et al., 2000; Kawka et al., 2014). This means that HPFB, together with HPMC, have a role in antigen presentation and amplification of inflammation in PD.

1.2.5.5 Epithelial to mesenchymal transition

Long term PD exposure of peritoneal membrane to PDE and recurrent episodes of peritonitis can cause chronic peritoneal inflammation leading to peritoneal fibrosis, angiogenesis and vasculopathy. Myofibroblasts together with activated leukocytes recruited or present at the site of the infection are the main cell type responsible for this structural alteration. However, new myofibroblasts can be generated by differentiation of mesothelial cells through a process called epithelial to mesenchymal transition (EMT). During this process, mesothelial cells lose their intercellular junctions, mainly by downregulation of the adhesion molecules E-cadherin, claudins, occludins and zona occludens-1 (ZO-1). Consequently, they lose the apical-basal polarity and connection with the cytoskeleton, which causes changes in signalling programmes specific for cells conformation and gene expression (*e.g.* Snail). EMT process involves: *i*) cell motility, ii) denudation of the mesothelium and *iii*) invasion of the fibrotic stroma with parallel increase of mesenchymal markers (*e.g.* α-SMA, collagen-I and fibronectin) (Figure 1.9).

TGF-β is the main pro fibrotic cytokine associated with EMT. It has been associated with fibrosis in different organs and its presence has been correlated with worse PD outcomes (Lai et al., 2000). The effect of this pro-fibrotic cytokine has been confirmed *in vivo* experiments where TGF-β was able to convert mesothelial cells into myofibroblast in the rat peritoneum (Loureiro et al., 2011; Margetts et al., 2001). Together with TGF-β, vascular endothelial growth factor (VEGF) has been associated with increased peritoneal capillary numbers and vessel permeability. It was confirmed that mesothelial cells undergoing EMT were the main source of this proangiogenic factor (Aroeira et al., 2005; Mandl-Weber et al., 2002). Other pro-inflammatory mediators such as TNF-α and IL-1β have also a role in the stimulation of resident cells and increase of extracellular matrix deposition (Aroeira et al., 2007). Moreover, advanced glycation end products (AGE) presents in the PDE can indirectly induce EMT inducing PD cells to release TGF-β and VEGF (García-López et al., 2012; Margetts Peter J. and Bonniaud Philippe, 2003).

EMT can be blocked at different stages. For instance, treatment of mesothelial cells with the anti-fibrotic protein bone morphogenic protein 7 (BMP-7) blocks EMT induced by AGE or TGF-β in a Smad-dependent manner (Loureiro et al., 2010). EMT can also be reversed by using molecules such as inhibitors of Rho-activated kinase or AngII (Liu, 2004; Strippoli et al., 2016; Wang et al., 2016). In addition, recently was found that vitamin D is able to

protect the peritoneal membrane against EMT reversing the effect of TGF-β inducing EMT (Lee et al., 2015).

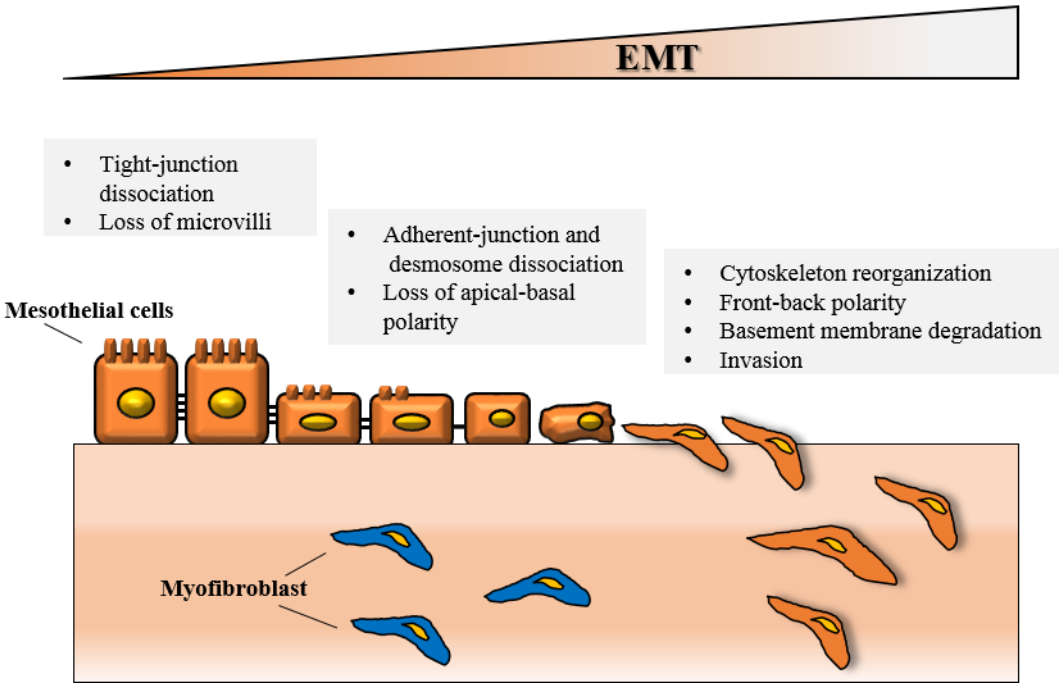

Figure 1.9 Key events during EMT.
Adapted from L. Aroeira et al.

1.3 Hypothesis and aims

Hypothesis

Vγ9/Vδ2 T cells and MAIT cells contribute to the amplification of local inflammation during acute infection by secretion of pro-inflammatory cytokines and stimulation of peritoneal epithelial and stromal cells.

Aims

The main aim of this study was to define the diagnostic, prognostic and therapeutic value of local immune responses triggered by unconventional T cells in PD patients during acute infection.

To achieve this aim the following objectives were defined:

- To study the responses of unconventional T cells from peripheral blood and peritoneal dialysis effluent in the presence of bacterial extracts *ex-vivo*;
- To analyse the frequencies of $V\gamma9^+$ T cells and MAIT cells during acute PD associated infections *in vivo*;
- To define clinical outcomes in the context of Vγ9/Vδ2 T cells and MAIT cells ligand production by the causative organism;
- To analyse the capability of activated unconventional T cells to induce peritoneal mesothelial cells and fibroblast activation in *ex-vivo* assays.

Chapter 2. Materials and Methods

2.1 Reagents

2.1.1 Complete RPMI 1640 medium

Complete RPMI 1640 medium used in cell culture was made by adding 10% foetal calf serum (FCS, Invitrogen), 50 mg/ml penicillin/streptomycin, 2 mM L-glutamine, 1% sodium pyruvate and 100 µM non-essential amino (NEAA) into RPMI-1640; all from Invitrogen.

2.1.2 Complete M-199 medium

Medium 199 (Life Technologies) was supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutammine (2 mM) and 10% foetal calf serum (FCS) from Invitrogen; and bovine pancreas insulin (5 µg/ml), hydrocortisone (0.4 µg/ml) and apo-transferrin (5 µg/ml) from Sigma-Aldrich.

2.1.3 Complete Ham's F12 medium

Ham's F12 nutrient mixture medium (Life Technologies) was mixed at a 1:1 ratio with DMEM medium and supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), 20% foetal calf serum (FCS) and 100 µM NEAA from Invitrogen; and bovine pancreas insulin (5 µg/ml), hydrocortisone (0.4 µg/ml) and apo-transferrin (5 µg/ml) from Sigma-Aldrich.

2.1.4 MACS buffer

To prepare magnetic-activated cell sorting (MACS) buffer, 2% FCS (Invitrogen) and 5 mM EDTA were added to sterile PBS and passed through 0.22 µm filter before use.

2.1.5 FACS buffer

To prepare fluorescent activated cell sorting (FACS) buffer, 2% FCS (Invitrogen) were added into sterile PBS and passed through 0.22 µm filter before use.

2.1.6 Freezing medium

To store leukocytes at -70°C in Cryo Tubes after cell isolation, cells were quickly re-suspended in complete medium supplemented with 40% FCS (Invitrogen) and 10% DMSO.

2.2 Cell isolation

2.2.1 Isolation of peripheral blood mononuclear cells (PBMC)

PBMCs were isolated from venous blood, collected from consented heathy volunteers. At the time of the collection, blood was mixed with anti-coagulant buffer made of 20 U/ml heparin and 15 mM EDTA (Fisher Scientific UK Ltd). Diluted blood was subsequently layered on top of Ficoll-Paque (Axis-Shield, Oslo, Norway) and centrifuged for 20 minutes at 1680 rpm at 18 °C with minimal acceleration and deceleration at lowest level. Afterwards, the white mononuclear cell layer (buffy coat) between the plasma and red blood cells (RBCs) was collected and washed three times with plain RPMI. The speed of centrifugation was gradually decreased from 1300 rpm, 1100 rpm to 900 rpm for three washes to reduce the number of platelets within the buffy coat. The cell pellet was then re-suspended in medium ready for the appropriate *in vitro* assays.

2.2.2 Isolation of monocytes from PBMCs

Monocytes were isolated by magnetic activated cell sorting (MACS) (Miltenyi) from PBMCs. In order to block non-specific binding of monoclonal antibodies to Fc receptors, PBMCs were firstly incubated with intravenous immunoglobulin (IvIg) (Kiovig; Baxter) at 1:1000 dilution in MACS buffer. Cells were subsequently washed with 20 ml MACS buffer and incubated with anti-CD14 microbeads (Miltenyi) at 4°C for 20 min. At this point cells were positively selected by passing through one LS columns (Miltenyi) to obtain purities of >95% $CD14^+$ monocytes, as determined by flow cytometry.

2.2.3 Isolation of MAIT cells from PBMCs

MAIT cells were isolated by MACS from monocyte-depleted PBMCs. Monocyte-depleted PBMCs were incubated with APC-conjugated anti-Vα7.2 mouse antibody (Biolegend) at 4°C for 20 minutes. Subsequently, cells were washed with 20 ml MACS buffer and incubated with anti-APC microbeads (Miltenyi) at 4°C for 20 min. Cells were then passed

through one LS columns to obtain purities of >95% Vα7.2$^+$ T cells, as determined by flow cytometry. The percentage average of MAIT cells Vα7.2$^+$ CD161$^+$ among CD3$^+$ Vα7.2$^+$ T cells was 79.8% ± 6.49 (average ± SD).

2.2.4 Isolation of γδ T cells from PBMCs

γδ T cells were purified by MACS from PBMCs, depleted of both monocytes and Vα7.2$^+$ T cells. The negative fraction of PBMCs following monocyte and MAIT cell purification was incubated with PE-Cy5-conjugated anti-Vγ9 mouse antibody (Immu360; Beckman Coulter) at 4°C for 20 minutes. Cells were then washed with 20 ml MACS buffer and incubated with anti-PE microbeads (Miltenyi) at 4°C for 20 min. Labelled cells were selected by LS columns (Miltenyi) to purities of >95%, as assessed by flow cytometry.

2.3 Bacteria extract preparation

Clinical bacterial isolates were kindly provided by Dr Mark Toleman, Division of Infection and Immunity, Cardiff University. These isolates were chosen based on their different capacity to utilise the non-mevalonate (HMB-PP) and vitamin B2 (DMRL) pathways (Table 2.1). The utilisation of each pathway was determined based on the absence or presence of HMB-PP synthase (EC 1.17.7.1) and/or DMRL synthase (EC 2.5.1.78) in the genomes of corresponding bacterial strains, according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg). Bacteria stored at -70°C in cryotubes with cryogenic medium (sterile PBS supplemented with sucrose, glycerol and peptone) were inoculated onto Colorex urinary tract infections (UTI) agar plate (E&O Laboratories Ltd) and incubated aerobically for 24h at 37°C (Figure 2.1). The following day, the growth of single species was confirmed by the presence of uniform colonies with the same colour (corresponding to the single organism and not to contamination with others, Figure 2.1). Then, bacteria were transferred from agar plates to 20 ml Luria Broth (LB, tryptone, yeast extract and NaCl) in an universal tube (Thermo Fisher Scientific) and incubated for 2.5h at 37°C with shaking to facilitate the bacterial division. Bacteria were harvested when the culture reached an optical density of 0.5-0.8 at a wavelength of 600 nm (OD_{600}). Each tube was subsequently centrifuged at 3,600 rpm for 1h and the bacterial pellet re-suspended in 1 ml of sterile PBS before sonication. Each bacterial suspension was kept on ice for sonication during the entire procedure using a Soniprep 150 sonicator for 10 min with 10 sec on and

20 sec off cycles. Bacterial extracts were then transferred to Eppendorf tubes (Fisher brand) and centrifuged at 20,000 *g* using an Eppendorf 5417R centrifuge, to pellet all cell debris. Next, collected supernatant was passed through Ultrafree-MC centrifugal filters (Durapore PVDF 0.1 µm, Millipore) and centrifuged for 10 min at 10,000 *g*, at 4°C, to remove larger aggregates. At this point a small aliquot was obtained to measure the protein concentration with BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific). To obtain fractions with lower molecular weight (<3kDa), the supernatant was then loaded on Ultracel YM-3 centrifugal cellulose filters (3 kDa NMWCO, Millipore) and centrifuged for 60-90 min at 10,000 *g*, 4°C. Bacterial extracts were then stored at -20°C and used for cell culture at dilutions corresponding to final protein concentrations of 60-100 µg/ml.

Table 2.1. Bacterial strains used in the study for Vγ9/Vδ2 and MAIT cells activation assay

	Strain	**Antibiotic resistance**		**Gram**	**Non-mevalonate pathway (Vγ9/Vδ2 T cell activation)**	**Vitamin B2 synthesis (MAIT cell activation)**
Klebsiella pneumoniae	156	NDM-1 and 16S rRNAmethylases positive	resistance to β-lactamases antibiotics (e.g. cephalosporins) and amynoglycosides	−	+	+
Escherichia coli	28	NDM-1 and 16S rRNAmethylases positive	resistance to β-lactamases antibiotics (e.g. cephalosporins) and amynoglycosides	−	+	+
Escherichia coli	17	NDM-1 and 16S rRNAmethylases positive	resistance to β-lactamases antibiotic (e.g. cephalosporins) and amynoglycosides	−	+	+
Pseudomonas aeruginosa	101-5178	SPM-1 positive	resistance to β-lactamases antibiotics (e.g. cephalosporins)	−	+	+
Enterococcus faecalis	12697	–	–	+	−	−
Corynebacterium striatum		–	–	+	−	−
Streptococcus pneumoniae	12-799	–	–	+	−	−
Staphylococcus aureus	12981	–	–	+	−	+
Listeria monocytogenes		–	–	+	+	−

*The listed details include relevant features introduced into bacteria such as strain identification numbers, Gram staining (Positive, + or Negative, −), HMB-PP and Vitamin B2 synthesis status (Positive,+ or Negative, −), and enzyme that confer bacteria antibiotic resistance such as New Delhi MBL (NDM-1) and Sao Paulo β-lactamase (SPM-1).

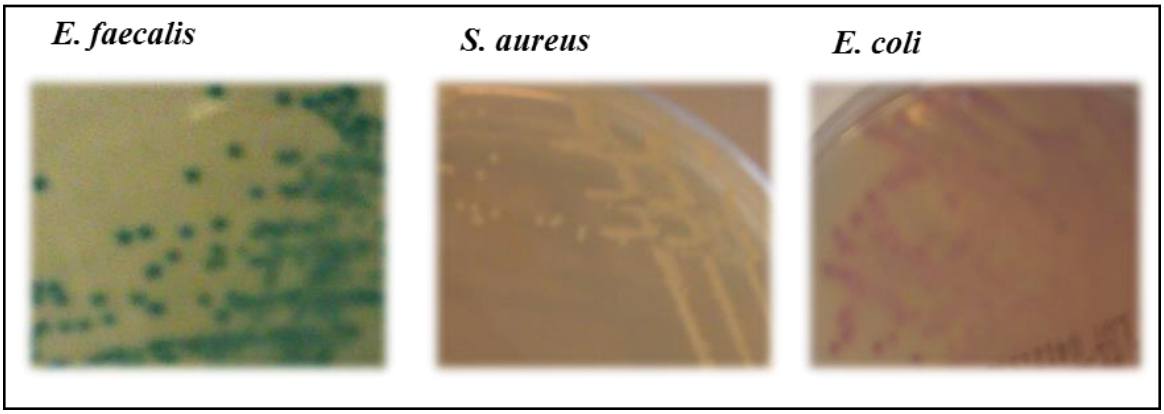

Figure 2.1. Bacteria identification on urinary tract infection (UTI) agar plate.
Bacteria were grown for single colony identification on UTI agar plates. This agar contains chromogens such as X-Gluc which is targeted towards β-glucosidase and allows the specific detection of E .faecalis through the formation of blue colonies. The other chromogen, Red-Gal, is cleaved by the enzyme β-galactosidase which is produced by E. coli and gives pink colonies, whereas S. aureus is characterized by a normal pigmentation.

2.4 Ethic statement

Recruitment of PD patients and healthy volunteers for this study was approved by the South East Wales Local Ethics Committee under reference numbers 04WSE04/27 and 08/WSE04/17, respectively. It was conducted according to the principles expressed in the Declaration of Helsinki. All individuals provided written informed consent. The PD study was registered on the UK Clinical Research Network Study Portfolio under reference numbers #11838 "Patient immune responses to infection in Peritoneal Dialysis" (PERIT-PD) and #11839 "Leukocyte phenotype and function in Peritoneal Dialysis" (LEUK-PD). Fresh omentum samples from consented patients were obtained from the Wales Kidney Research Tissue Bank.

2.5 Patient information and data collection

The local study cohort comprised 101 adults PD patients admitted to the University Hospital of Wales, Cardiff, on day 1 of acute peritonitis between September 2008 and October 2015. 45 stable individuals receiving PD for at least 3 months and with no previous infection served as non-infected controls or stable PD samples (Table 2.2). Subjects known to be positive for HIV or hepatitis C virus were excluded. Clinical diagnosis of acute peritonitis

was based on the presence of abdominal pain and cloudy peritoneal effluent with >100 white blood cells/mm^3. According to the microbiological analysis of the effluent by the routine Microbiology Laboratory, Public Health Wales, episodes of peritonitis were defined as culture-negative (with unclear aetiology) or as confirmed bacterial infections caused by specific subgroups of Gram-positive and Gram-negative organisms. The distribution of the non-mevalonate (HMB-PP) and vitamin B2 (DMRL) pathways across microbial species was determined based on the absence or presence of the enzymes HMB-PP synthase (EC 1.17.7.1) and DMRL synthase (EC 2.5.1.78) in the corresponding genomes, according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg). Cases of fungal infection and mixed or unclear culture results were excluded from this analysis.

Table 2.2. Characteristics of the PD patients recruited in this study.

PD patients characteristics, Cardiff UK	
Total number	146
Number of stable patients	45
Number of infected patients	101
Age (means ± SD)	68.4 ±13.6
Women among positively identified patients (%)	32.6
Culture positive episodes (%)	72.3
HMB-PP$^+$, Vitamin B2$^+$ organisms among positively identified species (%)	34.2
HMB-PP$^-$, Vitamin B2$^+$ organisms among positively identified species (%)	42.5
HMB-PP$^-$, VitaminB2$^-$ organisms among positively identified species (%)	23.2
Culture negative infection (%)	22.7
Fungal and mixed infections	4.9

2.6 T-cell culture

2.6.1 PBMC cultured with bacterial extract or with the ligands HMB-PP and DMRL

2.5 x 10^5 PBMCs per well were stimulated with bacterial extracts (Table 2.1) at a final protein concentration of 60 µg/ml in round-bottom, 96 well plates (Nunc plates; Thermo Scientific) and incubated overnight at 37°C. Similarly, PBMCs were stimulated with 0.1-100 nM HMB-PP or 0.1-100 µM DMRL (Table 2.4) in the presence or absence of 10 µg/ml anti-CD277 antibodies and/or 20 µg/ml anti-MR1 antibodies. Following overnight incubation, plates were centrifuged at 1300 rpm for 3 min, and the supernatant was collected for measurement of cytokines by ELISA, whereas the cells were re-suspended in PBS for measurements of cell activation by flow cytometry.

2.6.2 Co-culture of Vγ9$^+$ T cells or MAIT cells with monocytes

5 x 10^4 Vγ9$^+$ T cells or Vα7.2$^+$ T cells were co-cultured with autologous monocytes at 1:1 ratios, in the presence of bacterial extracts (Table 2.1) at a final protein concentration of 60 µg/ml in 96 well, round bottom plates (Nunc plates; Thermo Scientific) and incubated overnight at 37°C. For blocking experiments, anti-CD277 or anti-MR1 antibodies were added 30 min prior to stimulation of cultures. After overnight incubation, the plate was centrifuged at 1300 rpm for 3 min and the supernatant was collected for measurement of cytokines by ELISA, whereas cells were re-suspended in PBS for measurement of cell activation by flow cytometry.

2.6.3 Conditioned Medium generation

2.6.3.1 Preparation of Vγ9$^+$ T cell-Conditioned Medium

5 x 10^5 pure Vγ9$^+$ T cells were re-suspended in 1 ml of complete RPMI medium and cultured in a 24-well flat bottom culture plates (Nunc plates; Thermo Scientific). Conditioned Medium (CoM) was generated by adding 100 nM HMB-PP (Table 2.4) to the cultured cells for 24h in a humidified incubator at 37°C with 5% CO_2. Afterwards, the plate was centrifuged at 1400 rpm for 3 min and the supernatant (CoM) was collected carefully and stored at -70°C. Cell activation was confirmed by TNF-α and IFN-γ measurement in the CoM using ELISA.

2.6.3.2 Preparation of MAIT cell-Conditioned Medium

5×10^5 pure $V\alpha7.2^+$ MAIT cells were re-suspended in 1 ml of complete RPMI medium and cultured in a 24-well flat bottom culture plate (Nunc plates; Thermo Scientific). Conditioned Medium (CoM) was generated by adding Dynabeads® Human T-Activator CD3/CD28 (Life Technologies) at 1:2 bead-to-cell ratio to the cell cultures for 24h, in a humidified incubator at 37°C with 5% CO_2. The plate was then centrifuged at 1400 rpm for 3 min and the supernatant (CoM) was collected carefully and stored at -70°C. Cell activation was confirmed by TNF-α and IFN-γ measurement in the CoM using ELISA.

2.7 Isolation of peritoneal tissue cells and culture

2.7.1 Mesothelial cell isolation and culture

Human peritoneal mesothelial cells (HPMCs) were isolated from omental tissue of non-PD, consented patients undergoing abdominal surgery. Shortly after collection from surgery, tissue was stored in a sterile plastic container with PBS and incubated at 4°C until tissue dissection. In order to isolate HPMCs, omental tissue was subjected to 2 digestion cycles with 0.05% trypsin-EDTA solution (Invitrogen). Precisely, the tissue was placed in a petri dish and washed with PBS to remove blood and other material, and cut into small pieces depending on the tissue size. Each piece was then dissociated in a universal tube with 10 ml 0.05% trypsin-EDTA solution (Invitrogen) and positioned on a rotated wheel at 37°C for 15 minutes. Dissociated cells were pelleted by centrifugation at 1,300 rpm for 6 min at 15°C and supernatant was then carefully removed using a syringe. The cell pellet was re-suspended and washed with 20 ml of complete M-199 medium by centrifuging at 1,300 rpm for 6 min at 15°C.Washed cells were re-suspended in 5 ml of fresh complete M199 medium and transferred into T25 flask (NUNC, Thermo Scientific) (passage number 0) for further growth and expansion in culture. HPMCs were maintained at 37°C in a humidified incubator with 5% CO2. Cells were harvested and transferred to T75 flask when the cultures reached around confluency (≥80%) (passage 1). Prior to treatment, the growth of HPMCs was arrested by changing complete medium to serum-free M-199 for 48h. All the subsequent functional experiments were performed with the use of HPMCs expanded within 2 passages.

2.7.2 Fibroblast cell isolation and culture

Human peritoneal fibroblast cells (HPFBs) were isolated from omental tissue of consenting patients receiving abdominal surgery. Collected omental tissues were dissected into small pieces depending on the tissue size, and digested with 10 ml 0.05% trypsin-EDTA solution (Invitrogen) in tubes placed on rotated wheels at 37°C for 1h to facilitate the trypsinisation process. Digested cells were pelleted by centrifugation at 1,400 rpm for 6 min at 15°C and cell-free supernatant was then carefully removed. The cell pellet was re-suspended and rinsed with 20 ml of complete Ham's F12 medium and centrifuged at 1,300 rpm for 6 min at 15°C.Washed cells were re-suspended in 5 ml of fresh complete Ham's F12 medium and transferred into T25 flask (NUNC, Thermo Scientific) as passage 0 for further growth and expansion in culture. HPFBs were maintained at 37°C in a humidified incubator with 5% CO_2. When the cultures reached confluency (≥80%), cells were harvested and transferred

to T75 flask (passage 1). Prior to treatment, the growth of HPFBs was arrested by reducing the proportion of FCS in complete medium to 0.2% for 48h. All the subsequent functional experiments were performed with the use of HPFBs expanded within 2 passages.

2.7.3 Peritoneal mesothelial cells and fibroblast cell culture

HPMCs and HPFBs were maintained in flat-bottom 96 well plate (NUNC, Thermo Scientific) around confluency, and then were arrested from proliferation by depleting or reducing FCS within the complete medium for 48h prior to subsequent treatments. Thereafter, cells were cultured under one of the following conditions: *i*) bacterial extract at indicated protein concentration, *ii*) CoM derived from $V\gamma9^+$ T cells or $V\alpha7.2^+$ T cells at indicated dilution, *iii*) PD effluent derived from stable or infected PD patients at 1:1 dilution with cell medium, and *iv*) recombinant human TNF-α and IFN-γ, alone or in combination. For neutralisation of TNF-α and/or IFN-γ, CoM from unconventional T cells and PDE from infected patients were pre-treated with 10 μg/ml sTNFR p75-IgG1 fusion protein (Etanercept, Immunex) and anti-IFN-γ antibody together or alone for 30 mins before adding into cultures of HPMCs or HPFBs.

All reagents were diluted in complete medium with reduced FCS concentration. For treatments, cells were incubated at 37°C in humidified incubator with 5% CO_2 for 24h. Culture supernatant of HPMCs and HPFBs with various treatments was collected from plate centrifuged at 1,400 rpm for 5 min. Cytokines in collected supernatant were measured by ELISA according to manufacturers' instruction.

2.8 Leukocyte isolation from PD fluid

PD bags from stable patients (volume between 1 to 2.5 L from an overnight dwell) were collected as soon as possible in the morning after exchange and were placed on ice to reduce cell adhesion to the plastic bag. The date of collection, bag weight and patient details were recorded. Next, PD effluent was aliquoted in 500 ml centrifuge tube (Corning Inc., New York, USA) and centrifuged for 35 min at 2,000 rpm and 4 °C. 10 ml of cell-free supernatant was aliquoted into 2 ml labelled microcentrifuge tube (Fisher brand) and stored at -70 °C for cytokine analysis later. The remaining supernatant was decontaminated with chlorine tablets. The cells pellet was re-suspended in 50 ml centrifuge tube (Corning Inc., New York, USA) with cold phosphate buffered saline (PBS) (Invitrogen) and centrifuged for 8 min at 1300 rpm at 4 °C. Cells were counted using a dual chamber haemocytometer (Assistant, Sondheim, Germany). While counting, the rest of the cells were re-suspended with 10 ml PBS and centrifuge for the second time for 8 min at 1,300 rpm and subsequently re-suspended with appropriate volume of complete RPMI-1640 for further stimulation assays. Up to 14 x 10^6 cells were counted from one stable PD bag, among this 750,270 ± 1,3 x 10^6 were $CD3^+$ T cells.

PD bags from infected patients were processed as described above. This procedure was performed predominantly by Dr. Ann Kift-Morgan and Dr. Chan-Yu Lin (Division of Infection & Immunity, Cardiff University) as previously described (Lin et al., 2013).

2.8.1 Culture of peritoneal dialysis effluent cells

To activate peritoneal unconventional T cells, 2.5 x 10^5cells were seeded per well in round-bottom 96 well plate (Nunc; Thermo Scientific) and treated overnight with different bacterial extract positive and negative for HMB-PP and vitamin B2 (Table 2.1) and incubated overnight at 37°C in a humidified incubator with 5% CO_2. In the indicated conditions, cells were pre-incubated with blocking antibodies against CD277 and MR1 at 10 µg/ml for 30 min before addition of bacterial extracts. After overnight incubation, the plate was centrifuged at 1,300 rpm for 3 min and supernatant was collected for measurement of cytokines using ELISA, whereas the cells were re-suspended in PBS for measurement of cell activation by flow cytometry.

2.9 Flow cytometry

Flow cytometry was used to assess the expression of activation markers by cells on their surface or secreted by various immune cells. Cells were firstly washed with PBS and then stained with Live/dead fixable Aqua dead cell stain kit (Life Technologies) for 15 min at RT to distinguish live cells from dead cells during analysis. Subsequently, cells were washed with FACS buffer, centrifuged at 1,400 rpm for 3 min and incubated with IvIg (Kiovig; Baxter) at 1:1000 dilution for 15 min in fridge to block non-specific binding of Fc receptors to antibodies. In order to stain for markers expressed on cell surface, cells were washed with FACS buffer and incubated with a cocktail of monoclonal antibodies conjugated with different fluorochromes (Table 2.3) in the fridge for 20 min. To process intracellular staining, cells were fixed for 15 min at RT with fixation buffer (eBioscience), washed with permeabilisation buffer (eBioscience), and then incubated for 20 minutes at RT with cocktails of fluorochrome-conjugated monoclonal antibodies (Table 2.3) diluted in permeabilisation buffer. Cells were finally washed with FACS buffer and results were acquired using FACS canto II (BD Biosciences). All analysis was performed using FlowJo (version 10.0.8; TreeStar Inc.) and the cells of interest were gated based on the appearance on side scatter and forward scatter area/high (SSC-A/FSC-A) for intact cells, forward scatter area/high (FSC-A/FSC-H) for single cells and live-dead/FSC-A for live cells. T cells were defined as $CD3^+$ live cells. Among T cells, $V\gamma9^+$ T cells were defined as $CD3^+$ $V\gamma9^+$ and MAIT cells as $CD3^+$ $V\alpha7.2^+$ $CD161^+$ as previously described (Le Bourhis et al., 2010; Davey et al., 2011a).

2.9.1 Flow cytometry analysis of intracellular IFN-γ and TNF-α

Prior to staining, cells were simulated under different conditions in the presence of 5 µg/ml brefeldin A (Biolegend), which inhibits the intracellular protein transport, and incubated for 5 hours at 37°C in humidified incubator with 5% CO_2. Stimulated cells were then stained for surface markers and intracellularly for IFN-γ and TNF-α.

Table 2.3. Antibodies used in this study for surface and intracellular marker staining.

	Antigen	*Conjugate*	*Clone*	*Isotype*	*Company*	*Final concentration*
Surface marker staining	CD3	APC-H7	SK7	Mouse IgG1,κ	BD Bioscience	1:60
	CD8	PE-Cy7	SK1	Mouse IgG1,κ	BD Bioscience	1:200
	CD14	PE-Cy7	61D3	Mouse IgG1,κ	eBioscience	1:160
	CD15	APC	HI98	Mouse IgM	BD Bioscience	1:5
	CD25	PE	M-A251	Mouse IgG1, κ	BD Pharmingen	1:15
	CD69	FITC	FB50	Mouse IgG1,κ	BD Bioscience	1:20
	CD161	BV421	HP-3G10	Mouse IgG1,κ	Biolegend	1:50
	Vδ2	PE	B6.1	Mouse IgG1,κ	BD Bioscience	1:100
	Vγ9	PE-Cy5	Immu360	Mouse IgG1,κ	Beckman Coulter	1:400
	Vα7.2	PE	3C10	Mouse IgG1,κ	Biolegend	1:50
	Vα7.2	APC	3C10	Mouse IgG1,κ	Biolegend	1:50
	CCR2	PE-Cy5	TG5	Mouse IgG2b	Biolegend	1:20
	CCR2	APC	K036C2	Mouse IgG2a	Biolegend	1:40
	CCR5	PE	2D7	Mouse IgG2a	BD Pharmingen	1:20
	CCR6	PE	11A9	Mouse IgG1, κ	BD Pharmingen	1:20
Intracellular marker staining	IFN-γ	PE-Cy7	B27	mouse IgG1	BioLegend	1:100
	TNF-α	PE	IPM2	mouse IgG1	Coulter	1:30

Table 2.4. Soluble mediators and blocking antibodies used in functional assay.

	Reagent	***Supplier***
Functional assay	Recombinant human TNF-α	Miltenyi
	Recombinant human IFN-γ	Peprotech
	HMB-PP	A gift from Dr. Hassan Jomaa, Universitätsklinikum Giessen und Marburg, Germany
	DMRL	A gift from Dr Boris Illarionov, Hamburg School of Food Science, Germany).
Blocking assay	Purified anti-human IL-1β antibody	Clone H1b-27, Biolegend
	Purified anti-human IFN-γ antibody	Clone B27, Biolegend
	Etanercept (sTNFR)	Immunex

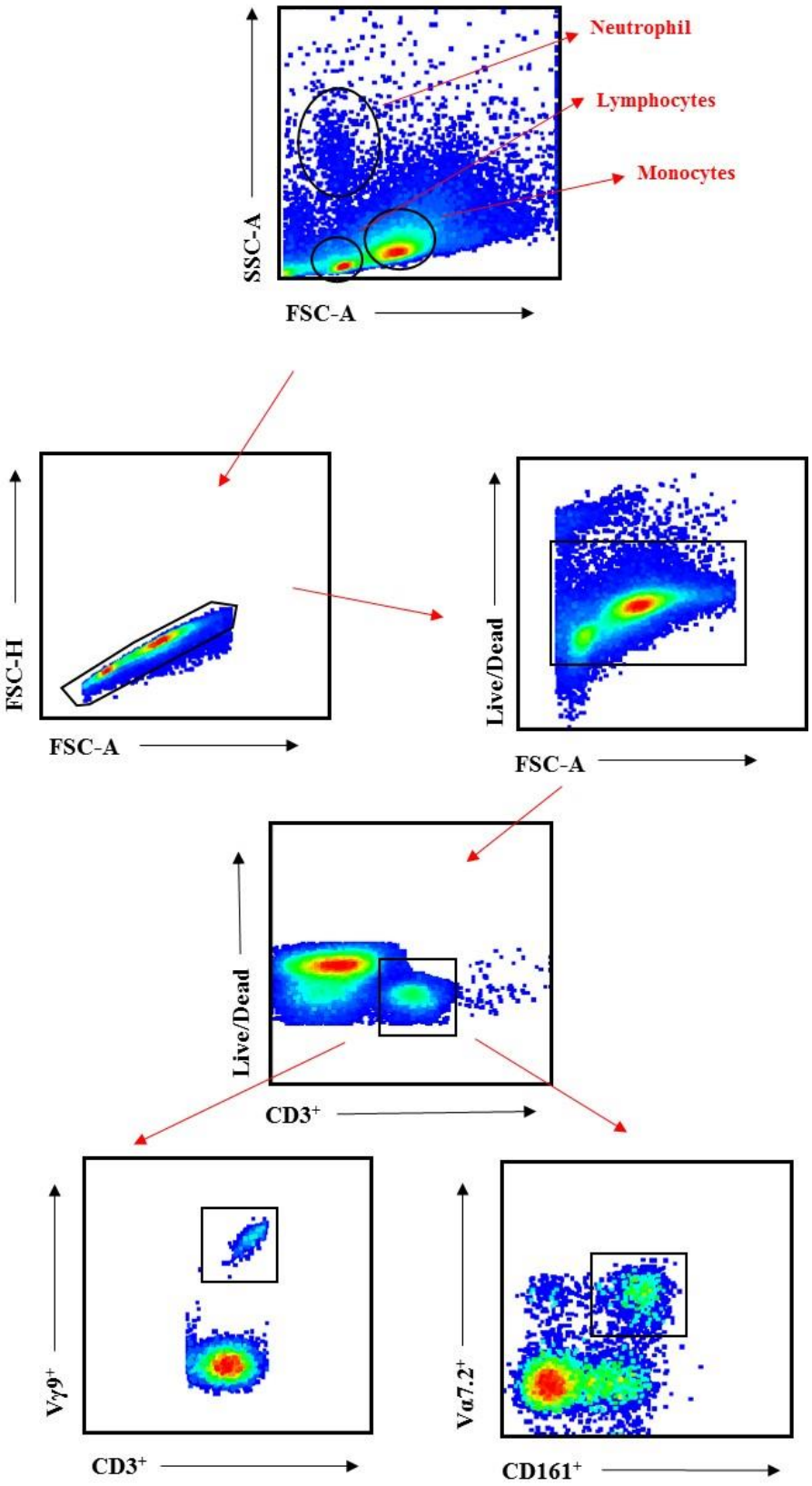

Figure 2.2. Flow diagram for gating strategy.
The cells of interest were gated based on the appearance on side scatter and forward scatter area/high (SSC-A/FSC-A); forward scatter area/high (FSC-A/FSC-H) were used to gate single cells and live-dead (fixable Aqua; Invitrogen) and FSC-A to select live cells. T-cells were defined as live cells and $CD3^+$. Among these cells $V\gamma9^+$ T cells were defined as CD3+ Vγ9+ and MAIT cells as $CD3^+V\alpha7.2^+CD161^+$.

2.10 Assessment of cytokines in culture supernatant by ELISA

Cell-free peritoneal effluents were analysed on a SECTOR Imager 6000 (Meso Scale Discovery, Maryland, USA) for TNF-α, IFN-γ, IL-1β, CCL3, CCL4 and CXCL8 in collaboration with Dr. Ann Kift-Morgan (Institute of Infection & Immunity, Cardiff University). Conventional ELISA kits were used for the measurement of CCL2 (eBioscience) and CCL20 (R&D Systems) in effluents with O.D. acquired using a Dynex MRX II reader. Cell culture supernatants were analysed using conventional ELISA kits for IFN-γ (Biolegend), TNF-α and CCL2 (eBioscience) as well as for CXCL8, CXCL10 and IL-6 (R&D Systems)with O.D. acquired using a Dynex MRX II reader.

2.11 Real-time PCR

2.11.1 RNA extraction from HPMCs

In order to measure the mRNA expression of specific genes by HPMCs with different treatments, cells were lysed with TRIzol reagent (Invitrogen), which combines phenol and guanidine thiocyanate in a single phase to quickly inhibit RNase activity. The homogenate was separated to aqueous and organic phases by addition of chloroform (Sigma) for 5 min at RT followed by centrifugation at 12,000 *g* for 15 min at 4°C. RNA was precipitated from collected upper aqueous phase by addition of isopropanol (Sigma) with following centrifugation at 12,000 *g* for 10 min at 4°C. After precipitation, RNA was washed 3 times with 75% ethanol (Sigma) with centrifugation at 12,000 *g* for 5 min at 4°C each wash. Afterwards, the RNA pellet was air-dried for 15 min and rehydrated in 10 µl nuclease-free water. 1 µl of sample were used to measure the purity and concentration of RNA by NanoDrop 2000 spectrophotometer (Thermo Scientific). RNA was stored in all cases at -80°C prior to subsequent experiments.

2.11.2 Generation of cDNA

mRNA was reverse-transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The RT master mix included: nuclease-free water, 10X RT buffer, 100 mM dNTPs, 40 U/µl RNase inhibitor (New England BioLabs ® Inc), 50 U/µl MultiScribe Reverse Transcriptase and 10X RT random primers. Next, 10 µl RT master mix were added to 10 µl RNA extract containing 0.5 µg RNA. The amplifications

were carried out with thermal cycles of 10 min at 25°C, 2 h at 37°C and 5 sec at 85°C, followed by a cooling step at 4°C. Generated cDNA was diluted with 60 µl water prior to use as a template for qPCR.

2.11.3 Real-time Quantitative PCR

mRNA quantitative polymerase chain reaction (qPCR) was carried out by mixing 300 nM forward and reverse mRNA specific PCR primer pairs (Table 2.5) with 10 µl Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific) and 4.8 µl water. 16 µl mRNA specific master mix and 4 µl of diluted cDNA were added to each wells of Optical 96-Well Fast Plate (Thermo Fisher Scientific). The reaction was performed on ViiA7 Real-Time PCR System (Thermo Fisher Scientific) and the cycling parameters were: 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C. Single PCR product amplification was confirmed by melting curve analysis.

The comparative $2^{-\Delta CT}$ method was used to calculate sample specific threshold cycle (Ct). The expression of target genes was normalized to the expressions of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control. Relative expression to 1,000 copies of the endogenous control was considered in this method.

Table 2.5. List of primer sequence used in real-time qPCR.

Marker	***Reverse and Forward Primers (5'-3')***
E-Cadherin	F: TCCCAATACATCTCCCTTCACA R: ACCCACCTCTAAGGCCATCTTT
ZO-1	F: GGAGAGGTGTTCCGTGTTGT R: GGCTAGCTGCTCAGCTCTGT
Occludin	F: TAAATCCACGCCGGTTCCTGAAGT R: AGGTGTCTCAAAGTTACCACCGCT
Claudin-1	F: CGGGTTGCTTGCAATGTGC R: CCGGCGACAACATCGTGAC
Fibronectin	F: CCGAGGTTTTAACTGCGAGA R: TCACCCACTCGGTAAGTGTTC
Collagen type I	F: CATGTTCAGCTTTGTGGACCTC R: TTGGTGGGATGTCTTCGTCT
Snail	F: TTTACCTTCCAGCAGCCCTA R: GGACAGAGTCCCAGATGAGC
α-SMA	F: AACTGGGACGACATGGAAA R: AGGGTGGGATGCTCTTCAG
GAPDH	F: CCTCTGACTTCAACAGCGACAC R: TGTCATACCAGGAAATGAGCTTGA
IL-6	F: CGAGCCCACCGGGAACGAAA R: GGACCGAAGGCGCTTGTGGAG

2.12 Statistical analysis

Statistical analyses were performed using Excel 2013, GraphPad Prism 6.0 or IBMS SPSS Statistics 20.0. All variables were tested for normal distribution using D'Agostino & Pearson omnibus normality test. Comparison between two non-paired normal distributed groups (parametric) was performed by one-sample *t* test, whereas paired *t* test was used for matched data. For the data that did not pass the normality test (non-parametric), Wilcoxon *t* test was performed for paired data and Mann-Whitney *t* test was used for unpaired data.

Comparison among three or more groups was performed with ordinary one way-ANOVA for unpaired parametric data. Repeated measure one-way ANOVA for paired parametric data. While the comparison between matched non-parametric groups was performed by Friedman test, Kruskal-Wallis test was used for the comparisons between unpaired non-parametric groups. Descriptive statistics were described as mean ± standard error of the mean (SEM) unless otherwise stated.

Survival analysis was performed using the Kaplan-Meier approach and differences in overall survival between groups was assessed using the log-rank test.

Outcomes associated with technique failure were predicted using multiple logistic regression analyses for both categorical and continuous variables. The analysis was conducted based on forward elimination of data and the chance of risk of a particular outcome was described as Odds Ratio. All statistical tests were two-tailed; differences were considered statistically significant as indicated in the figures and tables: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Chapter 3. Responses of unconventional T cells from peripheral blood and peritoneal dialysis effluent to bacterial extracts

3.1 Introduction

Bacterial infection with concomitant inflammation is a major risk factor limiting the choice of peritoneal dialysis (PD) as treatment for patients with end-stage renal failure. During this treatment the peritoneal membrane allows excess water and waste products to be discarded from blood to the dialysate solution by ultrafiltration. The permanent catheter placed in the peritoneal cavity allows regular exchange of PDE fluid. However, the catheter increases risk of bacterial infection, leading to peritoneal inflammation and membrane damage that result in progressive ultrafiltration failure (Cho and Johnson, 2014).

$V\gamma9^+$ T cells and MAIT cells represent significant populations of lymphocytes in blood that uniquely possess the ability to respond to a large array of microbes by the capacity to recognize the microbial metabolite HMB-PP and vitamin B2, respectively. Indeed, both cell types respond to the majority of $Gram^-$ pathogens (e.g. *E. coli*, *Klebsiella*, *Pseudomonas*) and most $Gram^+$ bacteria (e.g. *C. striatum*, *S. aureus*, *Mycobacterium*), whereas only MAIT cells became activated in presence of *Staphylococcus* species and $V\gamma9^+$ T cells in presence of *Listeria* species. However, both of them do not react to streptococci. Consequently, these two unconventional types of T cell complement each other in bacterial recognition.

Most importantly, previous work in the laboratory showed that Vγ9/Vδ2 T-cells represent a sizeable proportion of peritoneal leukocytes that are able not only to predict episodes of PD-related peritonitis caused by Gram-negative bacteria, but also to identify patients at increased risk of treatment failure (Davey et al., 2011a). In addition, MAIT cell numbers increase at sites of infection in diseases such as tuberculosis, and decrease in the blood of these patients (Le Bourhis et al., 2010; Gold et al., 2010).

To validate the diagnostic, predictive and therapeutic value of local unconventional T cell populations (γδ T-cells, MAIT cells) in PD patients during episodes of peritonitis, this study aimed to analyse the function of these cells, from both peripheral blood and peritoneal dialysis effluent, in response to a broad range of $Gram^-$ and $Gram^+$ pathogens.

The specificity of peritoneal Vγ9/Vδ2 T-cells and MAIT cells for HMB-PP and vitamin B2 metabolites, respectively, was demonstrated. Only bacteria positive for these two

metabolites (e.g. *E. coli* and *C. striatum*) activated γδ T cells and MAIT cells, and induced the release of TNF-α and IFN-γ, highlighting the importance of these cells in promoting inflammation during PD-associated infection. The data generated in the work described in this chapter provide insight into the relationship between microbial pathogens and inflammatory responses during peritoneal infection.

3.2 Aims

The aims of this chapter were:

1. To identify $V\gamma9^+$ γδ T cells and MAIT cells in PBMC and PDE fluid.
2. To study the responsiveness of peripheral blood and PDE $V\gamma9^+$ γδ T cells and MAIT cells to microbial ligands, and to a range of bacterial pathogens.
3. To study the role of BTN3 and MR1 in unconventional T cell responses to bacterial pathogens.

3.3 Results

3.3.1 Identification of Vγ9$^+$ T cells and MAIT cells in both PBMC and PDE

In order to study the role of unconventional T cells during PD-associated infections, I firstly tried to identify these lymphocytes in PBMC and in PDE of healthy and stable patients. In line with previous methods (Martin et al., 2009), I used an antibody against the invariant segment of the TCRα chain, Vα7.2-Jα33, together with an antibody against the marker CD161 to identify MAIT cells. HMB-PP responsive Vγ9/Vδ2 T cells were identified using an antibody against the Vγ9 chain of the TCR, since in the vast majority of cases the Vγ9 chains pair with Vδ2 (Bonneville et al., 2010). As shown in Figure 3.1, Vα7.2$^+$ CD161$^+$ MAIT cells were identified in both PBMC and PDE. The frequency of MAIT cells was higher in PBMC than in PDE (p=0.06, mean 7.0 % ± 2.0 and 3.0 % ± 0.5, respectively). Similarly, the frequency of Vγ9$^+$ γδ T cells was significantly higher in PBMC compared to PDE (p<0.0009, mean 6.5 % ± 1.0 and 2.6 % ± 0.6, respectively).

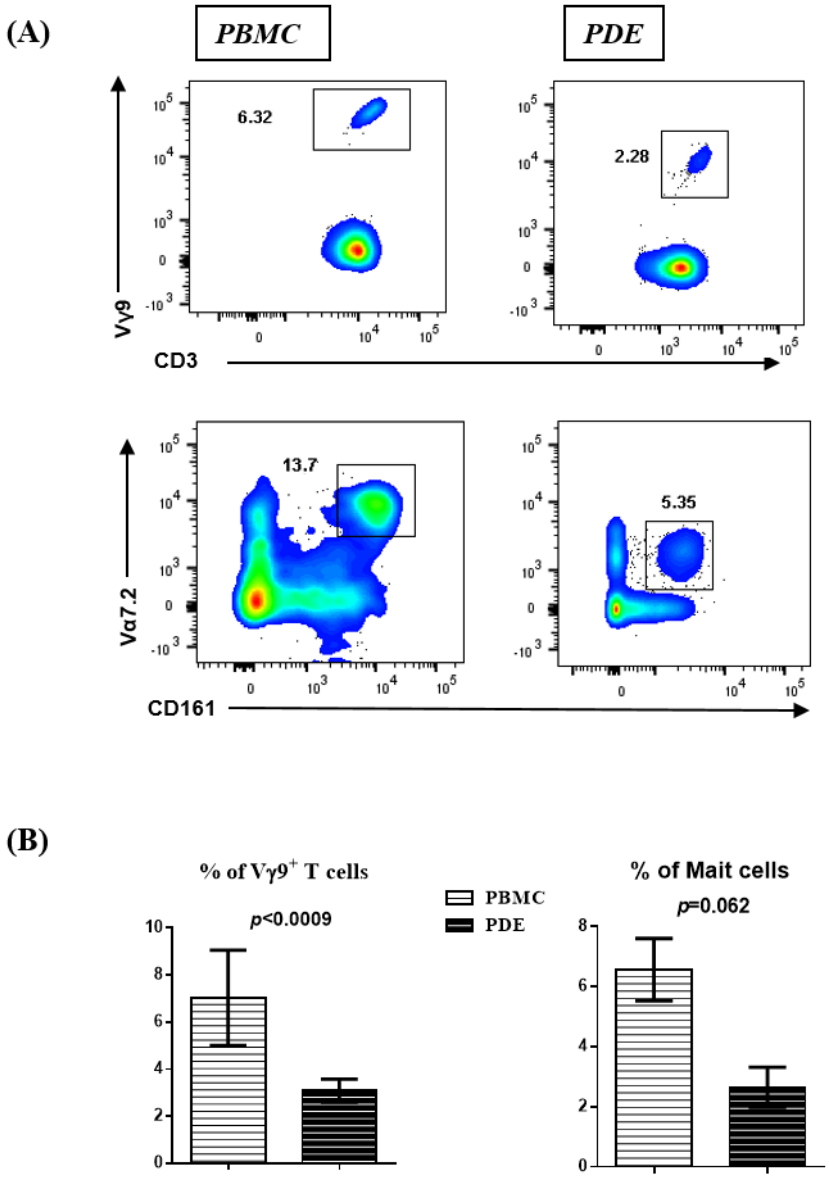

Figure 3.1. Identification of γδ T cells and MAIT cells in PBMC and PDE.
(A) Representative FACS plot of Vγ9$^+$ γδ T cells and Vα7.2$^+$ CD161$^+$ MAIT cells in peripheral blood of healthy volunteers (n=4) and in peritoneal effluent (PDE) of stable PD patients (n=45). (B) Frequencies of unconventional T cells amongst all T cells in PBMC and PDE are shown as ± SEM. Data were analysed by Mann Whitney t test.

3.3.2 Selective activation of γδ T cells and MAIT cells by HMB-PP and DMRL, respectively, in PBMC

To study the peritoneal unconventional T cell response to bacterial extract pathogens, I first analysed activation of these cells by microbial metabolites HMB-PP ((*E*)-4-hydroxy-3-methylbut-2-enyl pyrophosphate) and the vitamin B2 precursor DMRL (6,7-dimethyl-8-D-ribityllumazine). As shown in Figure 3.2, upregulated CD69 expression was observed in peripheral Vγ9$^+$ T cells, but not MAIT cells, in PBMC treated with ≥1 nM HMB-PP. In addition, γδ T cells alone showed a parallel (although less pronounced) increase in the CD25 expression. When culturing PBMC with ≥10 μM DMRL, only MAIT cells upregulated CD69. I also observed a significant MAIT cell CD25 expression, but only when these cells where exposed to high concentrations of DMRL (Figure 3.2). These findings agree with previous investigations demonstrating specific but modest activation of MAIT cells by DMRL. Unfortunately, high bioactivity ligands such as 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU; (Corbett et al., 2014)) were not available for the present studies.

As described previously, Vγ9$^+$ T cell response to HMB-PP and related compounds depends on the presence of the receptor CD277/BTN3A (Harly et al., 2012; Rhodes et al., 2015), whereas MAIT cells recognise antigens in association with the MHC class I related molecule MR1 (Le Bourhis et al., 2010; Treiner et al., 2003). Accordingly, the responses of Vγ9$^+$ T cells and MAIT cells to HMB-PP and DMRL were abrogated in the presence of monoclonal antibodies against CD277 and MR1, respectively (Figure 3.3), confirming that these accessory molecules were necessary for full activation of unconventional T cells.

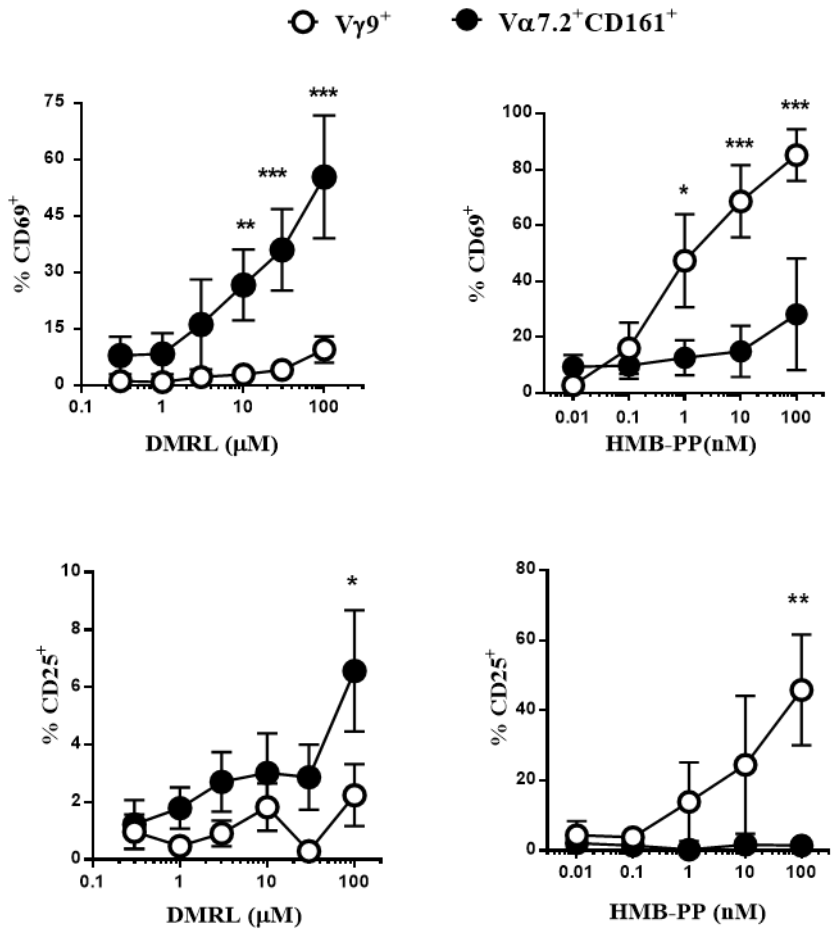

Figure 3.2. Response of peripheral unconventional T cells to microbial metabolites *in vitro*.
Surface expression of CD69 and CD25 by Vγ9$^+$ and Vα7.2$^+$ CD161$^+$ T-cells in PBMC stimulated overnight with HMB-PP (0.01-100 nM) or DMRL (0.3-100 µM). Data were analysed by two-way ANOVA with Bonferroni's post-hoc tests and are shown as mean values ± SD, n=5. Significant differences are indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

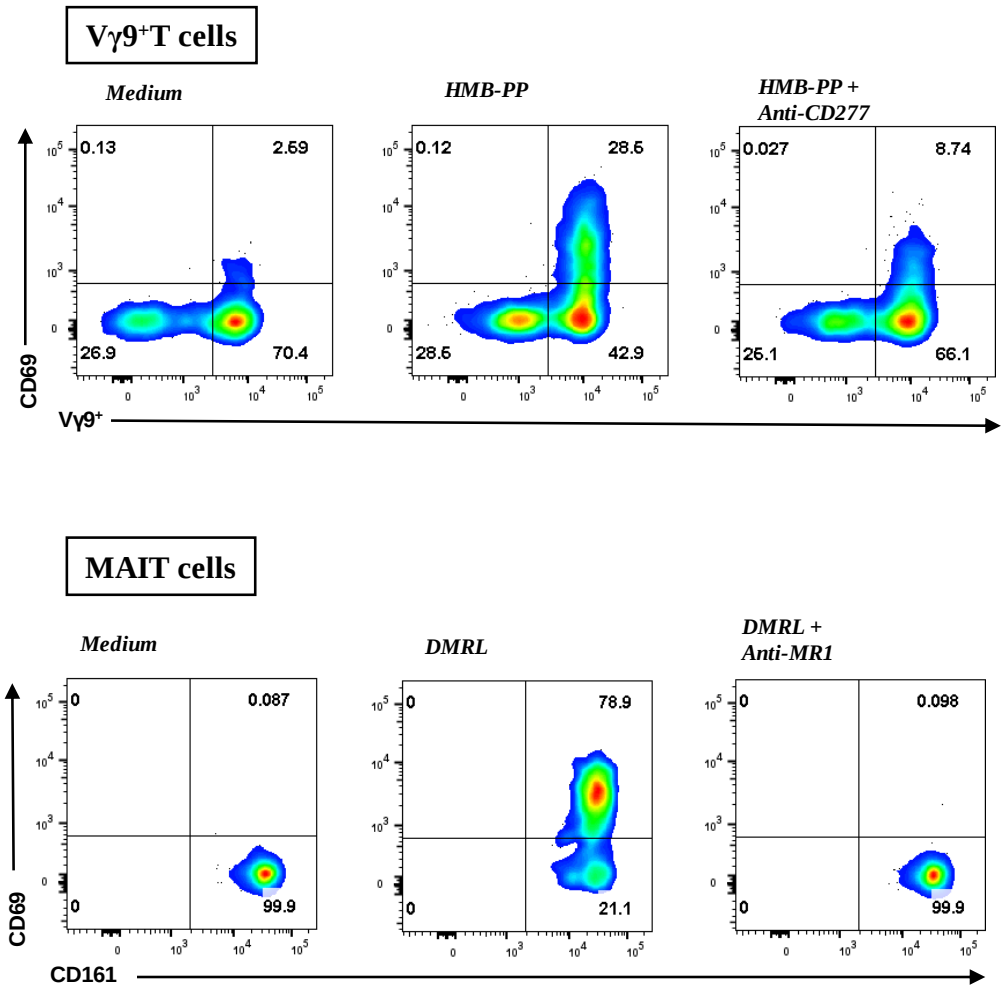

Figure 3.3. Vγ9$^+$ T cells and MAIT cells response to microbial metabolites is BTN3 and MR1 dependent, respectively.
Representative FACS plots of two donors showing CD69 expression by Vγ9$^+$ T-cells and Vα7.2$^+$ T-cells in PBMC stimulated overnight with 100 nM HMB-PP or 100 µM DMRL, in the absence or presence of anti-BTN3 or anti-MR1 monoclonal antibodies.

3.3.3 Selective activation of peripheral Vγ9/Vδ2$^+$ T cells and MAIT cells in the presence of bacteria producing HMB-PP and vitamin B2

To study the role of unconventional T cells in the peritoneal immune response during PD related infection, I prepared low molecular weight fractions of bacterial extracts from clinical isolates Gram$^+$ (*E. faecalis*, *C. striatum*, *S. aureus*, *L. monocytogenes*, *S. pneumoniae*) and Gram$^-$ (*E. coli*, *K. pneumoniae*, *P. aeruginosa*) species which possess or not the non-mevalonate and riboflavin pathways (Table 2.1, Materials and Methods).

Firstly the ability of these extracts to activate peripheral unconventional T cells was tested. PBMC were cultured overnight with bacterial extracts, and cell activation was then assessed by flow cytometry and ELISA. As shown in Figure 3.4, up to 60 % of Vγ9$^+$ γδ T cells upregulated CD69 in the presence of HMB-PP positive bacteria such as *P. aeruginosa* and *K. pneumoniae*, with a significant upregulation in response to *E. coli* and *C. striatum* ($p<0.05$ and $p<0.01$, respectively). A similar response to HMB-PP$^+$ bacteria was observed in Vγ9$^+$ T cells that co-expressed CD69 and TNF-α or IFN-γ. In contrast, activation of γδ T cells was not seen in the presence of extracts from the HMB-PP deficient species *E. faecalis* (Figure 3.4). However, although not significant, Vγ9$^+$ T cell activation was also observed in presence of the HMB-PP$^-$ bacteria *S. aureus*.

In the same PBMC cultures, up to 60% of MAIT cells upregulated CD69 and released TNF-α and IFN-γ in the presence of vitamin B2 positive bacteria *K. pneumoniae*, *E.coli*, *C. striatum* and *S. aureus*, but not *P. aeruginosa* (Figure 3.5). By contrast, only background activation of MAIT cells was observed in response to vitamin B2$^-$ *E. faecalis*.

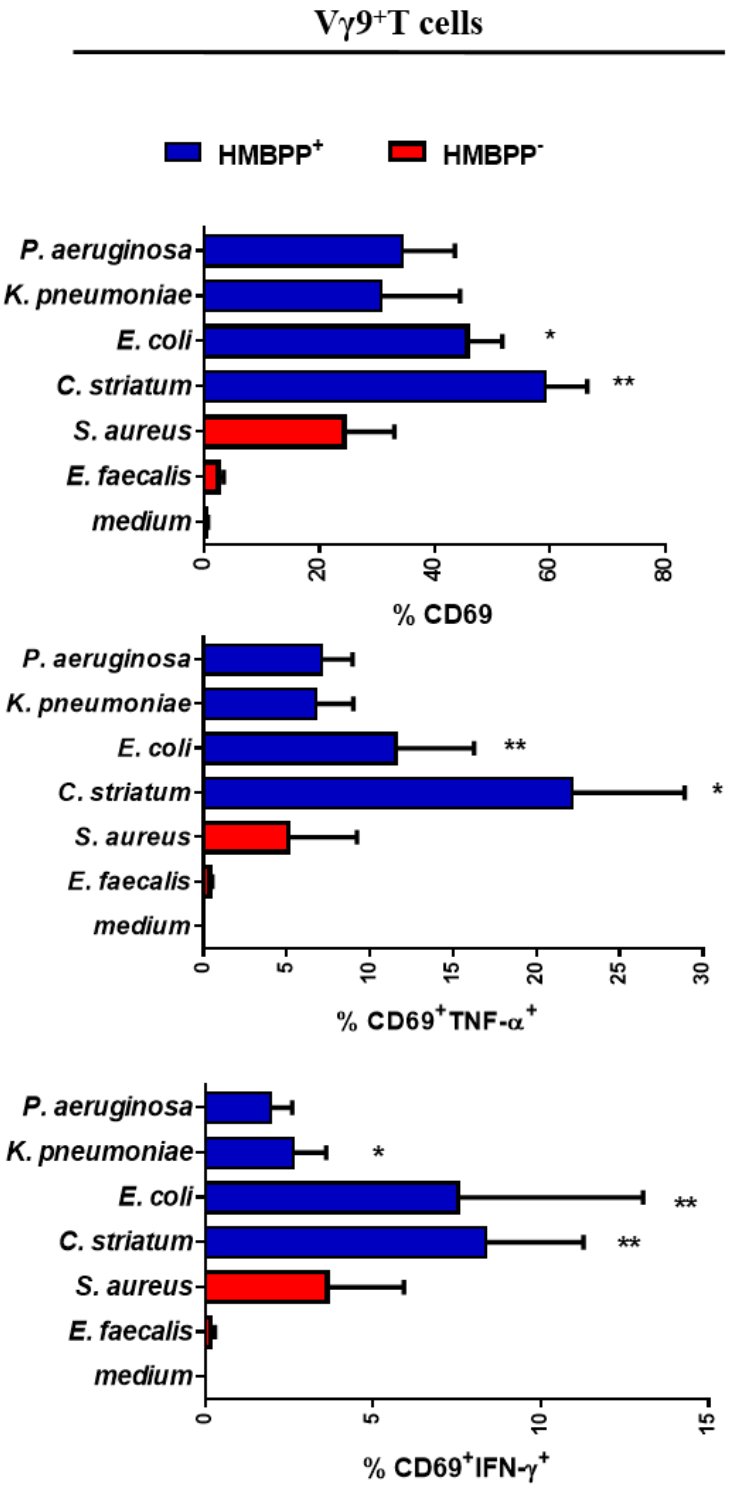

Figure 3.4. Peripheral Vγ9/Vδ2$^+$ T cells respond to HMB-PP producing bacterial extract, but not to HMB-PP deficient bacteria.
CD69 surface expression and intracellular TNF-α and IFN-γ expression by Vγ9/Vδ2 T cells in PBMC cultured overnight in the presence of bacterial extracts from *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *C. striatum*, *S. aureus* and *E. faecalis* , corresponding to final protein concentrations of 60 µg/ml (means ± SEM, n=4-5). Data were analysed by Kruskall Wallis test with Dunn's post-hoc tests, and significant differences are indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

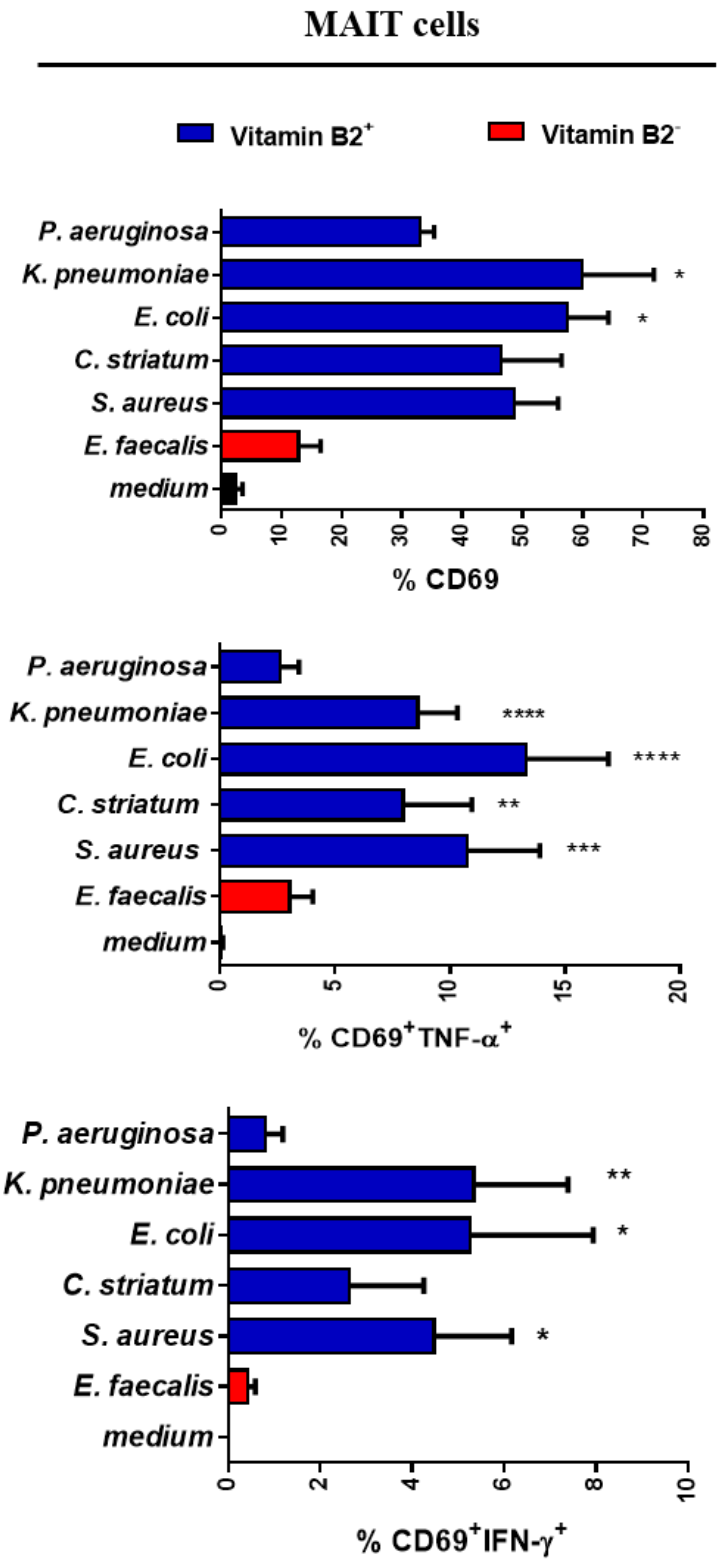

Figure 3.5. Peripheral MAIT cells respond to vitamin B2 producing bacterial extract but not to extract from vitamin B2 deficient bacteria.
CD69 surface expression and TNF-α and IFN-γ intracellular expression by MAIT cells in PBMC cultured overnight in the presence of bacterial extracts from *P. aeruginosa*, *K. pneumoniae*, *E.coli*, *C. striatum*, *S. aureus* and *E. faecalis* bacterial extracts, corresponding to final protein concentrations of 60 µg/ml (means ± SD, n=4-5). Data were analysed by Kruskall Wallis test with Dunn's post-hoc tests, and significant differences are indicated:: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

To confirm that full activation of unconventional T cells occurred in the context of antigen presenting cells, I isolated both Vγ9$^+$ T cells and Vα7.2$^+$ T cells from peripheral blood and cultured them with autologous monocytes. As shown in Figure 3.6, purified Vγ9$^+$ T cells upregulated CD69 and secreted TNF-α and IFN-γ, not only in response to HMB-PP, but also when cultured with the HMB-PP$^+$ bacteria *K. pneumoniae* and *E. coli* in the presence of autologous monocytes. By contrast, there was no such activation in the presence of HMB-PP$^-$ *E. faecalis*. Similarly, MAIT cells co-cultured with autologous monocytes upregulated CD69 and produced TNF-α and IFN-γ in response to DMRL as control, and to vitamin B2 producing bacteria. Of note, in these co-cultures purified MAIT cells also produced TNF-α, but not IFN-γ, in the presence of vitamin B2 deficient *E. faecalis* (Figure 3.7), possibly due to indirect activation by activated monocytes. Overall, the amount of IFN-γ secreted by MAIT cells was lower compared to that released by γδ T cells in the same conditions.

In summary, unconventional T cells were able to produce pro-inflammatory cytokines in response to bacterial extracts that contained HMB-PP and/or vitamin B2, but not in the presence of bacteria lacking these two metabolites (e.g. *E. faecalis*). These findings highlight the similar responsiveness of these two cell types to microbial metabolites.

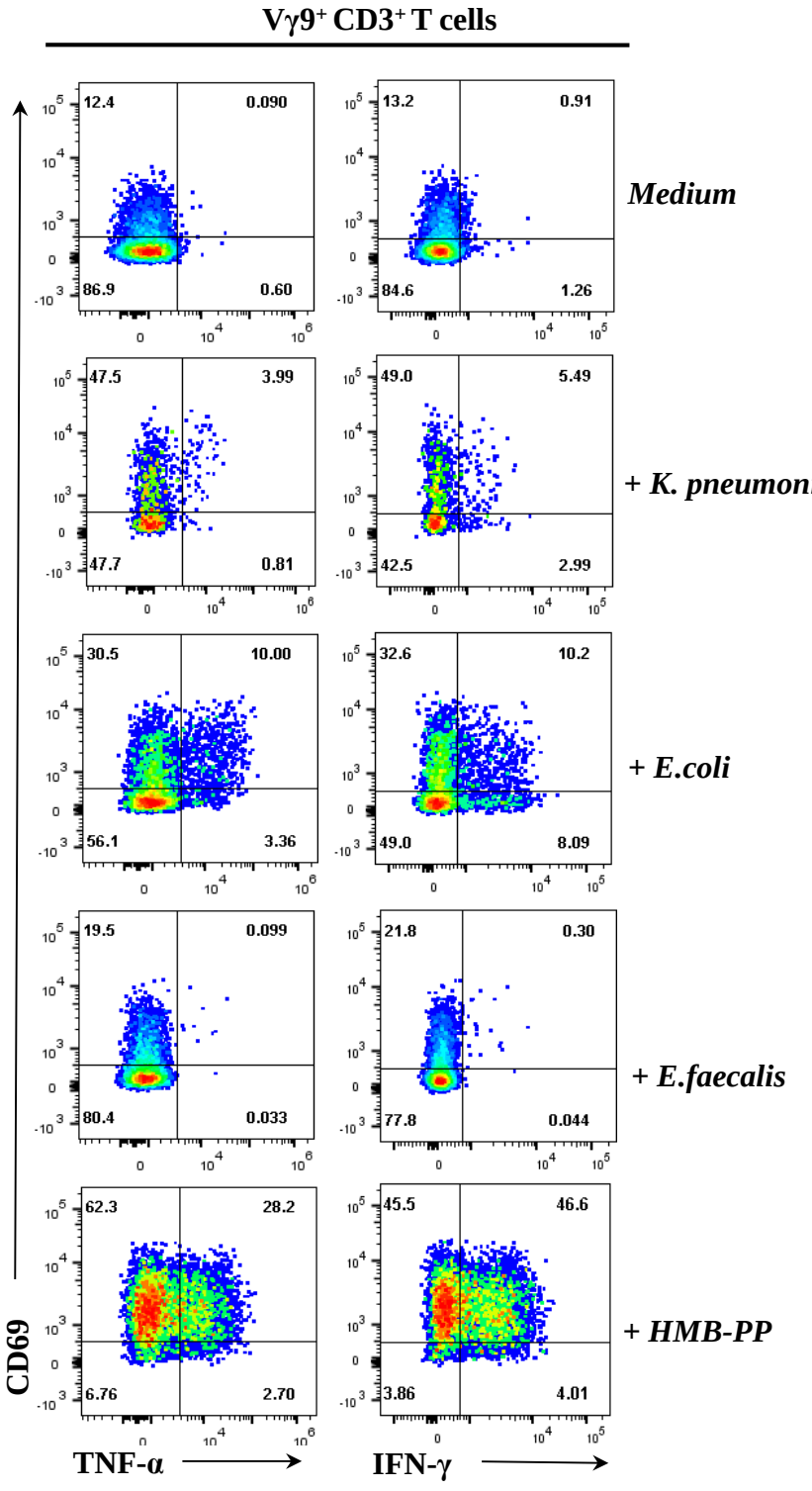

Figure 3.6. MACS-purified Vγ9/Vδ2 T cells co-cultured with autologous monocytes respond to HMB-PP producing bacteria but not to HMB-PP deficient bacteria.
FACS plots show expression of CD69 and either TNF-α or IFN-γ by Vγ9^{+} T-cells in response to *K. pneumoniae*, *E. coli* and *E. faecalis*, and 100 nM HMB-PP as control (representative of three donors).

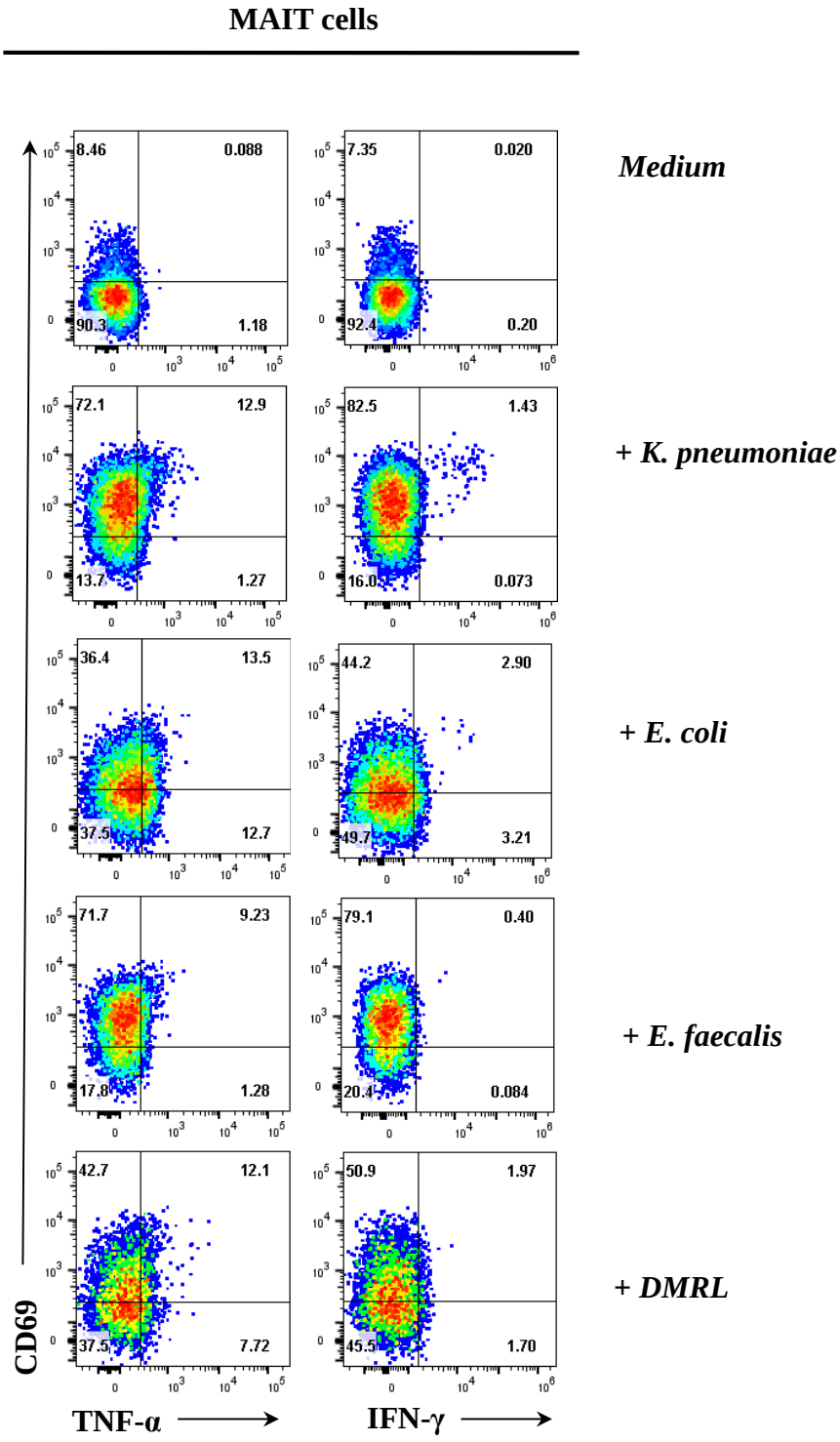

Figure 3.7. MACS-purified CD3$^+$ Vα7.2$^+$ T cells co-cultured with autologous monocytes respond to vitamin B2 producing bacteria but not to vitamin B2 deficient bacteria.
FACS plots show expression of CD69 and either TNF-α or IFN-γ by Vα7.2$^+$ CD161$^+$ MAIT cells in response to *K. pneumoniae*, *E.coli* and *E. faecalis*, and 100 µM DMRL as control (representative of three donors).

3.3.4 Selective activation of γδ T cells and MAIT cells by HMB-PP and vitamin B2 positive bacteria in PDE

To study the potential role of unconventional T cells during PD associated infection I next assessed the activation profile of these cells in the presence of different bacterial extract. Firstly, I showed that peritoneal γδ T cells, but not MAIT cells, were able to respond to increasing concentrations of synthetic HMB-PP as assessed by CD69 upregulation and TNF-α production. By contrast, peritoneal MAIT cells, but not γδ T cells, produced TNF-α in response to synthetic DMRL. Due to the high background of CD69 expression on peritoneal MAIT cell, I did not observe significant upregulation of CD69 expression in response to DMRL (Figure 3.8).

Next, peritoneal leukocytes derived from stable PD patients were cultured with different bacterial extracts. As shown in Figure 3.9, peritoneal Vγ9/Vδ2 T cells responded readily to HMB-PP producing Gram-negative (*E. coli*, *K. pneumoniae*, *P. aeruginosa*) and Gram-positive bacteria (*C. striatum*, *L. monocytogenes*), but not to HMB-PP deficient *E. faecalis* and *S. pneumoniae*. Similarly to the peripheral $V\gamma9^+$ T cells, peritoneal Vγ9/Vδ2 T cells also responded to *S. aureus*. Failure to block this response with anti-BTN3 neutralizing antibodies (data not shown) suggested an indirect recognition of *S. aureus*, possibly via superantigens (Morita et al., 2001).

Peritoneal MAIT cells responded to vitamin B2 positive bacteria *E. coli*, *K. pneumoniae*, *C. striatum* and *S. aureus*, but not to the vitamin $B2^-$ species *L. monocytogenes*, *E. faecalis* and *S. pneumoniae* with respect to TNF-α production (Figure 3.10). Peritoneal MAIT cell responses to vitamin $B2^+$ *P. aeruginosa* failed to reach statistical significance. Compared to TNF-α production, IFN-γ responses generally followed similar trends but were less pronounced and at times did not reach statistical significance (Figure 3.10). Overall, these findings confirm that both peritoneal γδ T cells and/or peritoneal MAIT cells are able to respond to a broad range of bacterial pathogens that are associated with PD-related infections.

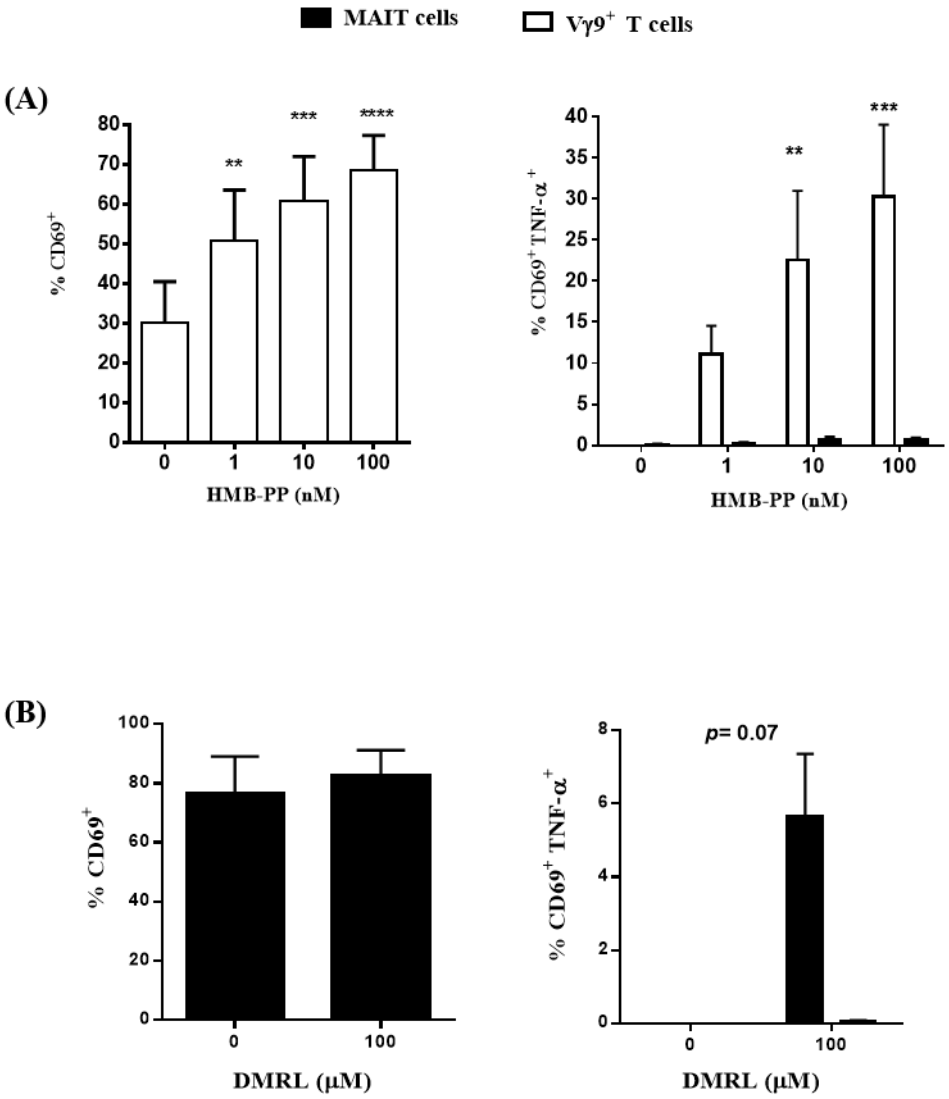

Figure 3.8. Peritoneal effluent derived unconventional T cell responses to microbial metabolites *in vitro*.

Activation of Vγ9$^+$ γδ T cells and Vα7.2$^+$ CD161$^+$ MAIT cells in peritoneal cells from stable PD patients following overnight stimulation with HMB-PP (n=4 individual patients) or with the vitamin B2 precursor DMRL (n=3), as analysed by flow cytometry and expressed as proportion of γδ or MAIT cells co-expressing CD69 and TNF-α (means ± SEM). Comparisons with the control group were made by repeated measures one way ANOVA, whereas multiple comparison between Vγ9$^+$ T cells and MAIT cells were made by two-way ANOVA with Sidak's multi comparison post hoc test. Significant differences are indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

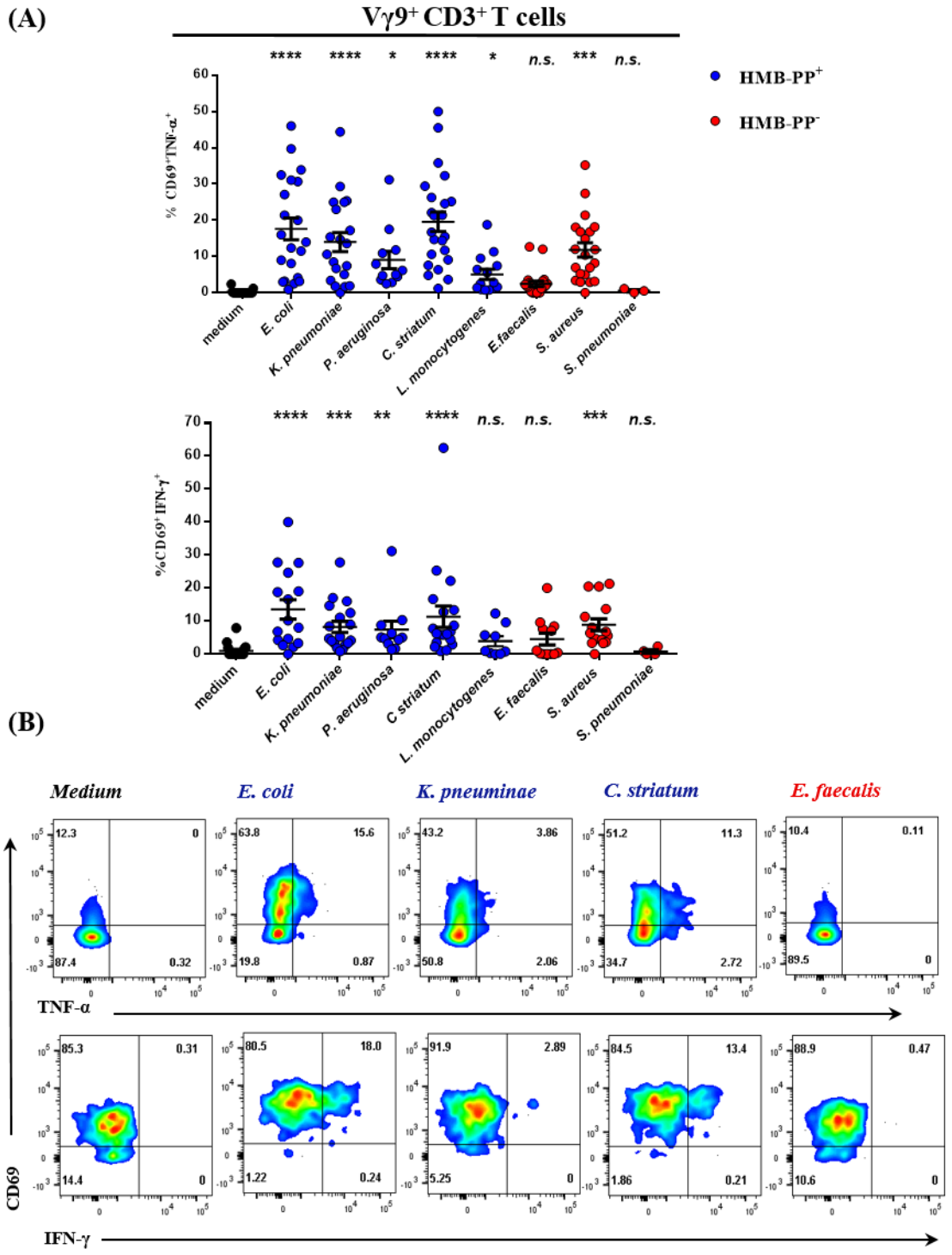

Figure 3.9. Peritoneal γδ T cell responses to microbial metabolites.
Activation of peritoneal Vγ9$^+$ γδ T cells following overnight stimulation in the presence of extracts from different clinical isolates: *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *C. striatum* (all HMB-PP$^+$, in blue); and *L. monocytogenes*, *S. aureus*, *E. faecalis* and *S. pneumoniae* (all HMB-PP$^-$, in red). (*A*). Summary of all data. Each data point represents an individual patient. Data were analysed by Kruskall Wallis test with Dunn's post-hoc tests (mean ± SEM, n= 21-27), significant differences are indicated: *, p<0.05; **, p<0.01; ***, p<0.001. (*B*) Representative FACS plots showing CD69 surface expression and intracellular TNF-α and IFN-γ expression by peritoneal Vγ9$^+$ γδ T cells (representative of 21-27 individual donors).

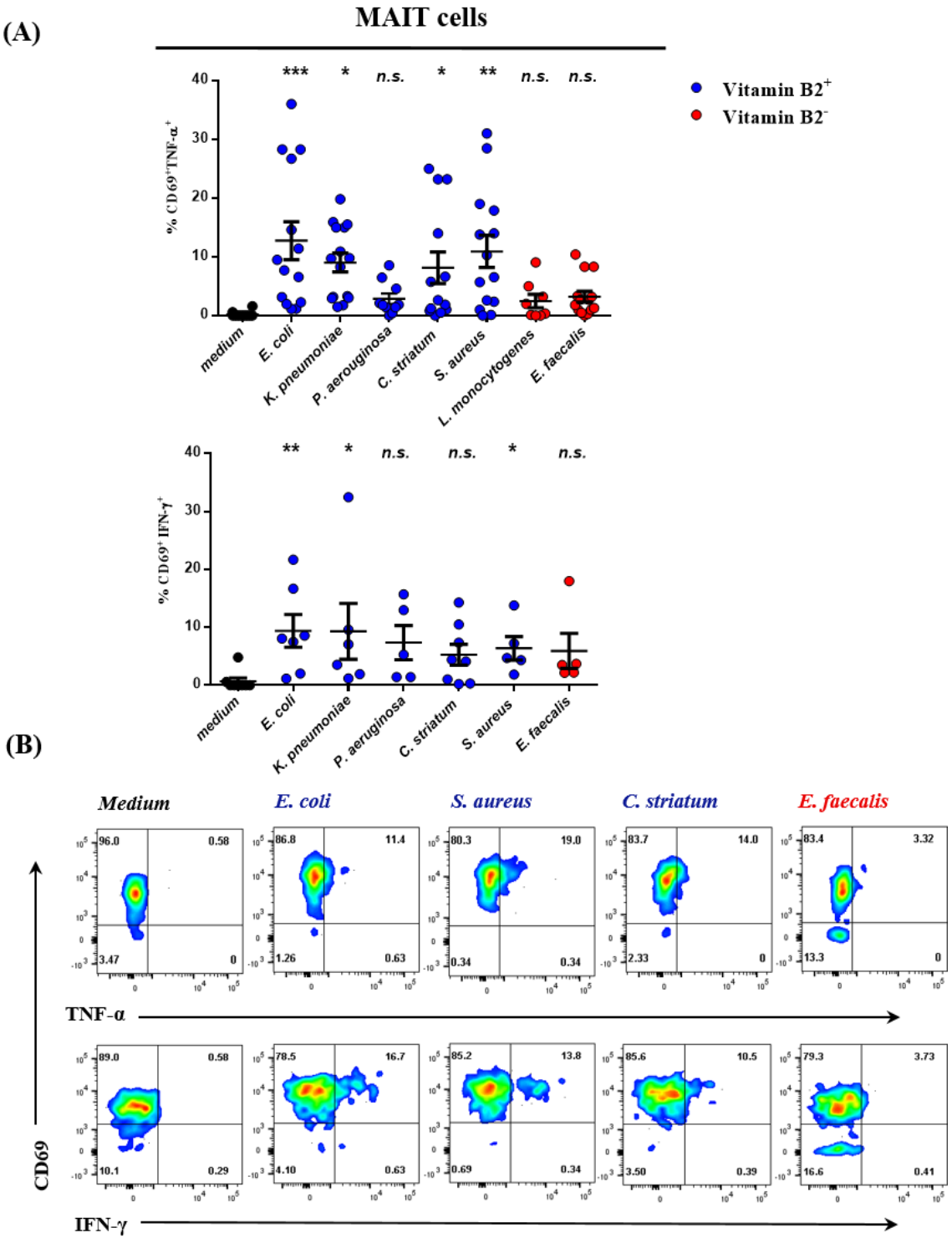

Figure 3.10. Peritoneal MAIT cell responses to microbial metabolites.
Activation of peritoneal MAIT cells following overnight stimulation in the presence of extracts from different clinical isolates *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *C. striatum*, *L. monocytogenes* and S. *aureus* (all vitamin B2^{+}, in blue); and *E. faecalis* and *S. pneumoniae* (vitamin B2^{-}, in red). (*A*). Summary of all data. Each data point represents an individual patient. Data were analysed by Kruskall Wallis test with Dunn's post-hoc tests (mean ± SEM, n=8-17). Differences were considered significant as indicated: *, p<0.05; **, p<0.01; ***, p<0.001. (*B*) Representative FACS plot of one donor showing CD69 surface expression and intracellular TNF-α and IFN-γ expression by peritoneal MAIT cells (representative of 8-17 individual donors).

3.3.5 Peritoneal unconventional T cells are major producers of TNF-α and IFN-γ in response to microbial pathogens

TNF-α is a pro-inflammatory cytokine that drives acute and chronic inflammation during episodes of peritonitis (Aroeira et al., 2007; Davey et al., 2011a). Previous work from our laboratory has also shown that, of all soluble factors present in peritoneal effluent, elevated levels of TNF-α at day 1 of peritonitis are associated with high rates of technique failure and mortality within three months following infection (Davey et al., 2011a). Here, after treatment of PDE leukocytes with *E.coli* extract (as example of an organism producing both HMB-PP and vitamin B2), responding Vγ9/Vδ2$^+$ T cells and MAIT cells together made up a large fraction of TNF-α (median of 31.7%) and IFN-γ (median of 39.2%) producing T cells, despite considerable variability across individual PD patients (Figure 3.11 and 3.12A). Of note, IFN-γ was produced principally by γδ T cells compared to MAIT cells (median of 33.0% vs 6.2 %, Figure 3.11 and 3.12A). By contrast, both cell types represented far lower proportions of lymphocytes amongst *E. coli* stimulated TNF-α$^-$ and IFN-γ$^-$ T cells (median of 7.7% and 8.2%, respectively, Figure 3.11 and 3.12A). Similar results were obtained using *C. striatum* extracts (data not shown).

Next, I measured the amount of IFN-γ released by PDE cells into the culture medium in response to different Gram$^+$ bacteria, and demonstrated that this cytokine was secreted only when the cells were exposed to extracts from bacteria possessing both HMB-PP and vitamin B2 (*S. aureus* and *C. striatum*), but not in the presence of *E. faecalis* extracts (HMB-PP$^-$ vitamin B2$^-$, Figure 3.12B). Similar results were obtained for IFN-γ-induced chemokine CXCL10. By contrast, levels of CXCL8 were comparable in response to all three Gram$^+$ bacteria (Figure 3.12B), confirming equal potential of the different organisms to stimulate peritoneal leukocytes other than unconventional T cells. Secretion of TNF-α was not assessed in these experiments, as any unconventional T cell-derived TNF-α would have been masked by TNF-α produced by peritoneal macrophages and neutrophils. In summary, unconventional T cells, while representing only a minor population of peritoneal T cells, are major producers of pro-inflammatory cytokines in response to a wide range of microbial pathogens.

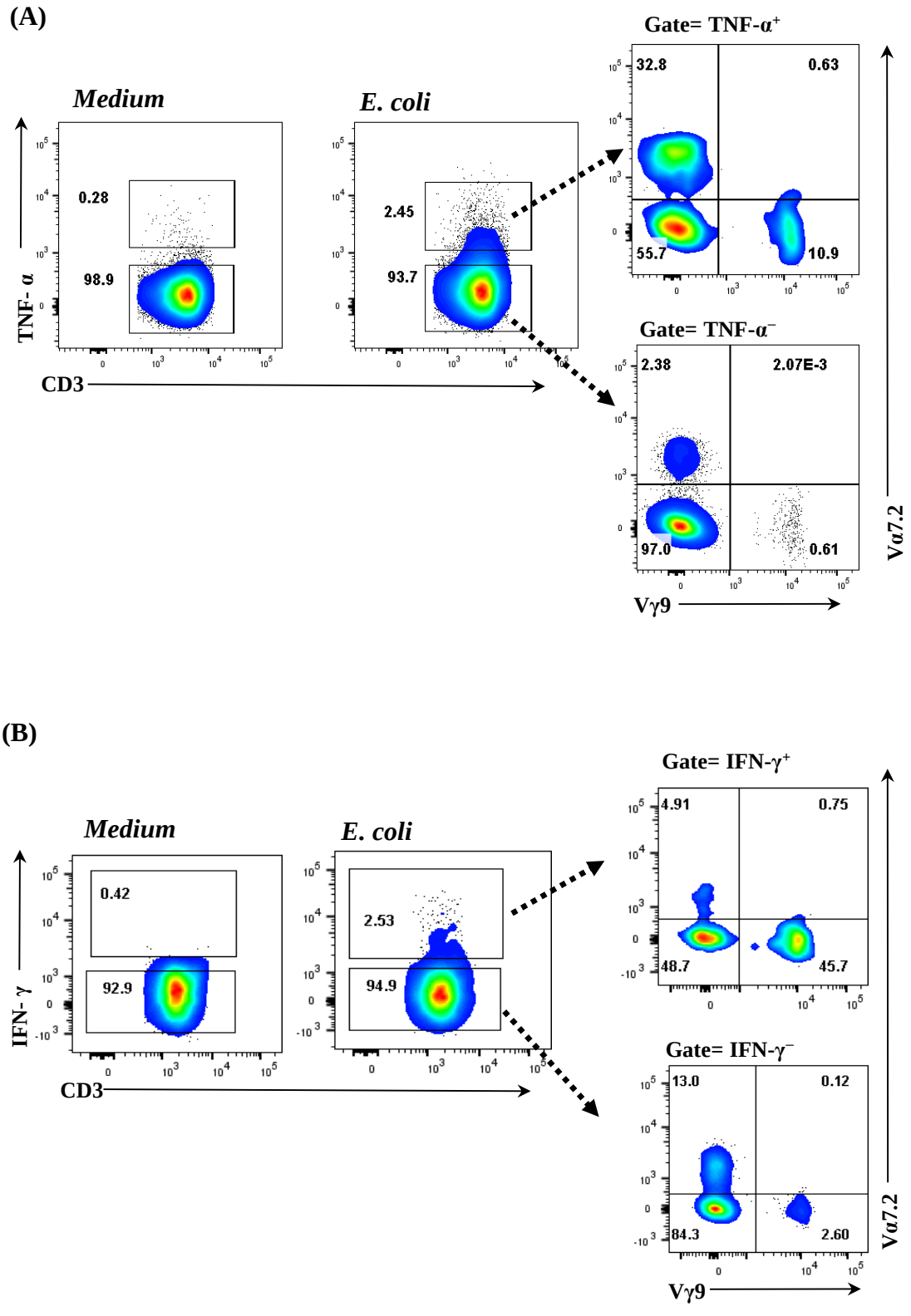

Figure 3.11▪ *In vitro* responsiveness of peritoneal leukocytes to pathogenic bacteria.
Intracellular staining of (*A*) TNF-α and (*B*) IFN-γ in peritoneal leukocytes cultured in the absence (medium; left panel) or presence of *E. coli* extract (middle panel), as analysed by flow cytometry within the $CD3^+$ gate. Right panel, distribution of $V\alpha7.2^+$ and $V\gamma9^+$ cells within all $CD3^+$ $cytokine^+$ peritoneal cells after stimulation with *E. coli* extract. Data shown are representative of 8-27 individual donors.

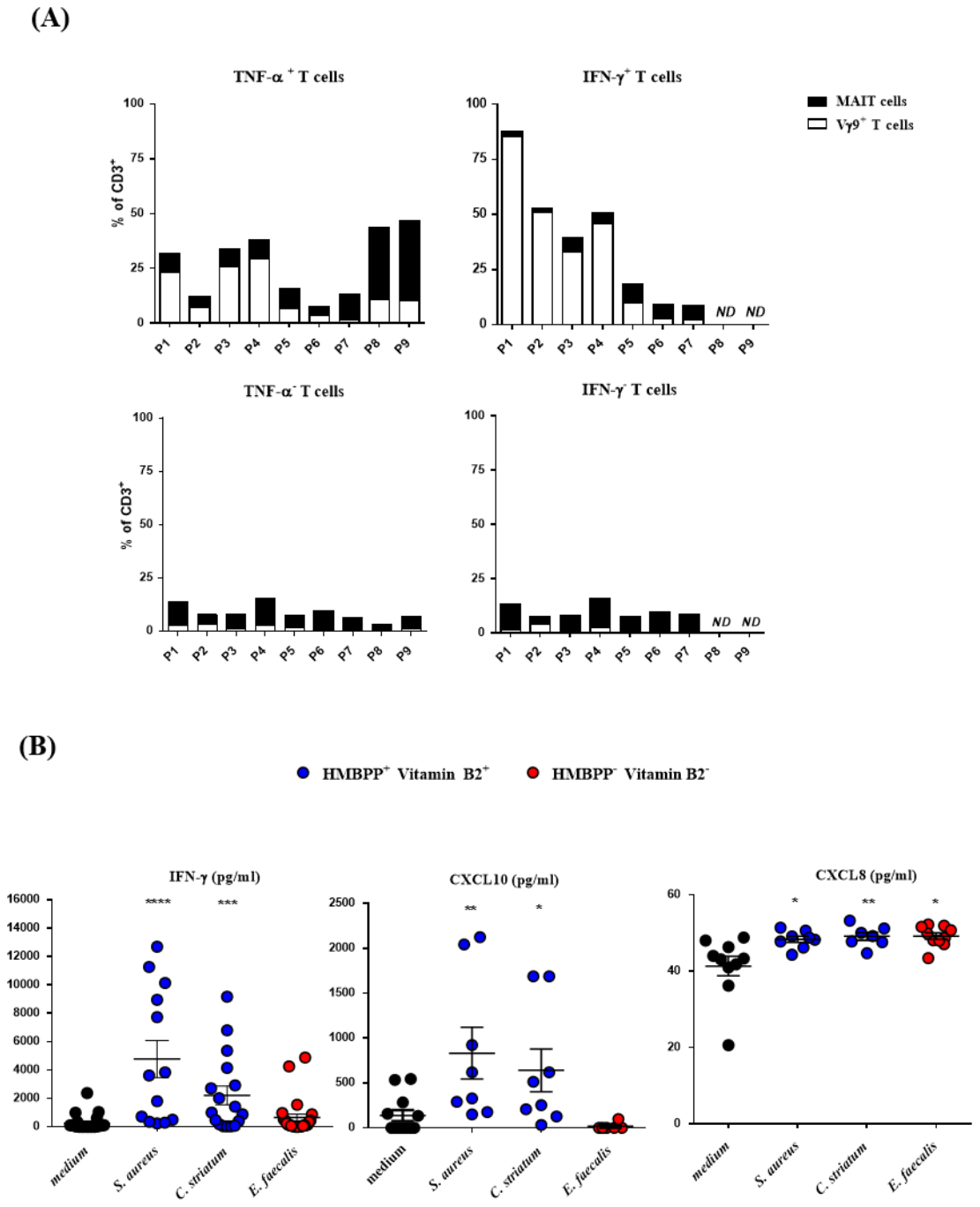

Figure 3.12. *In vitro* responsiveness of peritoneal leukocytes to pathogenic bacteria.
(*A*) Proportion of Vα7.2$^+$ and Vγ9$^+$ cells amongst peritoneal T cells producing TNF-α and IFN-γ in response to *E. coli*, as analysed by flow cytometry in nine stable PD patients. ND, not done. (*B*) Overnight secretion of IFN-γ, CXCL10 and CXCL8 by peritoneal leukocytes in response to *S. aureus*, *C. striatum* and *E. faecalis*, as analysed by ELISA (median ± SEM, n=27). Data were analysed using Kruskal-Wallis tests combined with Dunn's multiple comparisons tests. Each data point represents an individual patient; asterisks indicate significant differences compared to medium controls: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.3.6 Blocking antibodies against BTN3 and MR1 modulate pro-inflammatory cytokine production by unconventional T cells

During acute peritonitis, TNF-α and IFN-γ levels are frequently elevated (Lin et al., 2013). TNF-α and IFN-γ synergistically activate peritoneal tissue cells, thereby creating a positive feedback loop amplifying local chemokine and cytokine production during acute inflammation (Kawka et al., 2014; Visser et al., 1998). However, IFN-γ can have both pro- and anti-inflammatory effects, for instance in combination with IL-6, and regulate the recruitment of leukocytes to the site of infection (Fielding et al., 2014; McLoughlin et al., 2003). Together with TGF-β and IL-1β, TNF-α drives epithelial to mesenchymal transition (EMT) of mesothelial cells, which in the long term may lead to inflammation-associated damage of the peritoneal membrane (Aroeira et al., 2007; Wang, 2013).

As shown above, peritoneal γδ T cells and MAIT cells produced substantial amounts of TNF-α and IFN-γ in response to microbial pathogens. Given the impact of these two cytokines on leukocyte recruitment, resolution of the inflammatory response and local tissue damage, I performed blocking experiments to manipulate the amount of cytokines released by these cells in response to microbial pathogens. In the presence of anti-BTN3/CD277 mAbs there was significantly reduced peritoneal $V\gamma9^+$ T cell CD69 expression, either alone or in combination with TNF-α or IFN-γ, in response to HMB-PP^+ bacterial extracts (e.g. *E. coli, K. pneumoniae, P. aeruginosa, C. striatum*) (Figure 3.13 and 3.14) .

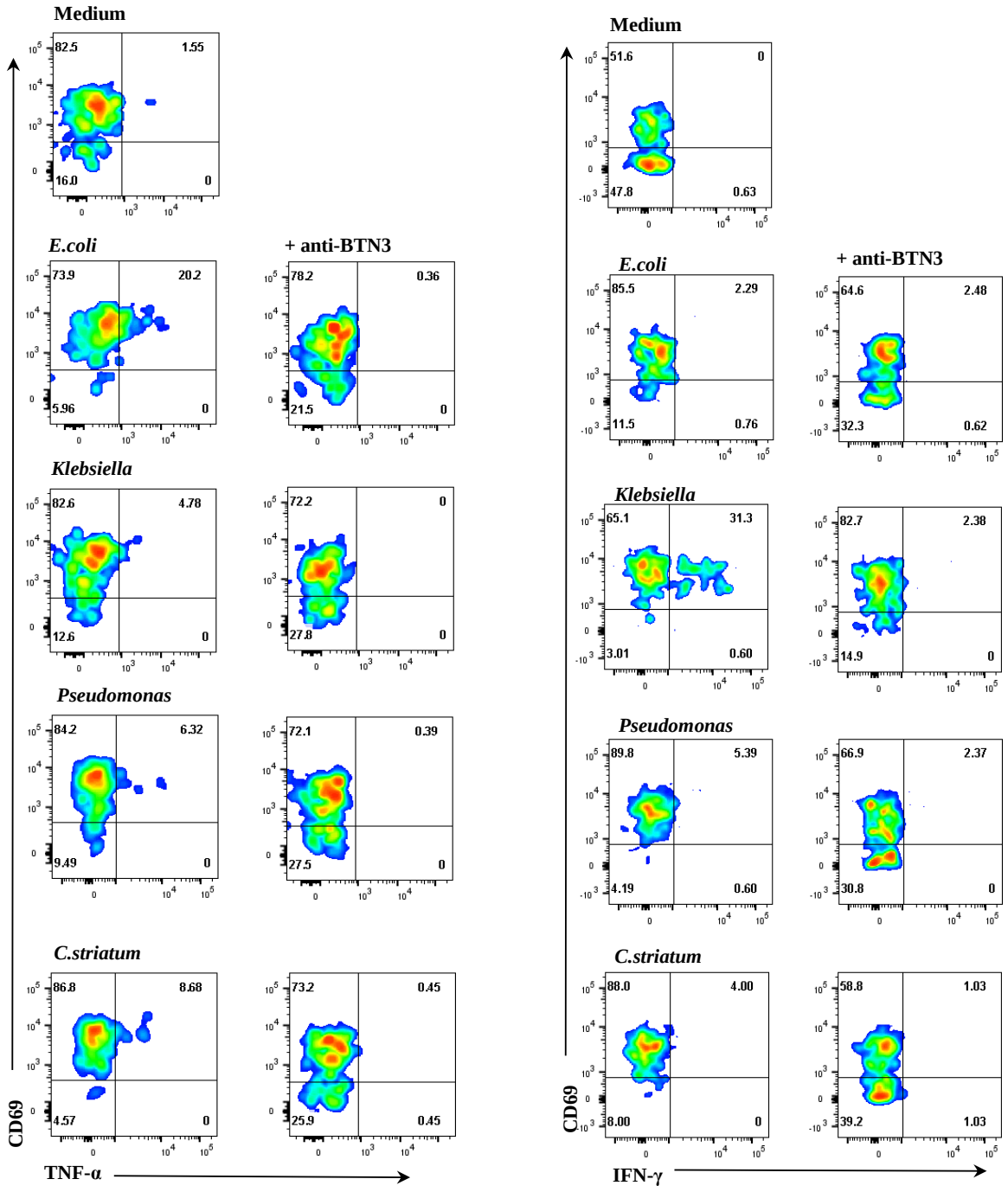

Figure 3.13. BTN3 dependent responses of peritoneal γδ T cells to Gram-positive and Gram-negative bacteria.

Activation of total peritoneal leukocytes by bacterial extracts, in the presence or absence of anti-BTN3 blocking antibodies, as analysed by flow cytometry within the Vγ9$^+$ CD3$^+$ cell gate. FACS plots show surface expression of CD69 and intracellular expression of TNF-α (left) or IFN-γ (right) after overnight stimulation; data are representative of independent experiments using five different donors.

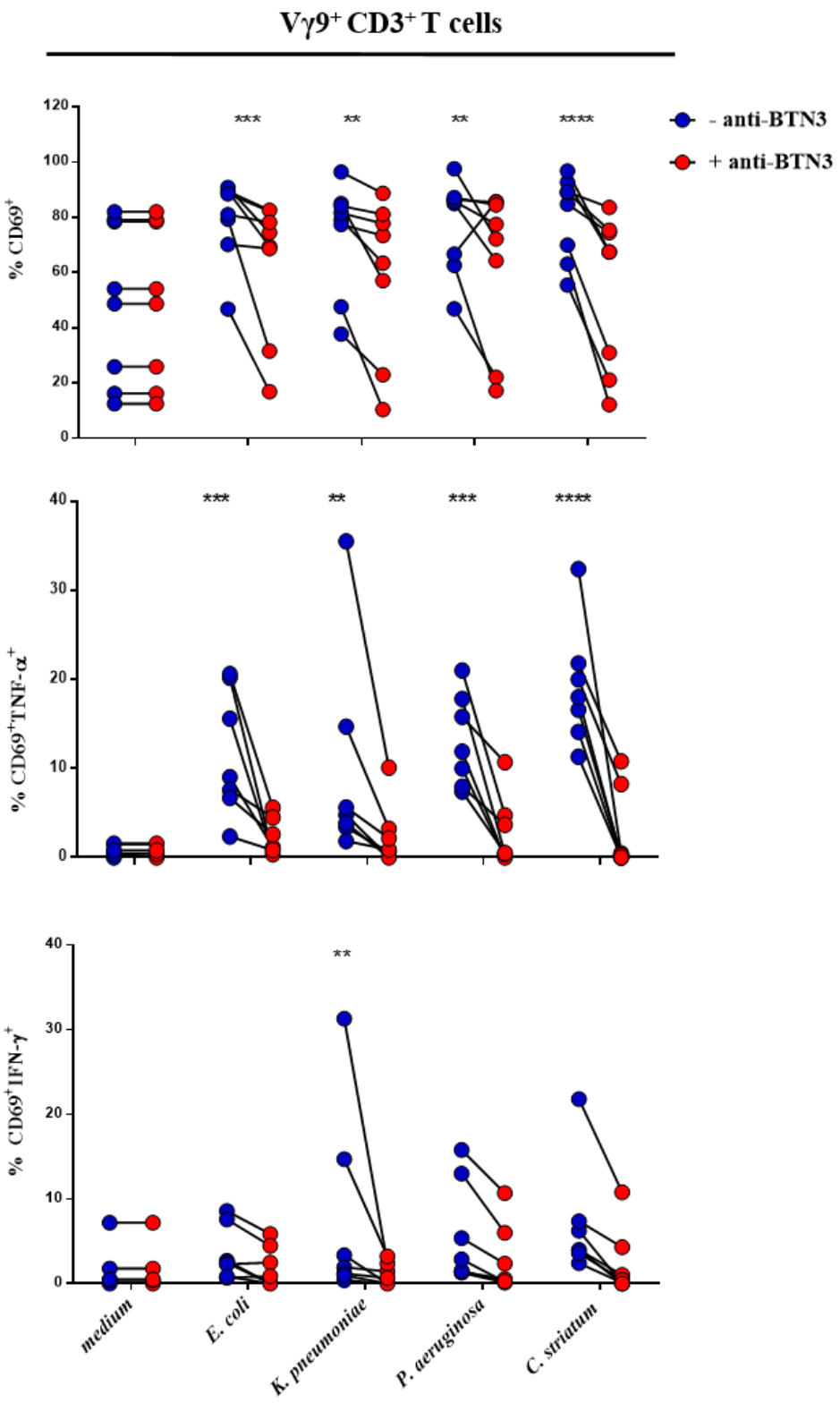

Figure 3.14. BTN3 dependent responses of peritoneal γδ T cells to Gram-positive and Gram-negative bacteria.

Summary of all BTN3 blocking experiments performed, shown as expression of CD69 (top), co-expression of CD69 and TNF-α (middle) or CD69 and IFN-γ (bottom) by Vγ9+ T cells after overnight stimulation with bacterial extracts. Data were analysed using two-way ANOVA and Bonferroni's post hoc multiple comparisons tests. Each data point represents an individual patient; asterisks depict significant differences of anti-BTN3 treated samples compared to untreated controls. Significant differences are indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

In the above paragraph I showed that PDE leukocytes secreted IFN-γ in response to HMB-PP and vitamin B2 positive bacteria but not in the presence of bacteria negative for these metabolites, indicating that unconventional T cells may have been responsible for these patterns. Supporting this hypothesis, blocking of BTN3 induced a significant reduction of IFN-γ release by PDE cells (Figure 3.15). IFN-γ levels were also reduced following the addition of anti-MR1 antibodies, but not significantly. However, at least in the case of *C. striatum*, the addition of anti-BTN3 and anti-MR1 antibodies together had an additive effect on IFN-γ inhibition (Figure 3.15), indicating a minor but key contribution of peritoneal MAIT cells to the overall IFN-γ production. Collectively, these experiments showed that, of all leukocytes present in PDE, specific inhibition of the small population of unconventional T cells reduced the production of pro-inflammatory cytokines by these cells.

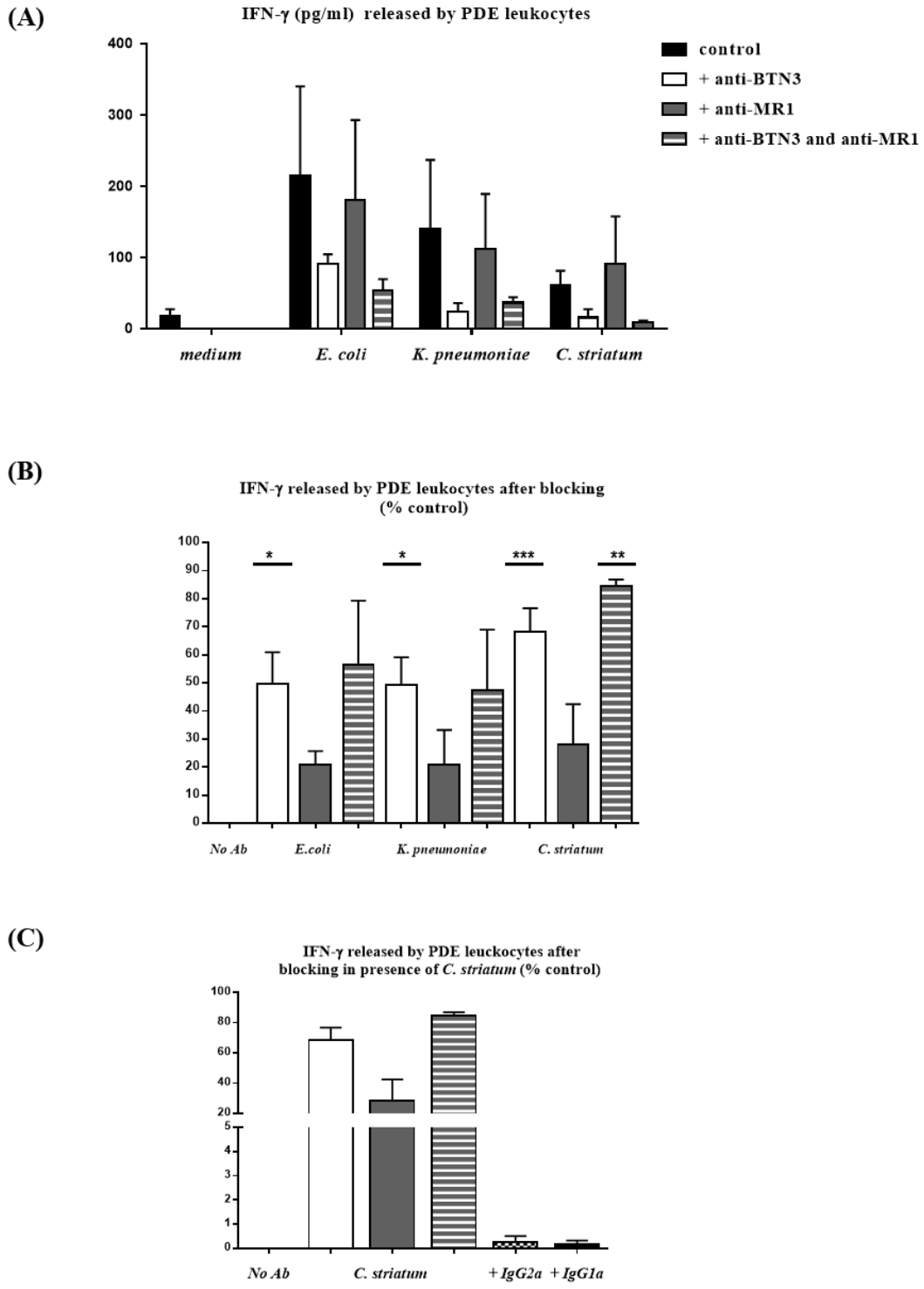

Figure 3.15. BTN3 and MR1 dependent secretion of IFN-γ by unconventional peritoneal T cells in presence of Gram-positive and Gram-negative bacteria.

(*A*) IFN-γ ELISA of cell culture supernatants derived from PDE leukocytes cultured for 20 h with extracts from HMB-PP$^+$ and vitamin B2$^+$ bacteria *K. pneumoniae*, *E.coli* and *C. striatum* in the absence or presence of blocking antibodies against BTN3 and MR1, alone or in combination. (*B*) Effect of the presence of anti-BTN3 and anti-MR1 blocking antibodies measured as relative inhibition of IFN-γ released by PDE leukocytes. (*C*) Effect of the presence of anti-BTN3 anti-MR1 antibodies and their respective isotype control measured as relative inhibition of IFN-γ released by PDE leukocytes in presence of *C. striatum*. Data were analysed by Kruskall Wallis test with Dunn's post-hoc test (mean ± SEM). Significant differences are indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.3.7 Pro-inflammatory cytokines released by PDE leukocytes in response to Gram$^+$ and Gram$^-$ bacteria

In addition to the clear association of peritoneal MAIT cells and Vγ9$^+$ T cell responses in the presence of distinct groups of bacterial pathogens, local immune fingerprints at the site of infection are likely to comprise immune mediators produced by different types of immune and non-immune cells. Previous work in the host laboratory showed that peritoneal levels of cytokines IL-10, IL-β, TNF-α and IL-6 predicted culture-positive infections at day 1 of acute peritonitis (Lin et al., 2013). Elevated levels of IL-10, TNF-α and IL-β were detected during episodes of peritonitis caused by Gram$^-$ bacteria.

To validate this distinct pattern of cytokine secretion in the presence of Gram$^-$ infections, PDE leukocytes from stable patients were cultured in the presence of different Gram$^-$ and Gram$^+$ bacterial extracts. As shown in Figure 3.16, the most pronounced synthesis of IL-10 by PDE cells occurred in the presence of Gram$^-$ *K. pneumoniae*, followed by *E. coli* and Gram$^+$ *C. striatum*. TNF-α and IL-β production was similar for Gram$^-$ and Gram$^+$ species. However, compared to the other Gram species, I did not observe a significant response in other cytokines release by cells in the presence of *P. aeruginosa*, one of the main bacteria responsible for Gram$^-$ infection (Figure 3.16).

IL-17 is another pro-inflammatory cytokine produced during PD associated infection that is synthesized mainly by resident cells at the site of infection, such as CD4$^+$ Th17 cells. A recent study by Rodrigues-Diez et al. showed that mice exposed to IL-17A develop long term peritoneal inflammation and fibrosis. Furthermore, IL-17A was present in peritoneal biopsies of inflamed tissue but absent in healthy individuals (Rodrigues-Díez et al., 2014). Given the importance of this cytokine during peritonitis, I analysed the presence of IL-17A in supernatant derived from PDE cells cultured in the presence of Gram$^+$ and Gram$^-$ bacterial extracts. My data show that IL-17A production increased in response to each bacterial extract (*E. coli, K. pneumoniae, P. aeruginosa, S. aureus, C. striatum, E. faecalis*) with the greatest increase in response to Gram$^+$ *S. aureus*. Collectively, these data confirm a contribution of peritoneal leukocytes to immune fingerprints in the presence of Gram$^+$ and Gram$^-$ infections.

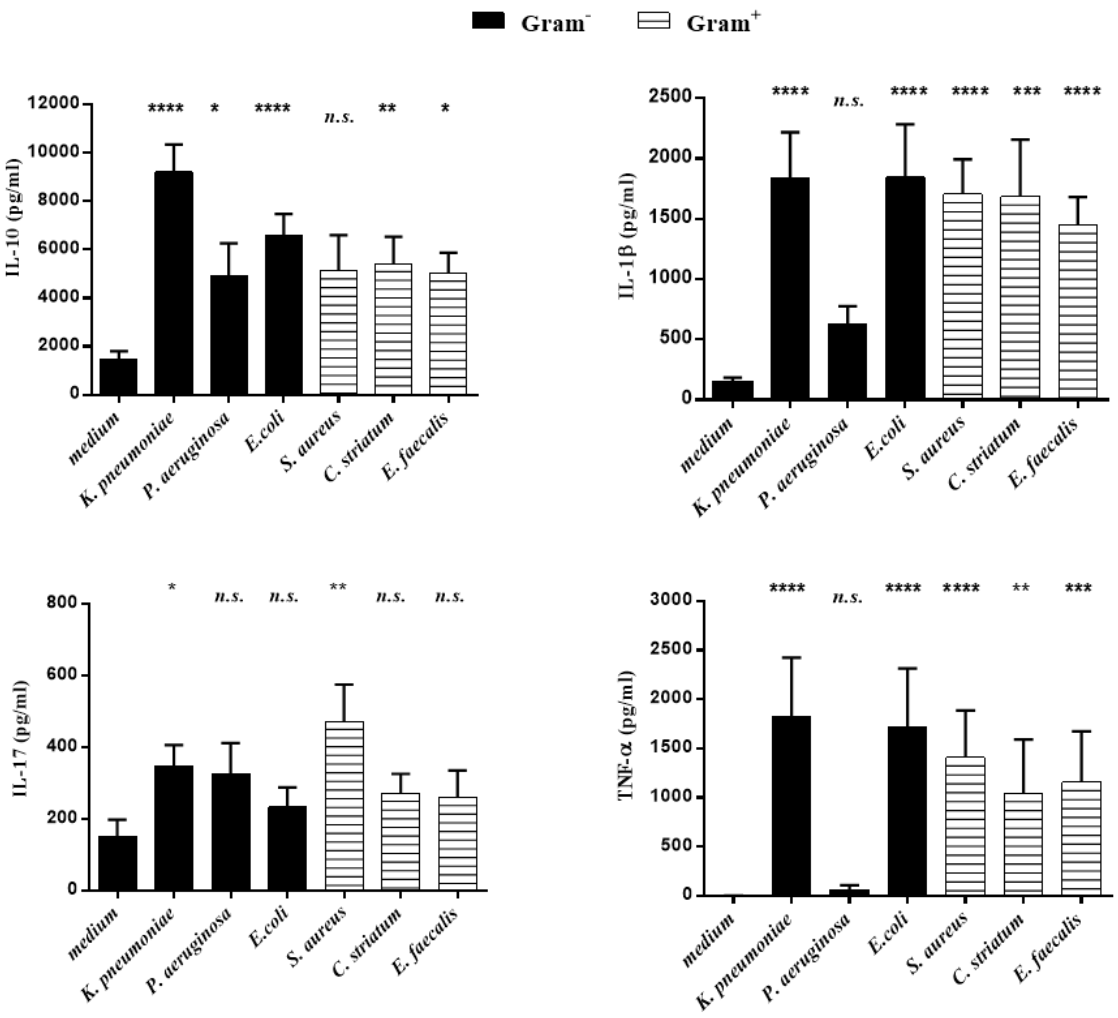

Figure 3.16. Cytokine secretion by PDE leukocytes in response to Gram-positive and Gram-negative bacteria.

Overnight secretion of IL-10, TNF-α, IL-17 and IL-1β by peritoneal leukocytes in response to Gram-positive (*S. aureus*, *C. striatum* and *E. faecalis*) and Gram-negative (*K. pneumoniae*, *P. aeruginosa* and *E. coli*) bacterial extracts, as determined by ELISA. Data were analysed using Kruskal Wallis test with Dunn's post-hoc test (mean ± SEM, n=3-28). Significant differences are indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.4 Discussion

In this Chapter I show for the first time how unconventional γδ T cells and MAIT cells may contribute to the amplification of inflammation during episodes of PD associated infection.

PD infections caused by Gram$^-$ bacteria (e.g. *E. coli*, *K. pneumoniae*, *Enterobacter*) are typically less frequent than Gram$^+$ infections (Ghali et al., 2011), but are associated with worse outcome (Chapter 1). A recent study has shown that peritoneal Vγ9/Vδ2 T cell frequency predicted Gram$^-$ infection at day 1 of infection, and the presence of these cells was associated with technique failure in 90 days (Lin et al., 2013). Here I confirmed that peritoneal Vγ9$^+$ T cells were activated by HMB-PP$^+$ bacteria but not by HMB-PP$^-$ pathogens, and release pro-inflammatory cytokines such as TNF-α and IFN-γ. Unexpectedly, γδ T cells were also activated in the presence of the HMB-PP$^-$ bacterium *S. aureus*, which might be BTN3 independent and instead mediated by superantigens such as staphylococcal enterotoxin A (SEA) (Morita et al., 2001). The vast majority of Gram$^-$ organisms causing peritonitis in PD patients are positive for both HMB-PP and vitamin B2, and are therefore likely to activate Vγ9$^+$ T cells as well as MAIT cells. Here, both peripheral and peritoneal MAIT cells were specifically activated by the vitamin B2 metabolite, DMRL, and in response to extracts from vitamin B2 producing Gram$^-$ and Gram$^+$ bacteria. MAIT cell responses to DMRL were not as pronounced as γδ T cell responses to HMB-PP. These modest responses were most likely due to the relatively modest bioactivity of DMRL compared to the true MAIT cell activators, 5-OP-RU 5-OE-RU (Corbett et al., 2014; Kjer-Nielsen et al., 2012), but these molecules are not commercially available and could not be tested in the present studies. Nevertheless, I observed that in some patients MAIT cells also became positive for CD69 and IFN-γ in the presence of vitamin B2$^-$ bacteria such as *E. faecalis*. This is possibly due to activation of MAIT in the presence of IL-12 and IL-18 in an MR1 independent manner (Ussher et al. 2014). In this system *E. faecalis* may induce a TLR2 dependent activation of peritoneal monocytes, which in turn activates MAIT cells by secretion of IL-12 and IL-18.

Davey *et al.* showed that elevated TNF-α at day 1 of peritonitis episodes is associated with technique failure and mortality (Davey et al., 2011a). In this chapter I demonstrated that peritoneal Vγ9/Vδ2 T cells and MAIT cells together represent up to 50% of TNF-α producing T cells of the PDE cells following stimulation with *E.coli* extract. This suggests that unconventional T cells, even if only present at small frequencies, are major producers

of pro-inflammatory cytokines in response to microbial stimulation. This has important implications for the orchestration of early immune responses at the site of infection.

Another pro-inflammatory cytokine released by activated unconventional T cells is IFN-γ, which is responsible for both leukocyte recruitment to the site of infection and their subsequent clearance to ensure resolution (Glik and Douvdevani, 2006; McLoughlin et al., 2003). Repeated episodes of infection or overproduction of IFN-γ will lead to a prolonged presence of neutrophils at the site of infection. In turn, neutrophils contribute to pathogen clearance via secretion of anti-microbial molecules, but the persistent presence of these cells can lead to the damage of the peritoneal membrane (Nathan, 2006). This means that the presence of IFN-γ at the wrong time and for too long at high concentration can lead to ultrafiltration failure (Davies, 2014). Furthermore, IFN-γ can induce fibrosis by excessive STAT1 activation and dysregulation of metalloprotease production (Fielding et al., 2014). I here showed that γδ T cells alone were able to contribute up to 50% and more of IFN-γ producing $CD3^+$ T cells in the presence of bacterial extracts, and that blocking of BTN3 was able to reduce this IFN-γ production substantially.

Alone or in combination TNF-α and IFN-γ produced by unconventional T cells are able to induce *i*) long-term survival and differentiation of neutrophils at the site of infection, which in turn are able to process antigens and prime $CD4^+$ and $CD8^+$ T cells (Davey et al., 2014); *ii*) differentiation of local monocytes into APCs (Eberl et al., 2009) and *iii*) activation of peritoneal tissue cells (Loghmani et al., 2002; Visser et al., 1998). All these processes may play a role in regulating local inflammatory responses at the site of infection with consequences for long term PD therapy failure, implying that the inactivation of this small population of unconventional T cells might have a significant impact on processes linked to fibrosis and ultrafiltration failure.

Previous findings in our laboratory showed Gram-negative infections were associated with increased levels of IL-1β, IL-10 and TNF-α in the PDE of infected patients compared to culture-negative and Gram-positive infection (Lin et al., 2013). I here tested whether or not it was possible to confirm this pattern of activation *in vitro* and determine the underlying mechanisms.

$Gram^-$ bacteria such as *E. coli* and *K. pneumoniae* induced more IL-10 in comparison to the $Gram^+$ bacteria analysed. However, PDE leukocyte released similar amounts of IL-1β and TNF-α in the presence of $Gram^+$ and $Gram^-$ bacteria. This can be explained by the difference

in PDE cell composition in stable patients and during Gram-negative infection. The latter is characterized by the presence of the potent bacterial ligand LPS, larger numbers of neutrophils and a lower proportion of monocytes/macrophages, which bear TLRs that respond promptly to LPS by releasing large quantities of cytokines. By contrast, $Gram^+$ infections are characterized by the presence of $Gram^+$ lipopeptides and large proportions of CD4 and CD8 T cells with lower TLR expression (Lin et al., 2013). This scenario is different to that in the stable patient, where the principal cell types are monocytes/macrophages followed by T cells and comparatively few neutrophils (Sharon Lewis and Clifford Holmes, 1991). Therefore, the similar release of TNF-α and IL-1β observed in the presence of $Gram^+$ and $Gram^-$ bacteria can be explained by similar monocyte TLR activation (Kumar et al., 2009) in presence of these bacteria (Davey et al. 2011).

IL-17 is produced during PD associated infection, secreted mainly by $CD4^+$ Th17 cells that are associated with inflammatory conditions such as rheumatoid arthritis and psoriasis (Niebuhr et al., 2011; Pène et al., 2008). The main function of this cytokine is to recruit neutrophils to the site of infection by regulating the release of CXCL8 and peptides with anti-microbial proprieties. Earlier studies showed association between IL-17 and peritoneal fibrosis in both mice and humans (Rodrigues-Díez et al., 2014). In addition, it has been shown that the balance between Th17 and regulatory T cells is crucial to maintain the integrity of the peritoneal membrane (Liappas et al., 2015). I here show that most $Gram^+$ and $Gram^-$ bacteria induced IL-17 production by PDE cells, and *S. aureus* was particularly effective. These results are supported by previous studies reporting elevated IL-17 production by PBMC cultured in the presence of *S. aureus* derived enterotoxins or supernatants (Islander et al., 2010). This effect was dependent on the presence of IL-1 receptor and induced chiefly by $CD4^+$ $IL\text{-}17^+$ T cells (Haileselassie et al., 2013; Islander et al., 2010; Niebuhr et al., 2011). Moreover, since *S. aureus* is a vitamin B2 positive bacterium, another source of IL-17 may be MAIT cells. Indeed, once activated, these cells release IL-17 and express CCR6, RORC and IL-23R, which are associated with Th17 profile (Dusseaux et al., 2011).

In summary in this chapter I showed a specific activation of peritoneal unconventional T cells in response to $HMB\text{-}PP^+$ and vitamin $B2^+$ organisms, resulting in the production of pro-inflammatory cytokines that can be modulated using monoclonal antibodies targeting the unconventional T cell presenting molecules BTN3 and MR1.

Chapter 4. *In vivo* analysis of Vγ9$^+$ T cells and MAIT cells during acute PD associated infections

4.1 Introduction

In Chapter 3, pro-inflammatory cytokine release by peritoneal Vγ9$^+$ T cells and MAIT cells in response to HMB-PP and/or vitamin B2 positive bacteria was shown. Moreover, it has been reported that in a cross-sectional cohort of PD patients Vγ9/Vδ2 T cells increase in frequency and number during infections caused by Gram-negative and HMB-PP producing bacteria (Davey et al., 2011a; Lin et al., 2013) confirming the ability of these cells to respond rapidly in acute infections. However, it is unclear whether this increase is caused by a preferential recruitment of Vγ9/Vδ2 T cells to the peritoneal cavity in certain infections and/or a result of ligand-specific local activation and expansion in response to the respective pathogens.

In this Chapter, I performed an *in vivo* analysis of Vγ9$^+$ T cells and MAIT cells during acute infections. Given the considerable biological variation between patients and the underlying pathologies, I analysed matched blood and PDE samples to examine systemic responses in blood and local responses in the peritoneal cavity, in stable PD patients before infection and again in the same individuals when presenting with acute peritonitis. Such matched investigations have never been attempted before, and highlight a unique advantage of studying individuals receiving PD.

4.2 Aims

The aims of this Chapter were:

1. To analyse expression of chemokines associated with cell migration during episodes of infection in PDE at day 1 of the infection.
2. To analyse unconventional T cells in the peripheral blood of stable PD patients for expression of chemokine receptors implicated in migration.
3. To analyse the frequencies of Vγ9/Vδ2 T cells and MAIT cells in the peritoneal cavity and in blood during episodes of PD associated infections.
4. To analyse unconventional T cell composition in both blood and peritoneal cavity with respect to patient age.

4.3 Results

4.3.1 Peripheral unconventional T cells express inflammatory chemokine receptors

PD associated peritonitis is characterised by a significant influx of leukocytes into the peritoneal cavity including unconventional T cells (Davey et al., 2011a, 2014; Glik and Douvdevani, 2006). This process involves adhesion of peripheral leukocytes to the endothelium, trans-endothelial migration and chemotaxis through the peripheral tissue (Bromley et al., 2008). This is mainly caused by the production of inflammatory chemokines by tissue resident cells. The balance among these chemokines is fundamental to avoid tissue fibrosis (Bromley et al., 2008; Charo and Ransohoff, 2006).

As shown in Figure 4.1, PDE from patients presenting with episodes of PD related peritonitis at the University Hospital of Wales showed an increase in chemokines including CXCL8, CCL2, CCL3, CCL4 and CCL20 at day 1 of the infection, compared to the effluent from stable non-infected patients. To test whether this chemokine increase depended on the causative pathogen, I divided the peritonitis group into subgroups according to the metabolic signature of the identified species (HMB-PP$^+$ vitamin B2$^+$, HMB-PP$^-$ vitamin B2$^+$ and HMB-PP$^-$ vitamin B2$^-$, and no growth). As shown in Figure 4.2, no significant differences between these groups and no growth was found for CCL20 release. By contrast, multiple comparisons showed that CCL2, CCL3 and CCL4 are significantly increased in infection caused by HMB-PP$^+$ and vitamin B2$^+$ microbes compared to the no growth culture results (Figure 4.2). Therefore, an increase of these chemokines during infection caused by

HMB-PP$^+$ and/or vitamin B2$^+$ bacteria might lead to migration of peripheral unconventional T cells to the infection site. Moreover, increased neutrophil chemotactic factor CXCL8 was observed, most clearly in the HMB-PP$^+$ and vitamin B2$^+$ groups (Figure 4.2), supporting a role for this chemokine in the recruitment of neutrophils at the site of infection during Gram negative (HMB-PP and vitamin B2 positive) infections (Lin et al., 2013).

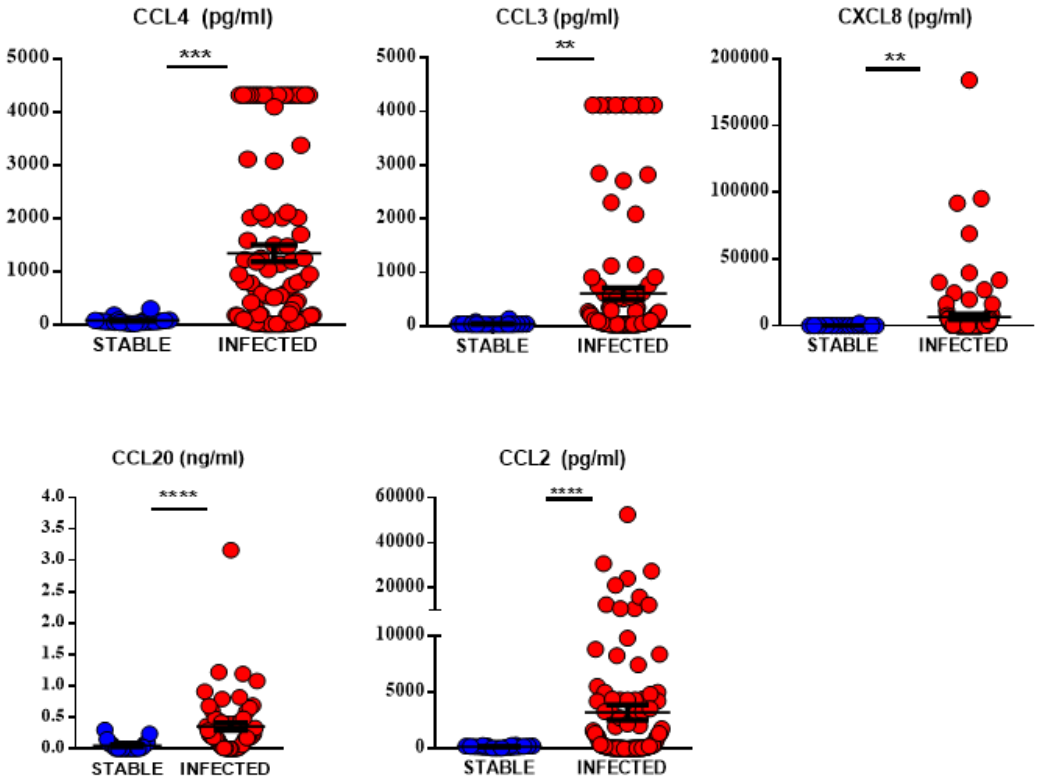

Figure 4.1. Peritoneal levels of pro-inflammatory chemokines in stable and infected PD patients.
PDE was collected from stable PD patients and from patients presenting with acute peritonitis, and analysed for the presence of the neutrophil-attracting chemokine CXCL8 and the T cell and monocyte attracting inflammatory chemokines, CCL2, CCL3, CCL4 and CCL20. Data were analysed using Mann Whitney tests. Each data point represents an individual patient, error bars depict the median ± interquartile range. *, $p<0.05$; **, $p<0.01$; ***, $p< 0.001$; ****, $p< 0.0001$.

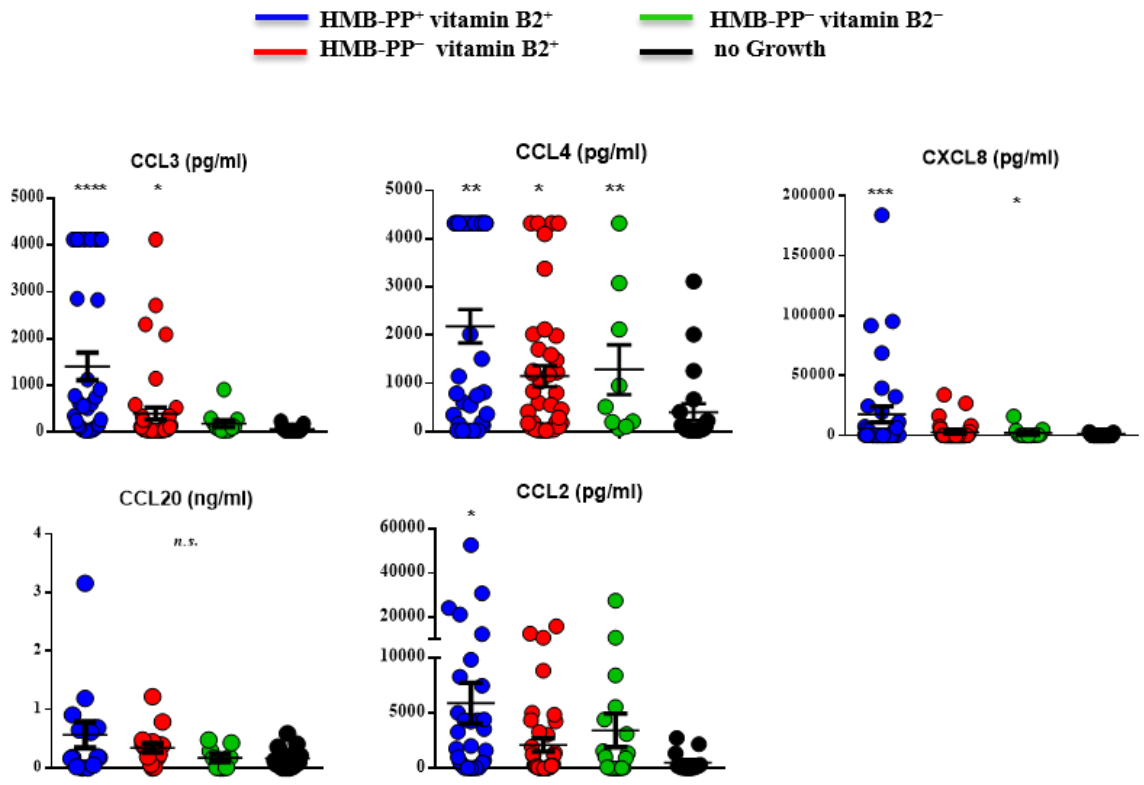

Figure 4.2. Peritoneal levels of inflammatory chemokines in infected PD patients.
PDE was collected from stable PD patients and from patients presenting with acute peritonitis and analysed for neutrophil-attracting chemokine CXCL8 and the lymphocyte and monocyte attracting inflammatory chemokines CCL2, CCL3, CCL4 and CCL20. Infected patients were divided by the microbiological results in infections HMB-PP$^+$ vitamin B2$^+$ (blue), HMB-PP$^-$ vitamin B2$^+$ (red), HMB-PP$^-$ vitamin B2$^-$ (green) and no growth (black). Data were analysed using Kruskal Wallis test with Dunn's post hoc test, and comparisons were made with the no growth group. Each data point represents an individual patient, error bars depict the mean±SEM. *, p<0.05; **, p<0.01; ***, p< 0.001; ****, p< 0.0001.

Next, to test if Vγ9$^+$ T cells and MAIT cells could potentially migrate to the infection site in response to these chemokines, expression of chemokine receptors CCR5 (for CCL4 and CCL3), CCR2 (for CCL2) and CCR6 (for CCL20) was measured on γδ T cells (CD3$^+$ Vγ9$^+$) and on MAIT cells (Vα7.2$^+$ CD161$^+$) from peripheral blood of stable PD patients. The flow cytometry analysis in Figure 4.3A shows significant upregulation of CCR5, CCR2 and CCR6 expression on Vα7.2$^+$ CD161$^+$ MAIT cells compared to their Vα7.2$^-$ CD161$^+$ T cell counterparts. Similarly, Vγ9$^+$ T cells showed a significantly increased expression of CCR5 and CCR6 compared to the non-Vγ9$^+$ cells. CCR2 was also present on γδ T cells, but the expression level was comparable to other CD3$^+$ T cells (Figure 4.3A).

These migratory profiles highlight the potential of blood Vγ9$^+$ T cells and MAIT cells to migrate toward locally expressed CCL2, CCL3, CCL4 and CCL20 at the site of infection, in keeping with a substantial increase in absolute numbers of both Vγ9/Vδ2 T cells and MAIT cells during acute peritonitis compared to stable patients (Figure 4.3B). These findings indicate that unconventional T cells may be rapidly co-recruited from blood to the inflamed peritoneal cavity along with neutrophils, and complement the local pool of tissue-resident Vγ9/Vδ2 T cells and MAIT cells already present in stable PD patients.

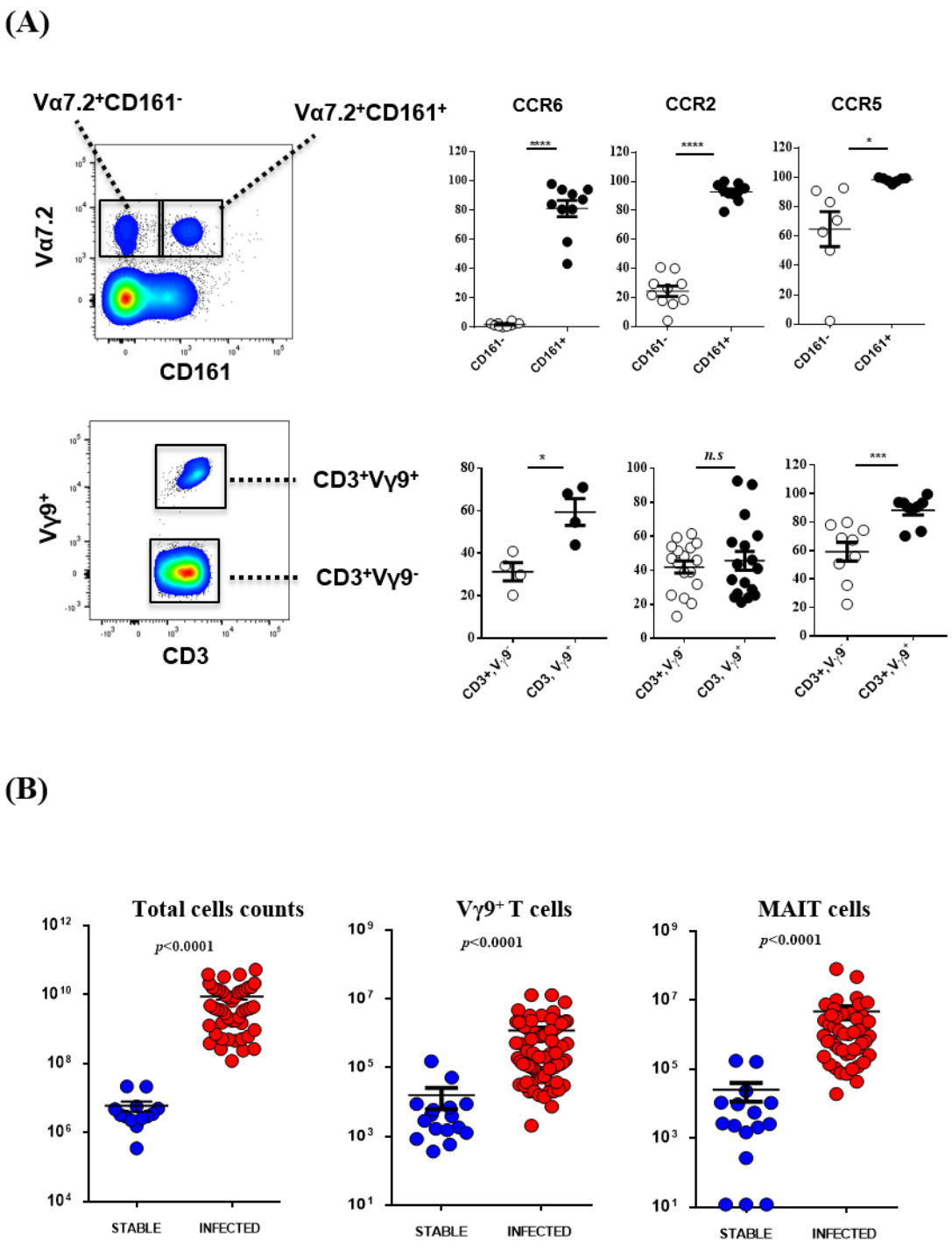

Figure 4.3. Migratory profile of peripheral blood Vγ9$^+$ T cells and MAIT cells.
(A) Peripheral blood samples from stable PD patients were analysed for chemokine receptor expression by flow cytometry on circulating Vγ9/Vδ2 T cells and MAIT cells. Panels show the percentage of CCR2$^+$, CCR5$^+$ and CCR6$^+$ cells in Vα7.2$^+$ CD161$^-$ and Vα7.2$^+$ CD161$^+$ CD3$^+$ T cells (upper panels), and in Vγ9$^-$ and Vγ9$^+$ CD3$^+$ T cells (lower panels). (B) Peritoneal cells were collected from stable PD patients and from patients presenting with acute peritonitis, and examined by flow cytometry. Data shown are total cell counts and the total numbers of Vγ9$^+$ CD3$^+$ T cells and Vα7.2$^+$ CD161$^+$ MAIT cells within the peritoneal cell population. Data were analysed using Mann Whitney tests. Each data point represents an individual patient, error bars depict the mean ± SEM. *, p<0.05; **, p<0.01; ***, p< 0.001; ****, p< 0.0001.

4.3.2 Local enrichment of γδ T cells and MAIT cells during acute infection caused by bacterial pathogens producing HMB-PP and vitamin B2

Previous work done in our laboratory showed a significant increase in the frequency of Vγ9/Vδ2 T cells in the peritoneal cavity of patients infected with HMB-PP$^+$ and/or Gram bacteria compared to HMB-PP$^-$ infections (Davey et al., 2011a; Lin et al., 2013). However, it is unclear whether this increase is caused by preferential recruitment of Vγ9/Vδ2 T cells to the peritoneal cavity during such infections and/or this is a result of ligand-specific local activation and expansion in response to the respective pathogens. To address this question, the frequencies of Vγ9$^+$ T cells and MAIT cells were measured in infected and stable patients and compared to the frequencies of the same cell types in blood.

As shown in Figure 4.4A, no differences were found in the frequency of peripheral Vγ9$^+$ T cells between stable and infected patients, nor in the frequency of Vγ9$^+$ T cells between the peritoneal cavity and blood in the absence of infection. However, there was a significant enrichment of Vγ9$^+$ T cells in the peritoneal cavity of patients presenting with peritonitis compared to the peripheral blood (median 1.74% vs 0.52%) but there was not difference in the frequency of peritoneal Vγ9$^+$ T cells between stable and infected patients (Figure 4.4A).

Unlike γδ T cells, MAIT cells were already present in a larger proportion in the peritoneal cavity of stable patients when compared with blood (median 1.8% vs. 0.56%). This proportion increased significantly during episodes of infection, but not in comparison with non-infection status in the peritoneal cavity (median 1.80% vs 1.5%) (Figure 4.4B).

This analysis confirmed a significant difference in the Vγ9$^+$ T cell frequency between HMB-PP$^+$ and HMB-PP$^-$ infection (median 2.6% vs 1.35%) suggesting increased recruitment and/or proliferation of these cells at the site of infection in response to HMB-PP$^+$ bacteria. However, I did not find a similar difference with Vγ9$^+$ T-cells in culture negative results (Figure 4.5A). The frequency of MAIT cells was measured in patients presenting with peritonitis caused by vitamin B2$^+$ and vitamin B2$^-$ bacteria. Although not significantly different, the MAIT cell frequency was higher in vitamin B2$^+$ infection than vitamin B2$^-$ infection (median 2.7% vs 1.2%, Figure 4.5B). In addition, the frequency of MAIT cells in vitamin B2$^+$ infection was significantly higher compared to culture negative episodes of peritonitis (Figure 4.5B).

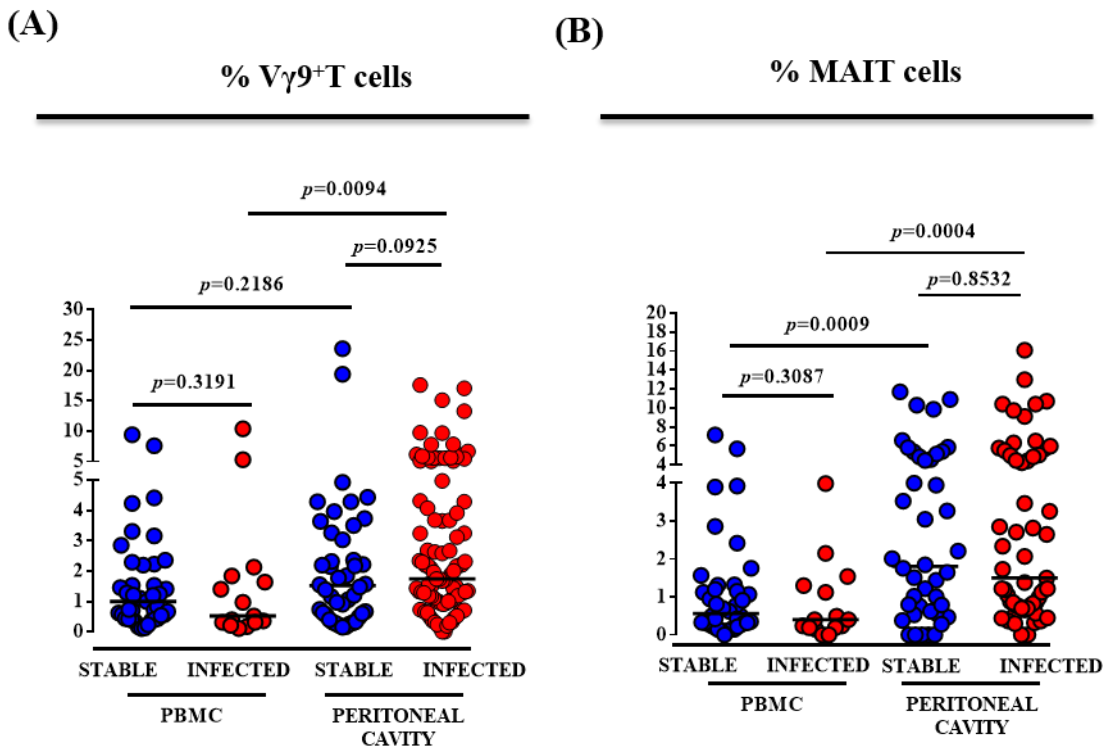

Figure 4.4. Vγ9$^+$ T cell and MAIT cell frequencies in peripheral blood and peritoneal cavity of stable patients and patients with acute peritonitis.
(A) Flow cytometry analysis of Vγ9$^+$ T cell frequency in blood (n=41) and peritoneal cavity (n=44) of stable patients (blue), and in patients with acute peritonitis (n=15 and n=99, respectively, shown in red). (B) Scatter dot plot of MAIT cell proportions in blood and peritoneal cavity of stable patients (n=40 and n=42, respectively) and during peritonitis (n=15 and n=57, respectively). Data were analysed using Mann Whitney tests. Each data point represents an individual patient, *, p<0.05; **, p<0.01; ***, p< 0.001; ****, p< 0.0001.

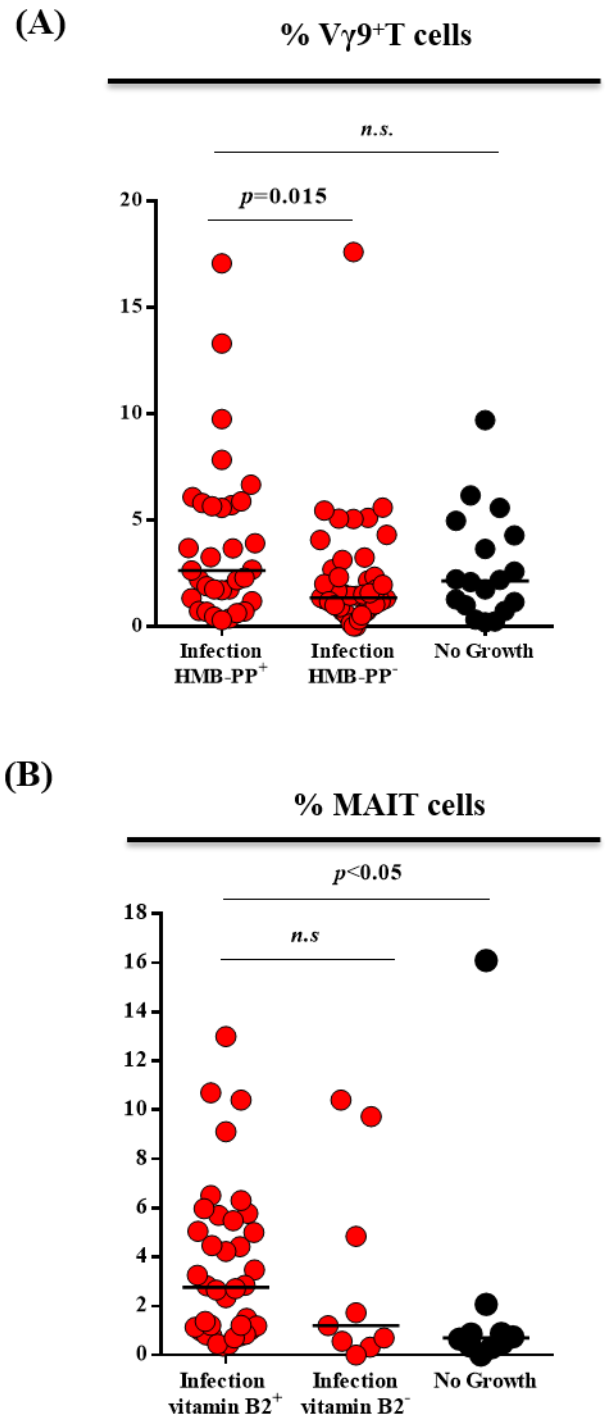

Figure 4.5. Vγ9$^+$ T cell and MAIT cell frequencies in the peritoneal cavity of patients with acute peritonitis.

Flow cytometry analysis of Vγ9$^+$ T cells and MAIT cells in the peritoneal cavity of PD patients with episodes of acute peritonitis that was either culture positive (red) or culture negative (black). (A) Scatter dot plot of Vγ9$^+$ T cells in the peritoneal cavity during acute peritonitis according to the HMB-PP status of the causative organism (HMB-PP$^+$, n=33; HMB-PP$^-$, n=49; culture negative episodes, n=18). (B) MAIT cell frequency in the peritoneal cavity during acute peritonitis according to the vitamin B2 status of the causative organism (vitamin B2$^+$, n=38; vitamin B2$^-$, n=9; culture negative episodes, n=10). Data were analysed using Kruskal Wallis test with Dunn's post hoc test. Each data point represents an individual patient, *, p<0.05; **, p<0.01; ***, p< 0.001; ****, p< 0.0001.

Mindful of the biological variation between individuals (Figures 4.4 and 4.5) frequencies of $V\gamma9^+$ T cells and MAIT cells in the blood and peritoneal cavity of PD patients from a longitudinal study were then examined before and after episodes of acute peritonitis by studying matched samples from the same individuals.

The proportions of Vγ9/Vδ2 T cells among all $CD3^+$ T cells in blood and peritoneal cavity were comparable in the absence of infection, and on the day of presentation with acute peritonitis there was a trend towards elevated levels of Vγ9/Vδ2 T cells compared to blood (median 1.19% vs 0.52%, Figure 4.6). Although not statistically significant due to the low number of matched samples available for this study, this increase of local Vγ9/Vδ2 T cell levels compared to blood was apparent in patients infected with HMB-PP^+ bacteria (4/4) but not in patients with HMB-PP^- infections (Figure 4.6, upper panel), despite comparable peritoneal chemokine profiles between HMB-PP^+ and HMB-PP^- infections (Figure 4.2).

There was a significant increase in local Vγ9/Vδ2 T cell levels in patients who developed acute peritonitis compared to the stable state (median 3.7% vs 1.56%, Figure 4.7). When the infected group was subdivided into infections caused by HMB-PP^+ and HMB-PP^- organisms, significantly increased levels of Vγ9/Vδ2 T cells were observed in matched patients with HMB-PP^+ infection (median 5.6% vs. 1.9%) but not in patients infected with HMB-PP^- bacteria (Figure 4.7). These findings suggest that Vγ9/Vδ2 T cells accumulate locally at the site of infection in response to HMB-PP^+ but not HMB-PP^-, organisms confirming the notion of a ligand-induced local expansion.

Similar results were observed for peritoneal MAIT cells. While in stable PD patients the local MAIT cell levels in the peritoneal cavity were significantly higher than those in blood (median 1.85% vs. 0.77%), such differences between anatomical sites were much more pronounced in acutely infected individuals (median 3.30% vs. 0.40%, Figure 4.6). Further, peritoneal MAIT cells increased significantly in matched patients affected by vitamin $B2^+$ infection but not in vitamin $B2^-$ compared to the blood levels (Figure 4.6). Similarly, peritoneal cavity MAIT cells increased during infection compared to the stable state (5.6% vs. 1.65%, Figure 4.7). This increase was significant in vitamin $B2^+$ infection (5.64% vs 1.53%) but not in infections by vitamin $B2^-$ organisms (Figure 4.7).

In summary, these results confirm that $V\gamma9^+$ T cells and MAIT cells not only are able to enrich the site of infection during PD associated peritonitis but that they appear to expand and increase in the peritoneal cavity in response to bacteria producing the corresponding

ligands. The specificity of this responsiveness by local Vγ9$^+$ T cells and MAIT cells implies that their frequency might be used as biomarker to discriminate HMB-PP and/or vitamin B2 positive infection from infections where these metabolites are absent.

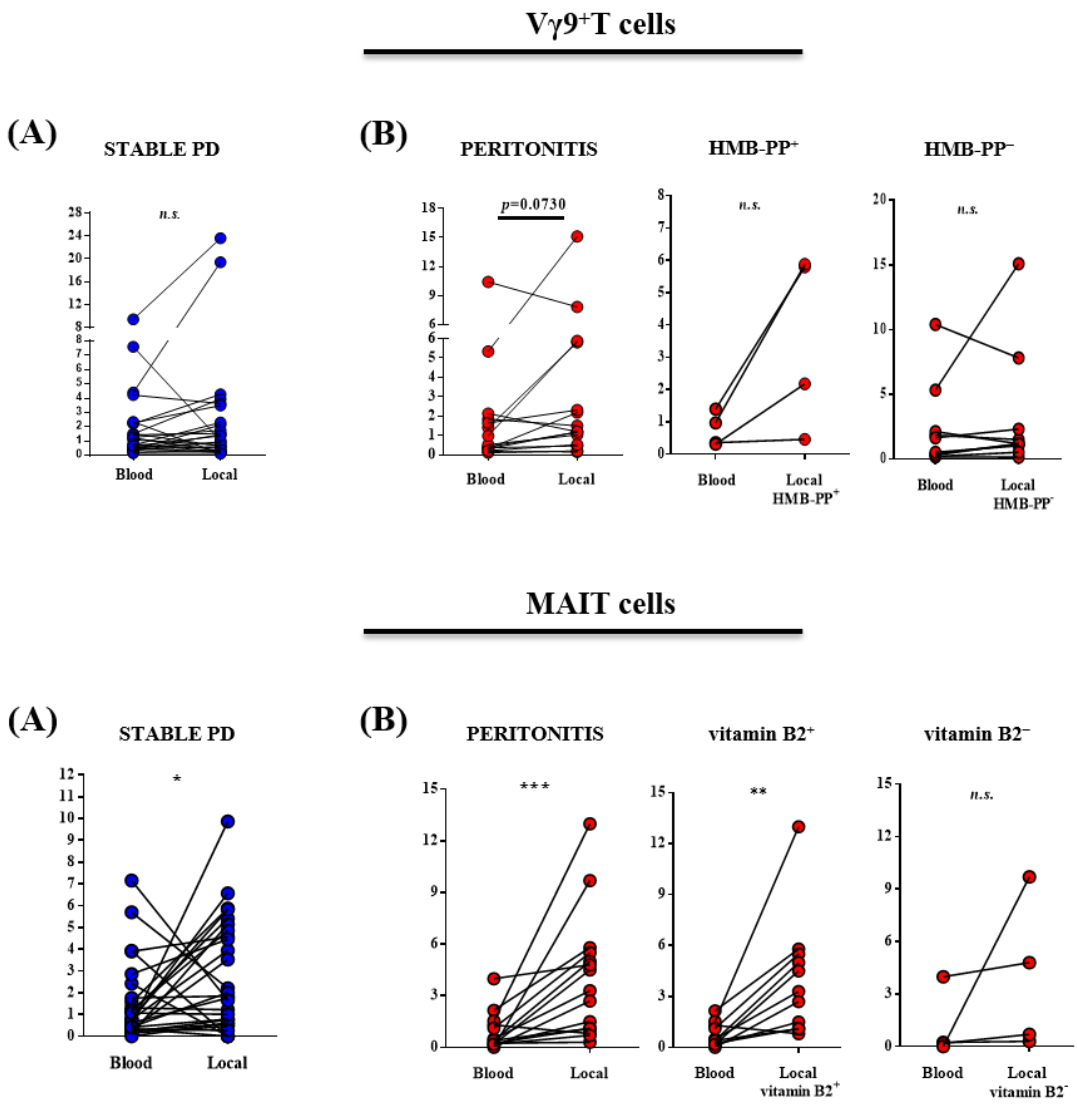

Figure 4.6. Unconventional T cells in matched blood and PDE samples in stable PD patients and during acute peritonitis.
Unconventional T cell levels in blood and effluent of stable (A, blue) and during episodes of acute peritonitis (B, red). Matched samples from the same individuals were analysed by flow cytometry for the proportion of Vγ9/Vδ2 T cells (identified as Vγ9$^+$; *upper panels*) and MAIT cells (Vα7.2$^+$ CD161$^+$; *lower panels*), expressed as percentage of all CD3$^+$ T cells. Samples were collected whilst patients were stable and again when they presented with acute peritonitis (day 1), before commencing antibiotic treatment. Vγ9$^+$ T cells were measured in 26 stable and 16 matched infected patients, MAIT cell data are from 27 and 15 matched infected patients. Patients with confirmed infections were divided into HMB-PP and vitamin B2 status subgroups. Data were analysed using Wilcoxon matched-pairs signed rank tests. Each data point represents an individual patient. *, p<0.05; **, p<0.01; ***, p< 0.001; ****, p<0.0001.

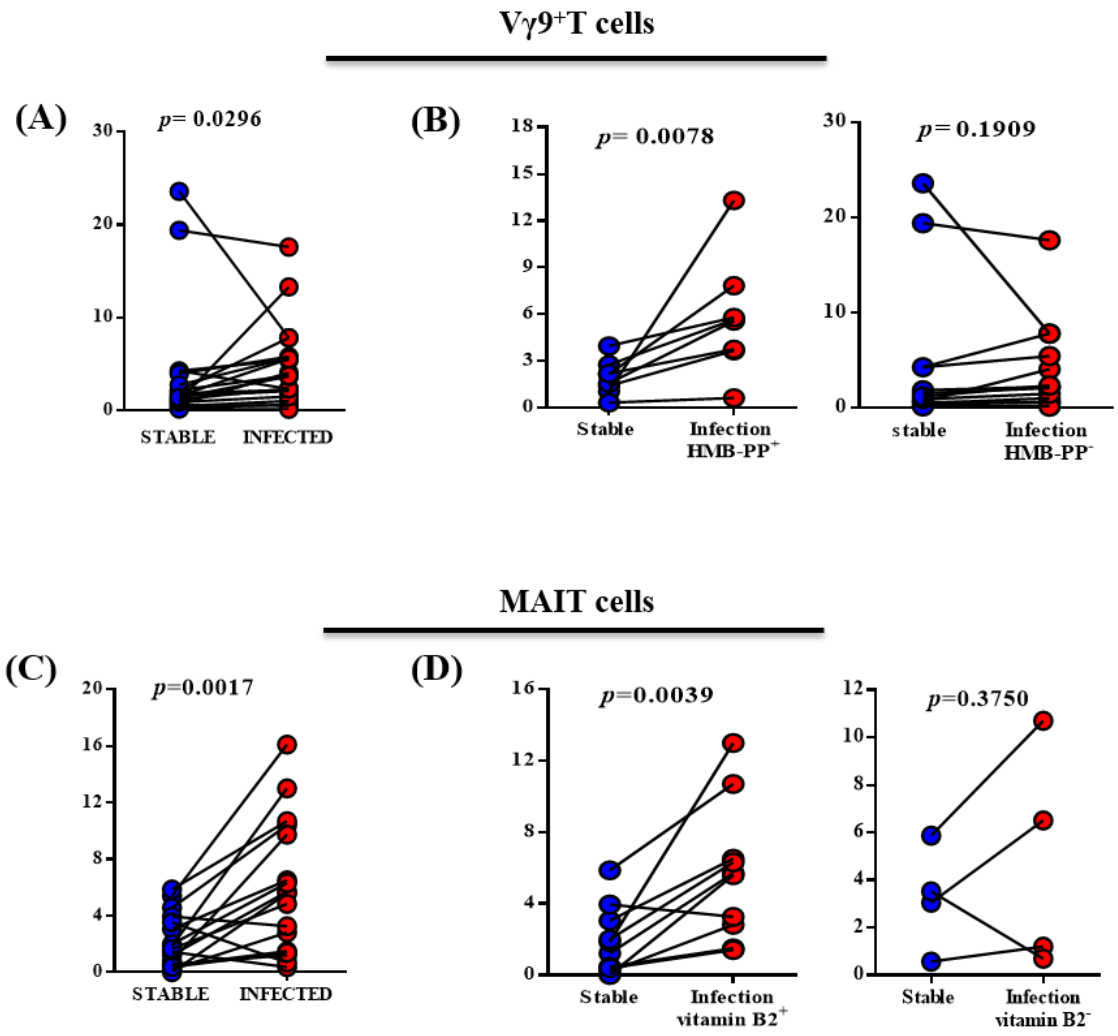

Figure 4.7. Unconventional T cells in matched PDE samples from PD patients before and during acute peritonitis.

Unconventional T cell levels in peritoneal effluent of individuals when they were stable (blue) and when they presented with acute peritonitis (red), before commencing antibiotic treatment. Matched peritoneal effluents from the same individuals were analysed by flow cytometry for the proportion of Vγ9/Vδ2 T cells (identified as $V\gamma9^+$; *upper panels*, A and B) and MAIT cells ($V\alpha7.2^+$ $CD161^+$; *lower panels*, C and D), expressed as percentage of all $CD3^+$ T cells. $V\gamma9^+$ T cells were measured in 20 individuals, MAIT cells in 17 individuals. Patients with confirmed infections were divided into HMB-PP and vitamin B2 status subgroups. Data were analysed using Wilcoxon matched-pairs signed rank tests. Each data point represents an individual patient. *, $p<0.05$; **, $p<0.01$; ***, $p< 0.001$; ****, $p<0.0001$.

4.3.3 Decreased frequencies of peritoneal MAIT cells and Vγ9/Vδ2 T cells in elderly patients

Previous findings related to the measurement of γδ T cells and MAIT cells in peripheral blood showed an age-dependent decline of these cells in elderly individuals (Caccamo, 2006; Colonna-Romano et al., 2002; Novak et al., 2014). The patients recruited for the present study had an average age of 68.4 years with SD ± 13.6 (Chapter 2). I therefore investigated whether the age of these patients might affect the frequencies of unconventional T cells in both blood and peritoneal cavity of stable and infected patients, as possible confounders of pathogen-specific immune profiles.

As shown in Figure 4.8, the frequency of $V\gamma9^+$ T cells in blood of stable and infected patients were not correlated with age. The failure to confirm earlier findings in healthy individuals (Caccamo, 2006) may have been due to the skewed age profile of the PD patients in the present study as $V\gamma9^+$ T cells were reported to peak in younger adults at an age that was not represented sufficiently here. However, a significant negative correlation between $V\gamma9^+$ T cells in the peritoneal cavity in stable patients and age was observed (Figure 4.9).

As shown in Figure 4.8, like $V\gamma9^+$ T cells, MAIT cells in blood of stable and infected patients were not correlated with age. Although no correlation of MAIT cell frequencies with age was found in stable PD patients, MAIT cells showed a significant decrease with age in the peritoneal cavity of infected patients (Figures 4.9), indicating that patient age might affect the incidence of unconventional T cells present at the peritoneal site.

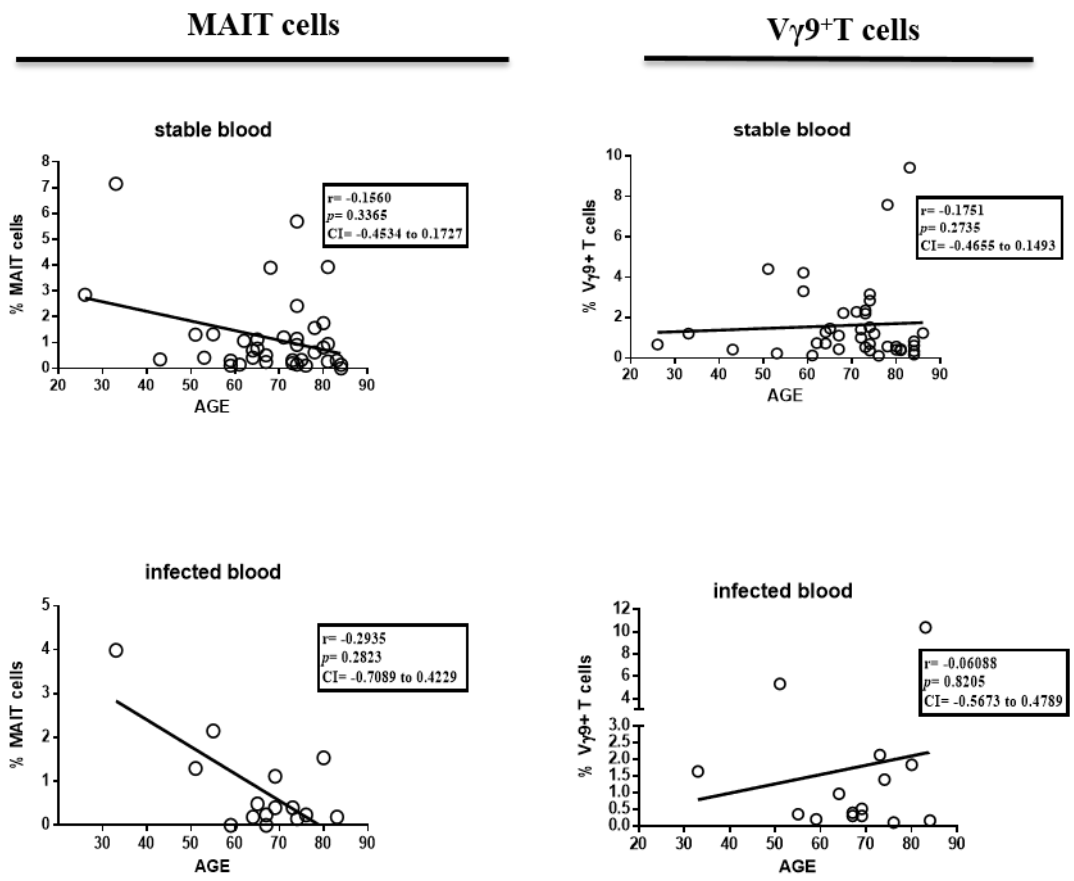

Figure 4.8. Correlation between frequencies of blood unconventional T cells and patient age.
Scatter plot showing correlations (Spearman's rank correlation coefficient) between frequency of blood MAIT cells (left column) and age in a cohort of stable patients (n=40, upper graph) and during acute peritonitis (n=15, lower graph). Right column, correlation of peripheral Vγ9/Vδ2 T cells with age in a cohort of stable patient (n=41, upper graph) and with acute peritonitis (n=15, lower graph).

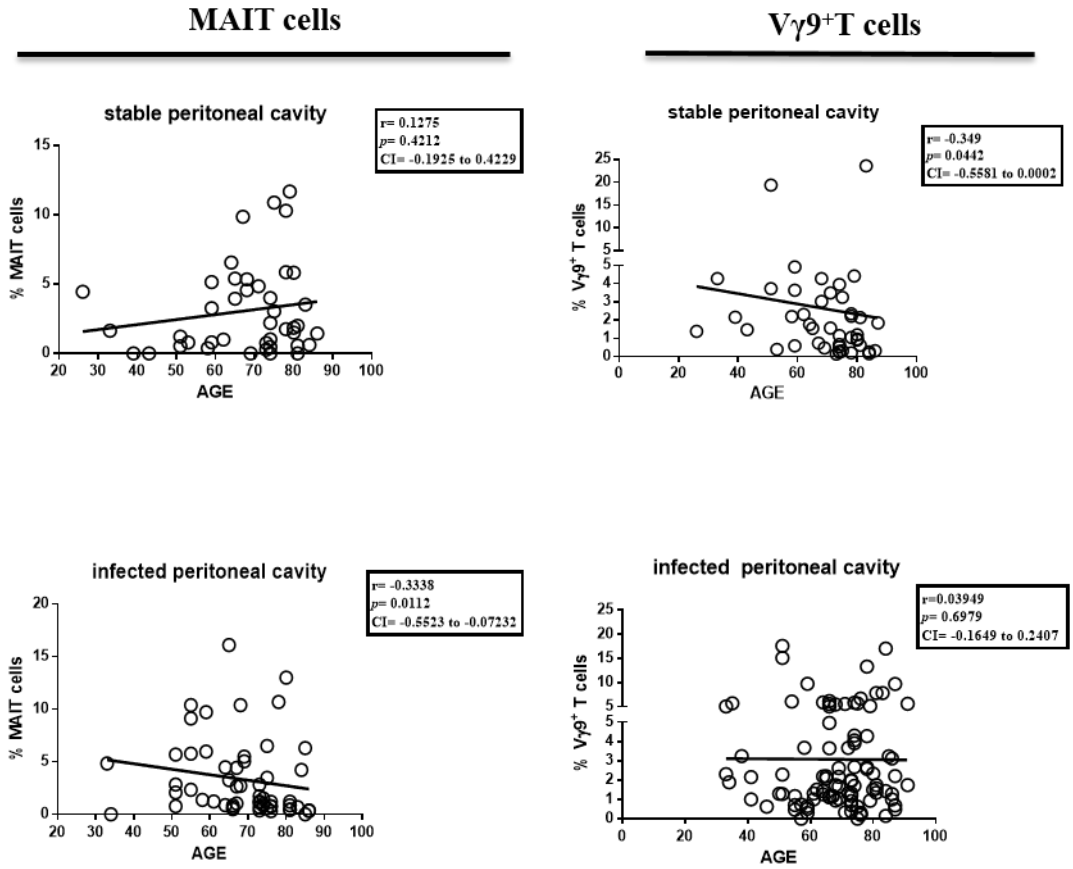

Figure 4.9. Correlation between frequencies of peritoneal unconventional T cells and patient age. Scatterplot showing correlations (Spearman's rank correlation coefficient) between the frequency of peritoneal MAIT cells (left column) and age in a cohort of stable patients (n=42, upper graph) and during acute peritonitis (n=57, lower graph). Correlation of peritoneal Vγ9/Vδ2 cells (right column) with age in a cohort of stable patient (n=44, upper graph) and with acute peritonitis (n=99, lower graph). Correlation was determined using Spearman correlation coefficient.

4.4 Discussion

Chemokines are small molecules that link local tissue activation to the recruitment of leukocytes including antigen specific T cells. At the time of infection, tissue cells and macrophages produce different chemokines upon triggering of pattern recognition receptors such as TLR2 and TLR4 (Luster, 2002). I observed an increase in expression of chemokines CCL2, CCL3 and CCL4 in HMB-PP and vitamin B2 positive infection, and parallel expression of chemokine receptor CCR5 and CCR2 on peripheral $V\gamma9^+$ and $V\alpha7.2^+$ $CD161^+$ T cells. The presence of these chemokine receptors on unconventional T cells and NKT cells has been described as a common feature of Th17 and effector $CD8^+$ T cells (Bromley et al., 2008; Dusseaux et al., 2011; Glatzel et al., 2002). Once recruited to the site of infection, innate T cells release large amounts of IFN-γ and TNF-α which in turn amplify the local immune response. This observation may therefore provide a link between recruitment and/or presence of these cells at the site of infection and poor outcome when these cells are persistently activated.

To further investigate the role of unconventional T cells in PD related infection, the frequency of both $V\gamma9^+$ T cells and MAIT cells was measured in two different cohorts of PD patients, before and after infection.

For the first time I looked at the frequency of immune cells in the peripheral blood and in the peritoneal cavity, not only in a cross-sectional cohort of patients but also longitudinally in individuals before and after episodes of peritonitis.

Previous studies have shown the capacity of unconventional T cells to migrate to perturbed tissue in different inflammatory diseases (Laggner et al., 2011; Magalhaes et al., 2015) and demonstrated a simultaneous reduction of these cells at the periphery. Here, an increase in unconventional T cells in the peritoneal cavity during episodes of infection was seen. The increase of pro-inflammatory chemokines like CCL3 and CCL4 during episodes of HMB-PP and/or vitamin B2 positive infections, and the expression of chemokine receptor (*e.g.* CCR5) on unconventional T cells, suggest a recruitment of circulating T cells to the site of infection. Moreover, I found that this increase was ligand specific, with an enrichment of $V\gamma9^+$ T cells and MAIT cells, respectively, in HMB-PP and vitamin $B2^+$ infections, but not in HMB-PP and vitamin B2 infections. This indicates that the increase is mainly antigen-specific, possibly due to local cell proliferation and expansion.

In contrast to previous studies in other inflammatory diseases (Dunne et al., 2013; Laggner et al., 2011; Magalhaes et al., 2015) I did not find a decrease in circulating unconventional T cells during episodes of PD associated infections, but this does not mean that these cells are not recruited from the blood. The absence of difference observed might be due to a combinations of factors such as the advanced age of the patient cohort studied and the relatively small sample size. A previous study on a cohort of young psoriasis patients showed a decrease in peripheral $V\gamma9^+$ T cells from 4.21% to 2.16% when compared with healthy donors (Laggner et al., 2011) and other reports suggest that circulating unconventional T cell frequency decreases with age (Caccamo, 2006; Colonna-Romano et al., 2002; Lee et al., 2014; Novak et al., 2014). In this study, most patients were between 50 and 80 years old, with a median of $V\gamma9^+$ T cells and MAIT cells in blood of 1.5% and 0.56%, respectively. Therefore the frequency of circulating unconventional T cells may already have dropped, and this could explain the absence of a further decrease of these cells in blood during episodes of peritoneal infection. Similarly *i*) the negative correlation between age and frequency of MAIT cells in the peritoneal cavity of infected patients, *ii*) the low percentage of these cells in the peritoneal cavity of stable patients (median 1.8%), and *iii*) the variability across PD patients could explain why I did not observe a significant difference between MAIT cells in stable and infected unpaired patients. To overcome some of these limitations I analysed matched patient samples and detected an overall increase of MAIT cells in the peritoneal cavity during episode of PD associated infection.

In summary, the results presented in this Chapter highlight a ligand-specific expansion of $V\gamma9^+$ T cells and MAIT cells at the site of infection and the specific contribution of these cells in infection caused by HMB-PP^+ and/or vitamin $B2^+$ pathogens. Consequently, these data suggest an association between the presence of these unconventional T cells and poorer outcomes in acute peritonitis.

Chapter 5. Clinical outcomes depending on the capacity of the causative organism to produce ligands for Vγ9/Vδ2 T cells and MAIT cells

5.1 Introduction

Infection remains a major cause of morbidity and technique failure in PD patients. PD related peritonitis accounts for 20% of PD technique failure cases and 2-6% of all deaths (Cho and Johnson, 2014). The mechanisms that underpin the clinical severity of peritonitis episodes and their association with clinical outcomes remain poorly defined.

The ANZDATA registry captures data from patients receiving renal replacement therapy in Australia and New Zealand, and is the largest and most comprehensive dataset of its kind in the world. The registry collects the incidence, prevalence and outcomes of dialysis and kidney transplant patients (Mcdonald and Russ, 2013; Mcdonald et al., 2012), and thereby supports health care planning and informs research (Mcdonald, Clayton, and Hurst 2012). In addition, the registry captures information about episodes of infection by recording the type of organism identified by microbiological culture and technique failure rates related to individual episodes. Around 13% of infections are recorded as culture negative, 11% are polymicrobial and the remaining 76% caused by a single organism. Single positive infections are mainly caused by $Gram^+$ organisms (around 60% of all cases) such as coagulase-negative staphylococci (CoNS) followed by *S. aureus*, streptococci and enterococci. $Gram^-$ infection account for around 23% of single organism peritonitis episodes. The most common $Gram^-$ organisms identified are *E. coli* followed by *Klebsiella*, *Pseudomonas*, *Acinetobacter* and *Enterobacter* (Cho and Johnson, 2014; Ghali et al., 2011; Jarvis et al., 2010). Moreover, similar levels and types of infections were also recorded in the UK registry (Chapter 1).

Technique failure is defined as any PD related complication that leads to the permanent cessation of the therapy. The end of therapy can be the consequence of catheter removal, permanent switch from PD to HD, switch to HD for a period of at least 1 month (interim HD) and death (Quinn et al., 2010). Type of infection is a major risk factor for PD outcome. Upon diagnosis of peritonitis, empirical antimicrobial therapy covering both $Gram^+$ and $Gram^-$ organisms is initiated before the nature of the causative pathogen is known, which generally takes several days due to the limitations of microbiological culture (Cho and

Johnson, 2014; Li et al., 2010). In many cases no organisms can be identified as result of suboptimal sample collection or culture method, or as consequence of a continued antibiotic use. Depending on the centre, culture negative results may account for up to 50% of all peritonitis cases (Fahim et al., 2010a). Conventional methods are therefore insufficiently robust and rapid to target therapy immediately when patients present with acute symptoms. As a result, patients may receive unnecessary and/or delayed treatment increasing the risk of opportunistic, chronic or recurrent infections and the spread of multidrug resistance.

Previous work in the host laboratory has shown that peritoneal γδ T cell levels on the first day of peritonitis predict the presence of $Gram^-$ organisms and the risk of subsequent technique failure within the next 3 months (Lin et al., 2013). Studying three different cohorts of PD patients, including patients from the ANZDATA registry, it was also observed that HMB-PP positive infections were associated with worse outcomes (Davey et al., 2011a).

Extending the earlier analysis by Davey 2011, which focused on catheter removal and mortality as outcome parameters and concentrated on the absence and presence of HMB-PP production by the causative pathogens as discriminator, in this study a larger dataset from the ANZDATA registry (n=4,003 patients compared to n=2,424 patients as done by Davey 2011) was analysed, allowing consideration of transfer to interim or permanent HD as additional outcome parameters. Most importantly, I analysed the outcome data with respect to the HMB-PP and vitamin B2 status of the causative organism. Given the importance of MAIT cells as contributors to peritoneal inflammation via production of pro-inflammatory cytokines in response to vitamin $B2^+$ bacteria (Chapter 3), this approach allowed investigation of infections caused by vitamin $B2^+$ bacteria as independent predictors of technique failure in 90 days.

This Chapter shows that bacteria able to stimulate Vγ9/Vδ2 T cells and MAIT cells are associated with worse clinical outcomes, highlighting the implication of unconventional T cells in PD associated inflammation.

5.2 Aims

The aims of this Chapter were:

1. To analyse association of HMB-PP and/or vitamin B2 metabolism in the causative pathogen with technique failure.
2. To analyse whether infections caused by $Gram^{-}$ or $Gram^{+}$ bacteria positive for HMB-PP and Vitamin B2 were independent predictor of technique failure.

5.3 Results

5.3.1 ANZDATA analysis: relation between technique failure and pathogen metabolic signature

The following analysis is based on the ANZDATA records of 5,073 Australian patients who received PD and experienced peritonitis between 15 April 2003 and 29 December 2012. A total of 4,003 patients with first-time episodes of peritonitis were included in this analysis, excluding all patients with mixed infections, fungal infections, unrecorded culture results or undefined reasons for technique failure (Figure 5.1 and Table 5.2)

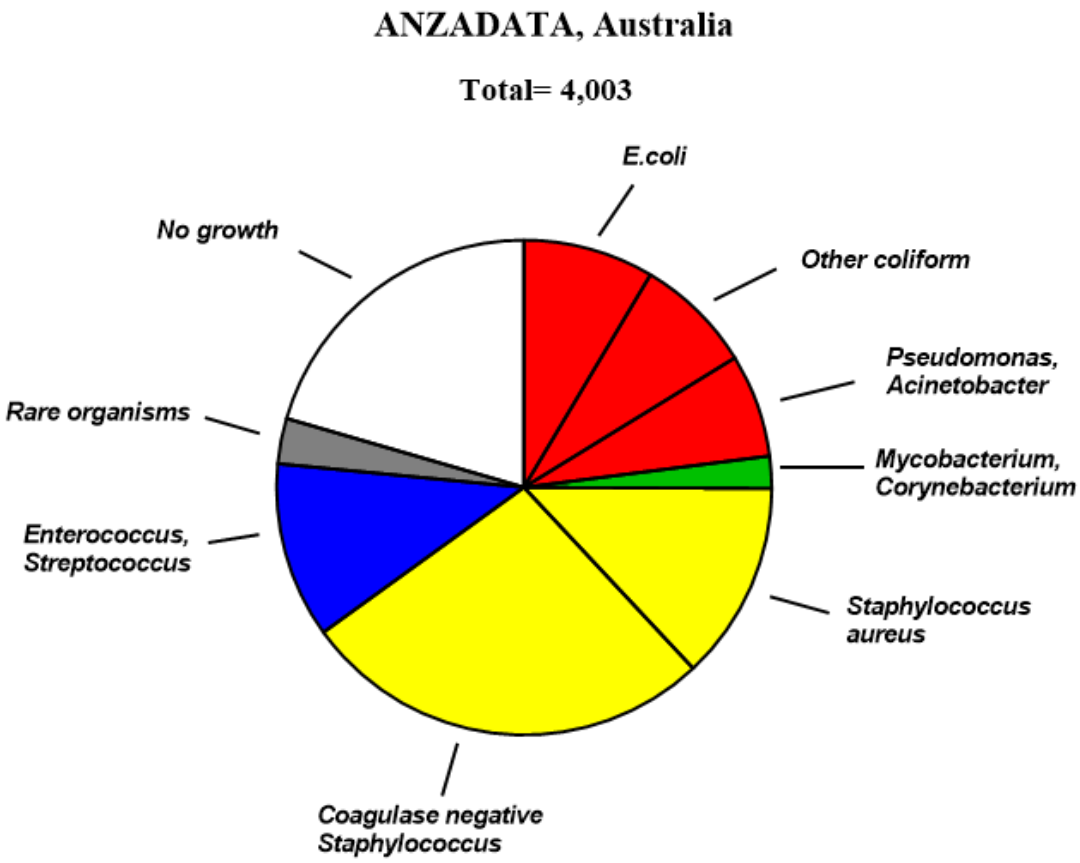

Figure 5.1. Types of infections included in the ANZDATA registry from 2003 to 2012.
4,003 patients from the ANZDATA registry grouped into culture-negative episodes (no growth, white) and confirmed infections caused by Gram^{+} HMB-PP^{+} vitamin B2^{+} bacteria (*Corynebacterium*, *Mycobacterium*, green), Gram^{+} HMB-PP^{-} vitamin B2^{+} bacteria (Coagulase-negative *Staphylococcus*, *Staphylococcus aureus*, yellow), Gram^{+} HMB-PP^{-} vitamin B2^{-} bacteria (*Streptococcus*, *Enterococcus*, blue), Gram^{-} HMB-PP^{+} vitamin B2^{+} bacteria (*E. coli*, *Klebsiella*, other coliform organisms, *Pseudomonas*, *Acinetobacter*, red) and rare organisms (e.g. *Proteus*, *Serratia*, *Neisseria*, *Rhodococcus*, *Rothia* and *Kocuria*, grey).

Table 5.1. ANZDATA registry bacteria grouped by Gram, HMB-PP and vitamin B2 status

Metabolic signature	Organism	Number of episodes
Culture negative		821
Gram$^+$, HMB-PP$^+$, vitamin B2$^+$	*Corynebacterium* species	67
	Mycobacterium tuberculosis	5
	Mycobacterium other	12
	Rhodococcus species	1
	Rothia mucilaginosa	1
	Kocuria kristinae	1
Gram$^+$, HMB-PP$^-$, vitamin B2$^+$	Coagulase-negative *Staphylococcus epidermidis*	618
	Coagulase-negative *Staphylococcus*, other	232
	Coagulase-negative *Staphylococcus*, unknown	231
	Staphylococcus aureus, methicillin resistant	86
	Staphylococcus aureus, non-MRSA	362
	Staphylococcus aureus, unknown sensitivity	70
Gram$^+$, HMB-PP$^-$, vitamin B2	*Enterococcus*	96
	Streptococcus viridans group	102
	Streptococcus, other	231
	Streptococcus, unknown	31
Gram$^-$, HMB-PP$^+$, vitamin B2$^+$	*Acinetobacter* species	81
	Citrobacter species	19
	E. coli species	342
	Enterobacter species	95
	Klebsiella species	194
	Neisseria species	14
	Proteus species	16
	Pseudomonas aeruginosa	111
	Pseudomonas maltophilia	5
	Pseudomonas cepacia	1
	Pseudomonas stutzeri	11
	Pseudomonas, other	44
	Pseudomonas, unknown	17
	Serratia species	80
	Roseomonas gilardii	2
	Pantoea agglomerans	1
	Pasteurella multocida	3

All patients presenting with first-time peritonitis were grouped according to the microbiological culture results into culture-negative and culture-positive episodes. The latter were subsequently divided into infections caused by $Gram^{+}$ and $Gram^{-}$ bacteria, respectively, and then further with regard to HMB-PP and vitamin B2 metabolic status.

The registry recorded 79.4% culture-positive episodes of peritonitis, amongst which 35.2% were infections caused by HMB-PP^{+} bacteria and 85.5 % by vitamin $B2^{+}$ bacteria (Table 5.2).

Table 5.2. Characteristic of PD patients with acute peritonitis for outcome analysis.

ANZDATA (Australia)	
Total number	4,003
Age (mean ± SD)	59.9 ± 17.0
Women (%)	43.0
Days on PD (mean ± SD)	1,044 ± 796.9
30th day mortality (%)	4.4
90th day catheter removal (%)	14.1
90th day transfer to permanent HD (%)	11.5
90th day transfer to interim HD (%)	2.0
90th technique failure in total (%)	17.9
Culture-positive infections (%)	79.4
of which HMB-PP^{+} bacteria (%)	35.2
of which Vitamin $B2^{+}$ bacteria (%)	85.5
of which $Gram^{+}$, HMB-PP^{+}, vitamin $B2^{+}$ bacteria (%)	2.7
of which $Gram^{+}$, HMB-PP^{-}, vitamin $B2^{+}$ bacteria (%)	50.2
of which $Gram^{+}$, HMB-PP^{-}, vitamin$B2^{-}$ bacteria (%)	14.4
of which $Gram^{-}$, HMB-PP^{+}, vitamin $B2^{+}$ bacteria (%)	32.5

I next assessed the clinical outcome after episodes of acute peritonitis. Cumulative survival curves for technique failure showed that up to 20% of the patients with culture positive infections had technique failure within 90 days. These results were significantly different from the survival curve of patients with culture negative peritonitis ($p<0.0001$, Figure 5.2). However, outcome mortality at 30 days was not significantly different between the two groups (Figure 5.2).

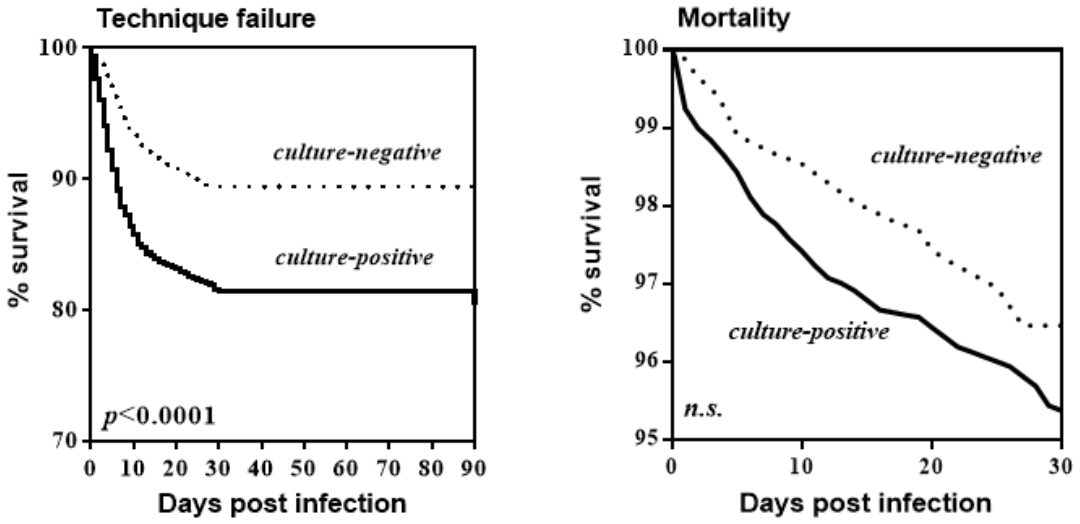

Figure 5.2. Association of culture-positive status with clinical outcome.
Australian PD patients from the ANZDATA registry with first-time peritonitis. Cumulative patient survival (right) and cumulative technique survival (left) of patients with acute peritonitis, grouped into culture-negative (dotted lines) and culture-positive episodes (solid lines).

Next, I divided patients into Gram, HMB-PP and vitamin B2 status groups. Patients presenting with infections caused by $Gram^{-}$ $HMB\text{-}PP^{+}$ vitamin $B2^{+}$ (e.g. *E. coli*, *Pseudomonas*) and $Gram^{+}$ $HMB\text{-}PP^{+}$ vitamin $B2^{+}$ bacteria (e.g. *Corynebacterium*, *Mycobacterium*) had overall technique failure rates of 35% and 23%, respectively, in 90^{th} days (Figure 5.3). By contrast, only 10% of patients infected with $Gram^{+}$ $HMB\text{-}PP^{-}$ vitamin $B2^{-}$ bacteria (e.g. *Streptococcus, Enterococcus*) and 12% of patients infected with $Gram^{+}$, $HMB\text{-}PP^{-}$, vitamin $B2^{+}$ bacteria (e.g. *Staphylococcus*) had to discontinue PD in 90^{th} days (Figure 5.3).

To determine specific association between technique failure and types of infection, I next looked at defined clinical outcome parameters including mortality after 30 days, catheter removal and transfer to permanent HD within 90 days after presenting with acute peritonitis. Figure 5.3 shows that in contrast to infections due to organisms lacking HMB-PP and vitamin B2, infections caused by HMB-PP and vitamin B2 positive bacteria were associated with high rates of catheter removal and subsequent HD transfer. Furthermore, I observed that patients with Gram$^-$ infections had higher 30th day mortality rates compared to patients infected with other organisms (Figure 5.3).

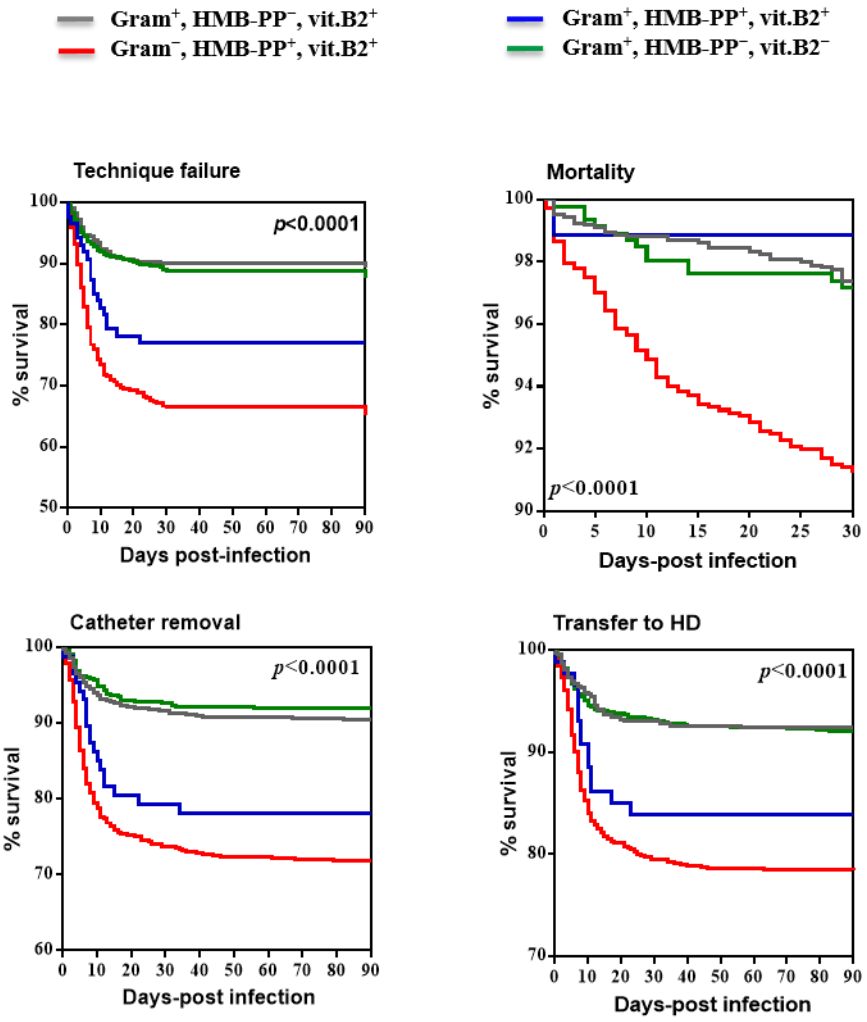

Figure 5.3. Episodes of peritonitis caused by HMB-PP$^+$ and vitamin B2$^+$ bacteria are associated with poor clinical outcome.
Cumulative technique survival (*left, middle*), cumulative patient survival (*right, middle*), catheter removal (*left, bottom)* and transfer to permanent HD (*right, bottom)* of patients with acute bacterial peritonitis, grouped into infections with Gram$^+$ HMB-PP$^-$ vitamin B2$^+$ (*grey*), Gram$^-$ HMB-PP$^+$ vitamin B2$^+$(*red*), Gram$^+$ HMB-PP$^+$ vitamin B2$^+$ (*blue*) and Gram$^+$ HMB-PP$^-$ vitamin B2$^+$ organisms (*green*). Comparisons were made using log-rank tests. *, p<0.05; **, p<0.01, ***, p< 0.001.

Collectively, these results suggested that the presence of HMB-PP and vitamin B2 can predict subsequent clinical outcome in patients presenting with acute PD associated infection.

5.3.2 Episodes of peritonitis caused by HMB-PP and vitamin B2 producing bacteria are associated with poor clinical outcome

To study the importance of HMB-PP and vitamin B2 status as predictors of peritonitis associated outcomes, I performed a binary logistic regression where Gram, HMB-PP and vitamin B2 status were considered as predictors of clinical outcome. As showed in Table 5.3 patients infected with $Gram^-$ bacteria (e.g. *E. coli, Klebsiella, Pseudomonas*) producing HMB-PP and vitamin B2 had a four times higher risk of technique failure in 90 days when compared to patients with culture negative episodes (OR=4.3). This risk was also significantly higher for patients infected with $Gram^+$ $HMB\text{-}PP^+$ vitamin $B2^+$ bacteria such as *Corynebacterium* and *Mycobacterium*, compared to patients with culture negative episodes (OR=2.4). However in this last group, as shown in Table 5.8, infections caused by *Mycobacterium* but not *Corynebacterium* species were associated with high risk of technique failure (OR= 19.2, $p< 0.001$).

Table 5.3. Risk of technique failure within 90 days after presentation with acute peritonitis, depending on the causative pathogen.

90th day technique failure	**Odds ratio** (95%CI)	***p***
Reference: culture-negative	**1**	
$Gram^+$ $HMB\text{-}PP^+$ $Vitamin\ B2^+$	2.4 (1.389-4.129)	0.002
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^+$	1.1 (0.866-1.468)	0.374
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^-$	0.9 (0.662-1.381)	0.812
$Gram^-$ $HMB\text{-}PP^+$ $Vitamin\ B2^+$	4.3 (3.318-5.500)	***
Reference: culture-negative	**1**	
$HMB\text{-}PP^+$	4.1 (3.194-5.271)	***
$HMB\text{-}PP^-$	1.0 (0.843-1.405)	0.515
Reference: culture-negative	**1**	
$Vitamin\ B2^+$	2.1 (1.703-2.734)	***
$Vitamin\ B2^-$	0.9 (0.662-1.381)	0.812
Reference: $HMB\text{-}PP^-$	**1**	
$Gram^+$ $HMB\text{-}PP^+$	2.2 (1.312-3.689)	0.003
$Gram^-$ $HMB\text{-}PP^+$	3.9 (3.263-4.721)	***
Reference: $Vitamin\ B2^-$	**1**	
$Gram^+$ $Vitamin\ B2^+$	1.2 (0.892-1.721)	0.201
$Gram^-$ $Vitamin\ B2^+$	4.5 (3.235-6.168)	***
Reference: $Gram^-$ $HMB\text{-}PP^+$ $Vitamin\ B2^+$	**1**	
$Gram^+$ $HMB\text{-}PP^+$, $Vitamin\ B2^+$	0.6 (0.335-0.939)	0.028
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^+$	0.3 (0.217-0.321)	***
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^-$	0.2 (0.162-0.309)	***

Compared to culture-negative episodes of peritonitis, infections caused by Gram$^-$ HMB-PP$^+$ vitamin B2$^+$ bacteria were associated with a higher risk of permanent PD cessation for all four different types of technique failure investigated: 30th day mortality (OR=2.6; Table 5.4), 90th day catheter removal (OR=4.4; Table 5.5), 90th day transfer to permanent HD (OR=3.4, Table 5.6) and 30th day transfer to interim HD (OR=9.0; Table 5.7).

Infections caused by Gram$^+$ HMB-PP$^+$ vitamin B2$^+$ bacteria were associated with higher risks of catheter removal (OR=3.2; Table 5.5) as well as transfer to permanent HD (OR=7.3; Table 5.6) and interim HD (OR=2.3; Table 5.7) but not with increased mortality (Table 5.4). By contrast, patients presenting infections caused by bacteria deficient for HMB-PP and vitamin B2 (e.g. *Enterococcus* and *Streptococcus*) did not carry a significantly enhanced risk of technique failure (Tables 5.4-5.8).

5.3.3 Contribution of HMB-PP producing bacteria to clinical outcome

The previous section identified an association of infections caused by Gram-negative and Gram-positive HMB-PP$^+$ vitamin B2$^+$ bacteria with poor clinical outcome. I next sought to determine the contributions of HMB-PP and vitamin B2 to overall outcome prediction.

Patients with HMB-PP$^+$ bacterial infection had a four times greater risk to discontinue PD therapy than HMB-PP$^-$ infection when compared to culture-negative results (Table 5.3). Taking different qualities of clinical outcomes as dependent variables and HMB-PP status as predictor, these analyses demonstrate that patients with infections caused by HMB-PP$^+$ bacteria had higher risks of catheter removal (OR=4.4), mortality (OR=2.4) and of being transferred to interim (OR=8.9) or permanent HD (OR=3.3) (Tables 5.4-5.7).

Using HMB-PP$^-$ infections as reference to assess outcomes in patients with either Gram$^-$ or Gram$^+$ HMB-PP$^+$ infections, HMB-PP$^+$ bacteria were significantly associated with increased risk of technique failure such as catheter removal and transfer to permanent HD (Tables 5.5-5.6). In addition, Gram$^-$ but not Gram$^+$ HMB-PP$^+$ infections were associated with a higher risk of mortality in 30 days and interim HD (Table 5.4 and 5.7).

5.3.4 Contribution of vitamin B2 producing bacteria to clinical outcome

I next tested whether or not the presence of vitamin B2 alone was an independent predictor of poor clinical outcome. To this end, I grouped culture positive infections into vitamin $B2^+$ and vitamin $B2^-$ infections, according to the metabolic signature of the causative organism. As shown in Table 5.3, the odds of patients having technique failure as a result of an infection by a vitamin $B2^+$ pathogen were two times greater than infections caused by vitamin $B2^-$ bacteria.

Using vitamin $B2^-$ infections as reference to assess outcomes in patients with either $Gram^-$ or $Gram^+$ vitamin $B2^+$ infections, my analysis shows that patients with infections caused by $Gram^-$ vitamin $B2^+$ bacteria had a higher risk of catheter removal (OR=4.4) as well as transfer to interim (OR=3.4) or permanent HD (OR=3.3) than patients with vitamin B2 negative infections.

Finally, when I used patients with infections caused by $Gram^-$ HMB-PP^+ vitamin $B2^+$ organisms as reference group, I found that all three $Gram^+$ groups were associated with a lower risk of mortality (Table 5.4). In particular, infections caused by $Gram^+$ HMB-PP^- vitamin $B2^-$ species (*Enterococcus, Streptococcus*) and $Gram^+$ HMB-PP^- vitamin $B2^+$ species (*Staphylococcus*) species were associated with a lower risk of catheter removal and transfer to HD compared to the $Gram^-$ group (OR=0.27-0.35, Tables 5.4-5.7). Moreover, as shown in Table 5.8, infections caused by the $Gram^+$ vitamin $B2^+$ *Coagulase-negative Staphylococcus* bacteria are associated with a significantly lower risk of technique failure including mortality, catheter removal and transferred to permanent HD.

Table 5.4. Risk of mortality within 30 days after presentation with acute peritonitis, depending on the causative pathogen.

30th day mortality	**Odds ratio** (95%CI)	***p***
Reference: culture-negative	**1**	
Gram$^+$ HMB-PP$^+$ Vitamin B2$^+$	0.262 (0.043-2.360)	0.262
Gram$^+$ HMB-PP$^-$ Vitamin B2$^+$	0.737 (0.455-1.192)	0.213
Gram$^+$ HMB-PP$^-$ Vitamin B2^{-}	0.794 (0.409-1.543)	0.497
Gram$^-$ HMB-PP$^+$ Vitamin B2$^+$	2.6 (1.713-4.037)	***
Reference: culture-negative	**1**	
HMB-PP$^+$	2.43 (1.589-3.737)	***
HMB-PP$^-$	0.75 (0.474-1.184)	0.217
Reference: culture-negative	**1**	
Vitamin B2$^+$	1.4 (0.939-2.129)	0.097
Vitamin B2$^-$	0.8 (0.409-1.543)	0.497
Reference: HMB-PP$^-$	**1**	
Gram$^+$ HMB-PP$^+$	0.424 (0.058-3.098)	0.398
Gram$^-$ HMB-PP$^+$	3.5 (2.488-4.947)	***
Reference: Vitamin B2$^-$	**1**	
Gram$^+$ Vitamin B2$^+$	0.9 (0.480-1.688)	0.742
Gram$^-$ Vitamin B2$^+$	3.3 (1.832-5.985)	***
Reference: Gram$^-$, HMB-PP$^+$ Vitamin B2$^+$	**1**	
Gram$^+$ HMB-PP$^+$ Vitamin B2$^+$	0.12 (0.017-0.877)	0.037
Gram$^+$, HMB-PP$^-$ Vitamin B2$^+$	0.3 (0.193-0.407)	***
Gram$^+$ HMB-PP$^-$ Vitamin B2$^-$	0.3 (0.167-0.546)	***

Table 5.5. Risk of catheter removal within 90 days after presentation with acute peritonitis, depending on the causative pathogen.

90th day catheter removal	**Odds ratio** (95%CI)	***p***
Reference: culture-negative	**1**	
$Gram^+$ $HMB\text{-}PP^+$ $Vitamin\ B2^+$	3.2 (1.812-5.637)	***
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^+$	1.2 (0.895-1.637)	0.215
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^-$	1.0 (0.658-1.523)	0.998
$Gram^-$ $HMB\text{-}PP^+$ $Vitamin\ B2^+$	4.4 (3.358-5.946)	***
Reference: culture-negative	**1**	
$HMB\text{-}PP^+$	4.3 (3.285-5.792)	***
$HMB\text{-}PP^-$	1.2 (0.868-1.558)	0.312
Reference: culture-negative	1	1
$Vitamin\ B2^+$	2.3 (1.789-3.074)	***
$Vitamin\ B2^-$	1.0 (0.658-1.523)	0.998
Reference: $HMB\text{-}PP^-$	**1**	
$Gram^+$ $HMB\text{-}PP^+$	2.7 (1.618-4.670)	***
$Gram^-$ $HMB\text{-}PP^+$	3.8 (3.141-4.700)	***
Reference: $Vitamin\ B2^-$	**1**	
$Gram^+$ $Vitamin\ B2^+$	1.3 (0.896-1.883)	0.167
$Gram^-$ $Vitamin\ B2^+$	4.4 (3.108-6.415)	***
Reference: $Gram^-$ $HMB\text{-}PP^+$ $Vitamin\ B2^+$	**1**	
$Gram^+$ $HMB\text{-}PP^+$ $Vitamin\ B2^+$	0.7 (0.423-1.211)	0.212
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^+$	0.3 (0.219-0.336)	***
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^-$	0.2 (0.156-0.322)	***

Table 5.6. Risk of transfer to permanent HD in 90 days after presentation with acute peritonitis, depending on the causative pathogen.

90th day transfer to permanent HD	**Odd Ratio** (95%CI)	***p***
Reference: culture-negative	**1**	
Gram^{+} HMB-PP^{+} Vitamin B2^{+}	2.3 (1.253-4.398)	0.008
Gram^{+} HMB-PP^{-} Vitamin B2^{+}	1.0 (0.763-1.437)	0.775
Gram^{+} HMB-PP^{-} Vitamin B2^{-}	1.0 (0.655-1.551)	0.971
Gram^{-} HMB-PP^{+} Vitamin B2^{+}	3.4 (2.506-4.550)	***
Reference: culture-negative	**1**	
HMB-PP^{+}	3.3 (2.449-4.425)	***
HMB-PP^{-}	1.0 (0.766-1.408)	0.808
Reference: culture-negative	1	
Vitamin B2^{+}	2.0 (1.403-3.049)	***
Vitamin B2^{-}	0.9 (0.493-1.689)	0.771
Reference: HMB-PP^{-}	**1**	
Gram^{+} HMB-PP^{+}	2.3 (1.248-4.095)	0.007
Gram^{-} HMB-PP^{+}	3.3 (2.614-4.046)	***
Reference: Vitamin B2^{-}	**1**	
Gram^{+} Vitamin B2^{+}	1.1 (0.748-1.617)	0.629
Gram^{-} Vitamin B2^{+}	3.3 (2.302-4.874)	***
Reference: Gram^{-} HMB-PP^{+} Vitamin B2^{+}	**1**	
Gram^{+} HMB-PP^{+} Vitamin B2^{+}	0.228 (0.385-1.255)	0.695
Gram^{+} HMB-PP^{-} Vitamin B2^{+}	0.3 (0.245-0.392)	***
Gram^{+} HMB-PP^{-} Vitamin B2^{-}	0.3 (0.205-0.434)	***

Table 5.7. Risk of transfer to interim HD in 30 days after presentation with acute peritonitis, depending on the causative pathogen.

Transferred to interim HD for at least 30 days	**Odd Ratio** (95%CI)	***p***
Reference: culture-negative	**1**	
$Gram^+$ $HMB\text{-}PP^+$ $Vitamin\ B2^+$	7.3 (1.606-33.143)	0.010
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^+$	3.2 (1.125-9.353)	0.029
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^-$	2.7 (0.758-9.615)	0.126
$Gram^-$ $HMB\text{-}PP^+$ $Vitamin\ B2^+$	9.0 (3.242-25.319)	***
Reference: culture-negative	**1**	
$HMB\text{-}PP^+$	8.9 (3.201-24.863)	***
$HMB\text{-}PP^-$	3.1 (1.099-8.873)	0.033
Reference: culture-negative	**1**	
$Vitamin\ B2^+$	5.5 (2.021-15.235)	0.001
$Vitamin\ B2^-$	2.7 (0.758-9.615)	0.126
Reference: $HMB\text{-}PP^-$	**1**	
$Gram^+$, $HMB\text{-}PP^+$	0.9 (0.700-7.796)	0.167
$Gram^-$ $HMB\text{-}PP^+$	2.9 (1.821-4.623)	***
Reference: $Vitamin\ B2^-$	**1**	
$Gram^+$ $Vitamin\ B2^+$	1.3 (0.526-3.105)	0.588
$Gram^-$ $Vitamin\ B2^+$	3.4 (1.420-7.933)	0.006
Reference: $Gram^-$ $HMB\text{-}PP^+$ $Vitamin\ B2^+$	**1**	
$Gram^+$ $HMB\text{-}PP^+$ $Vitamin\ B2^+$	0.805 (0.245-2.648)	0.721
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^+$	0.4 (0.218-0.589)	***
$Gram^+$ $HMB\text{-}PP^-$ $Vitamin\ B2^-$	0.3 (0.126-0.704)	0.006

Table 5.8. Risk of technique failure, catheter removal, transfer to HD and mortality after presentation with acute peritonitis, depending on the causative pathogen.

		Technique failure				Catheter removal				Mortality				Transfer to permanent HD				Transfer to interim HD			
	Episodes	Sig.	OR	95% C.I.		Sig.	OR	95% C.I.		Sig.	OR	95% C.I.		Sig.	OR	95% C.I.		Sig.	OR	95% C.I.	
Reference culture negative	821																				
Coagulase-neg. Staphylococcus	1081	0.001	0.581	0.421	0.802	0.001	0.532	0.362	0.780	0.017	0.489	0.272	0.878	0.002	0.544	0.367	0.806	0.371	1.715	0.526	5.588
Staphylococcus aureus-MR	86	***	4.521	2.767	7.389	***	4.957	2.934	8.375	0.122	2.048	0.826	5.082	***	3.472	1.96	6.148	***	12.608	3.32	47.885
***Staphylococcus aureus* species**	432	***	2.201	1.604	3.02	***	2.56	1.803	3.635	0.719	1.119	0.608	2.06	***	2.013	1.384	2.928	0.004	5.337	1.689	16.861
***Streptococcus-Enterococcus* species**	460	0.812	0.956	0.662	1.381	0.998	1.001	0.658	1.523	0.497	0.794	0.409	1.543	0.971	1.008	0.655	1.551	0.126	2.699	0.758	9.615
***Corynebacterium* species**	67	0.873	0.936	0.415	2.109	0.792	1.125	0.469	2.701	0.389	0.414	0.055	3.086	0.359	0.574	0.175	1.879	0.004	9.574	2.097	43.706
***Pseudomonas* species**	189	***	8.458	5.907	12.11	***	9.35	6.385	13.69	0.004	2.526	1.342	4.753	***	5.42	3.622	8.112	***	24.172	8.158	71.622
***Acinetobacter* species**	81	0.185	1.534	0.815	2.885	0.042	1.989	1.026	3.859	0.618	0.691	0.162	2.952	0.26	1.53	0.73	3.206	0.06	5.171	0.932	28.676
***E. coli* species**	342	***	4.858	3.568	6.615	***	4.999	3.554	7.031	***	3.313	2.002	5.483	***	4.049	2.834	5.784	0.005	5.52	1.688	18.049
***Klebsiella* species**	194	***	3.338	2.287	4.871	***	2.971	1.934	4.564	***	3.139	1.735	5.679	***	2.789	1.787	4.353	0.129	3.208	0.712	14.453
***Enterobacter* species**	95	***	3.185	1.939	5.232	***	3.051	1.753	5.307	0.027	2.511	1.113	5.664	0.008	2.295	1.248	4.221	0.002	8.978	2.208	36.507
***Serratia* species**	80	***	3.237	1.904	5.504	***	3.813	2.167	6.71	0.92	1.064	0.317	3.574	0.009	2.375	1.243	4.541	***	13.617	3.58	51.789
***Proteus* species**	16	0.019	3.646	1.239	10.73	0.024	3.813	1.196	12.154	0.081	3.901	0.847	17.968	0.018	4.081	1.278	13.028	0.999	0	0	.
***Citrobacter* species**	19	***	5.834	2.287	14.882	0.009	4.085	1.427	11.694	0.013	5.121	1.413	18.558	0.041	3.265	1.051	10.135	0.999	0	0	.
***Neisseria* species**	14	0.999	0	0	.	0.999	0	0	.	0.999	0	0	.	0.999	0	0	.	0.999	0	0	.
***Mycobacterium* species**	17	***	19.253	6.632	55.893	***	27.455	9.387	80.295	0.999	0	0	.	***	22.444	8.03	62.731	0.999	0	0	.
Other species	9	0.306	2.292	0.469	11.2	0.145	3.268	0.666	16.051	0.999	0	0	.	0.999	0	0	.	0.006	25.531	2.562	254.471

5.4 Discussion

PD is the principal form of home based dialysis therapy for patients with end-stage renal failure. Compared to haemodialysis, PD gives a better preservation of residual renal function and is more cost effective (Chaudhary et al., 2011; Tokgoz, 2009). Unfortunately, infection and associated inflammation remain the major reasons for treatment failure and death in PD patients (Cho and Johnson, 2014). However, the mechanisms that underlie the clinical severity of peritonitis episodes, and their link to outcomes, is poorly defined.

I here re-analysed the ANZDATA registry that contains data on the outcomes of 1,579 additional patients than a previous similar analysis on patient data recorded between 2003 and 2008 (Davey et al., 2011a). This larger dataset allowed me to look in detail at prediction of different types of technique failure by the metabolic signature of the causative bacterial pathogen, including transfer to interim HD and permanent HD. While the previous analysis considered 90th day mortality (Davey et al., 2011a), I here focused on death within 30 days from the time of presentation with acute peritonitis as the more relevant time point (Boudville et al., 2012). Furthermore, in the current analysis I also considered how different types of metabolic signature can affect outcomes.

As seen in Chapter 3, peritoneal Vγ9/Vδ2 T cell and MAIT cells are likely to contribute to peritoneal inflammation by production of large amounts of pro-inflammatory cytokines including TNF-α and IFN-γ. This conclusion is supported by the ANZDATA analysis. Indeed, Gram$^-$ HMB-PP$^+$ vitamin B2$^+$ are associated with poor clinical outcomes such as catheter removal, transfer to permanent and interim HD compared to infections by bacteria lacking these metabolites. This group of pathogens includes bacteria such as *Pseudomonas, E. coli*, *Klebsiella* and *Enterobacter*, which have previously been associated with worse outcomes (Jarvis et al., 2010; Siva et al., 2009). Moreover, Lin et al. showed that Gram$^-$ infections are characterized by increased pro-inflammatory cytokines IL-1β, IL-10 and TNF-α in the peritoneal cavity compared to Gram$^+$ infection or culture negative episodes of peritonitis (Lin et al., 2013). Considering that peritoneal Vγ9/Vδ2 T cell and MAIT cells produce up to 50% of TNF-α after treatment with *E. coli* (Chapter 3), it is conceivable that these cells may contribute to detrimental outcomes caused by Gram negative bacteria during PD associated infection.

It was also observed that Gram$^+$ HMB-PP$^+$ vitamin B2$^+$ bacteria are associated with elevated rates of catheter removal and transfer to permanent HD within 90 days after presentation with acute peritonitis. The poor clinical outcome in this patient group was due mainly to mycobacterial infections, whereas corynebacterial infections were not a significant predictor of technique failure. These findings concur with earlier reports on the relatively mild clinical course of coryneform infections (Barraclough et al., 2009), and on the high frequency of catheter removal in patients with mycobacterial infections (Ghali et al., 2011; Li et al., 2010).

Access to the largest dataset yet produced for such analyses, I here confirmed that infections caused by HMB-PP$^+$ bacteria are associated with poor outcomes in PD patients. This was true for episodes of peritonitis caused by both Gram$^+$ and Gram$^-$ species, thereby identifying the production of HMB-PP by the causative organism as an effective predictive marker, and implying that Vγ9/Vδ2 T cell-driven responses may contribute to overall clinical outcome.

In this analysis I also examined for the first time the impact of vitamin B2$^+$ organisms on outcomes from PD associated peritonitis. The data indicate that while the presence of vitamin B2 was a significant predictor of technique failure, the associated risk was lower overall than that with HMB-PP positive infections (OR=2.1 vs OR=4.1). In addition, patients with Gram$^+$ vitamin B2$^+$ infections were more likely to continue PD therapy without interruptions over the next 90 days compared to patients with Gram$^+$ HMB-PP$^+$ infections. This observation was valid for all outcomes considered. The relatively benign course due to HMB-PP$^-$ vitamin B2$^+$ peritonitis might be explained by the high prevalence of infections caused by the skin commensal bacterium *Staphylococcus epidermidis* and related coagulase-negative *Staphylococcus* species (CoNS) (67.6% of all infections in this group) and *S .aureus* species (32.2% of all infections in this group). *S. aureus* infections, in agreement with earlier studies (Fahim et al., 2010b; Govindarajulu et al., 2010), were associated with a considerably greater risk of technique failure and catheter removal than CoNS infections. Indeed, even if CoNS infections were more frequent, they were resolved with antibiotics within three weeks and were not associated with hospitalization, catheter removal and transfer to HD (Fahim et al., 2010b).

These last results may support the hypothesis that I should not consider Vitamin B2 metabolising organisms less pathogenic than HMB-PP$^+$ bacteria, but that the nature of the immune response depends strictly on the type of pathogen, which as a consequence defines

outcome. Moreover, these findings indicate that the presence of the vitamin B2 pathway alone may not be sufficiently predictive of clinical outcome in PD patients, and may be masked by the stronger predictive power of HMB-PP.

Chapter 6. Activation of peritoneal mesothelial cells and fibroblasts by unconventional T cells

6.1 Introduction

During PD therapy, preservation of peritoneal membrane integrity is fundamental to long term treatment survival. Human peritoneal mesothelial cells (HPMC) are the most numerous cells lining the peritoneal cavity, form the first line of defence against bacteria in the peritoneal cavity and are able to maintain a chemotactic gradient to allow leukocyte infiltration during peritoneal inflammation (Yung and Chan, 2012). Chronic exposure to PD fluid and episodes of peritonitis can induce detachment of HPMC from the basement membrane through a process called mesothelial to mesenchymal transition (MMT), and migration of the cells undergoing MMT to the submesothelium (Selgas et al., 2006).

With the loss of the mesothelium layer, PD fluid and bacteria invade the connective tissue and activate peritoneal fibroblasts (HPFB). The latter produce extracellular matrix components in the connective tissue, and persistent activation of these cells can contribute to peritoneal fibrosis (Witowski et al. 2015). In general, activated HPMC and HPFB contribute to increase pro-inflammatory cytokines and matrix proteins in the peritoneal cavity. Any structural changes to the peritoneal membrane will affect ultrafiltration capacity, and may ultimately lead to cessation of PD treatment (Lai and Leung, 2010).

Previous studies have shown that HPMC and HPFB produce chemokines (e.g. CXCL10, CXCL8 and CCL2) and pro-inflammatory cytokines such as IL-6 in response to pro-inflammatory cytokines like IFN-γ, IL-1β and TNF-α. As described in earlier chapters, $V\gamma9^+$ T cells and MAIT cells are major producers of TNF-α and IFN-γ in response to a range of bacterial pathogens. However, regulation of HPMC and HPFB by these unconventional T cells and the potential implications for clinical outcome have not yet been investigated.

By isolating HPMC and HPFB from fresh omental specimens and culturing them with conditioned medium derived from activated unconventional T cells, I here demonstrated that unconventional T cells induce the release of pro-inflammatory chemokines and cytokines by peritoneal tissue cells, and induce striking morphological changes and alterations in the expression levels of epithelial and mesenchymal markers. These results point to the involvement of peritoneal tissue cells in the amplification of local inflammatory

responses during PD associated infection and their possible regulation by unconventional T cells.

6.2 Aims

The aims of this Chapter were:

1. To examine activation of HPMC or HPFB by TNF-α and IFN-γ, and by conditioned medium (CoM) derived from activated γδ T cells and MAIT cells.
2. To study HPMC and HPFB cytokine and chemokine release in response to $Gram^+$ and $Gram^-$ bacteria, and PDE from stable or infected patients.
3. To analyse expression of epithelial and mesenchymal markers implicated in peritoneal membrane integrity in response to activated unconventional T cell CoM.

6.3 Results

6.3.1 Unconventional T cell CoM induced release of pro-inflammatory cytokines and chemokines by HPMC and HPFB.

As shown in previous Chapters, unconventional T cells are present in the peritoneal cavity of stable PD patients and increase during episodes of acute peritonitis. I also found these cells are major producers of TNF-α and IFN-γ in response to bacterial extracts. To analyse activation of HPMC and HPFB by unconventional T cell cytokines during PD associated infection, CoM was obtained from activated Vγ9/Vδ2 T cells and MAIT cells. $V\gamma9^+$ T cells and $V\alpha7.2^+$ T cells were isolated from PBMC and incubated with HMB-PP and anti-CD3/CD28 beads, respectively, and, after 24 hours, cell-free supernatants were collected. While activated γδ T cells and MAIT cells yielded different concentrations, both supernatants were confirmed to contain substantial amounts of TNF-α and IFN-γ (Figure 6.1A).

Confluent HPMC and HPFB were growth synchronised by serum deprivation and cultured in the presence of γδ T cell or MAIT cell CoM for 24 hours. Under these conditions, both HPMC and HPFB released significant amounts of chemokines CCL2, CXCL8, CXCL10, and IL-6, at CoM dilutions as high as 1:16 (Figures 6.1B-C and 6.2).

To confirm this chemokine and cytokine induction was not caused by the presence of FCS in the CoM, I cultured HPMC in the presence of defined concentrations of FCS as control. As shown in Figure 6.3, when I compared the amount of CCL2 and CXCL8 induced by CoM derived from unconventional T cells with the medium supplemented with 10% FCS, I observed that the latter induced 8 and 2 times less amount of these two chemokines, respectively. This confirmed that FCS did not have a major effect on chemokine production induced by CoM derived from unconventional T cells.

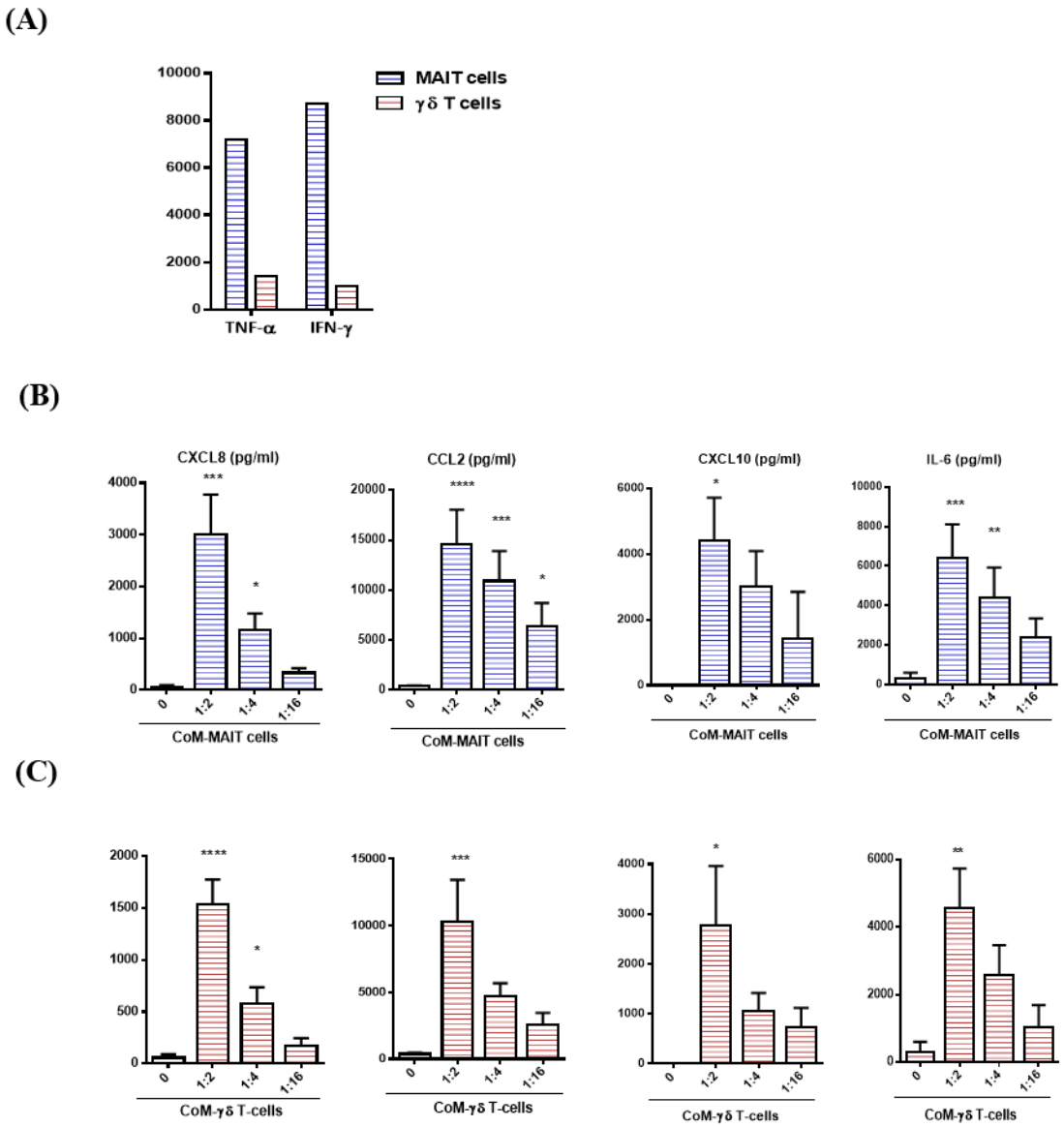

Figure 6.1. Effect of unconventional T cell CoM on CXCL8, CCL2, CXCL10 and IL-6 secretion by HPMC.

Secretion of TNF-α and IFN-γ by FACS sorted Vγ9$^+$ T cells and Vα7.2$^+$ T cells stimulated overnight in the presence of 100 nM HMB-PP or anti-CD3/CD28 beads for 24 h, respectively, as detected by ELISA (A). Quiescent cells were exposed to either control medium (0) or to MAIT cell CoM (B) or γδ T cell CoM (C) at the dilutions shown for 24 h (means ± SEM from independent experiments, n = 4-7 omental donors). Data were analysed by RM one-way ANOVA or Friedman test with Holm-Sidak or Dunn's post hoc test respectively. Comparisons were made with control medium. Differences were considered significant as indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

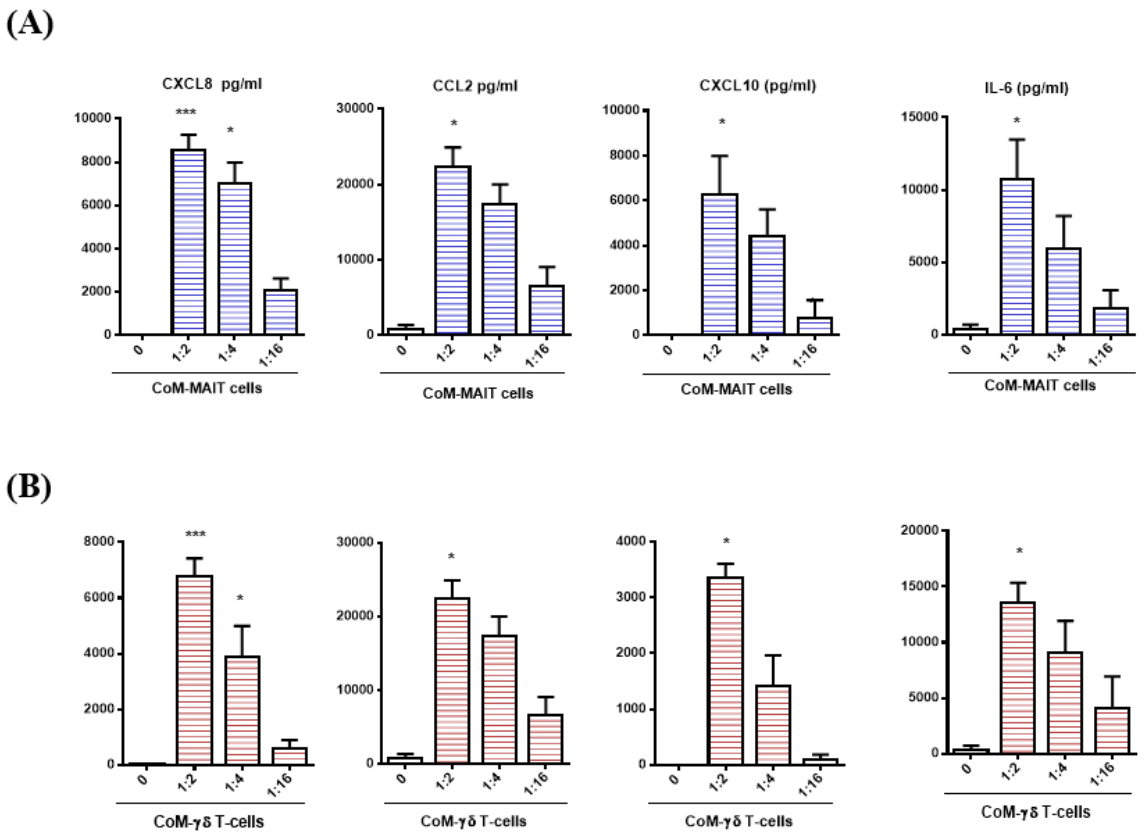

Figure 6.2. Effect of unconventional T cell CoM on CXCL8, CCL2, CXCL10 and IL-6 secretion by HPFB.

Quiescent cells were exposed to control medium (0), MAIT cell CoM (**A**) or γδ T cell CoM (**B**) at the dilutions shown for 24 h (means ± SEM from independent experiments, n=4-7 omental donors). Data were analysed by RM one-way ANOVA or Friedman test with Holm-Sidak or Dunn's post hoc test respectively. Comparisons were made with control medium. Differences were considered significant as indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

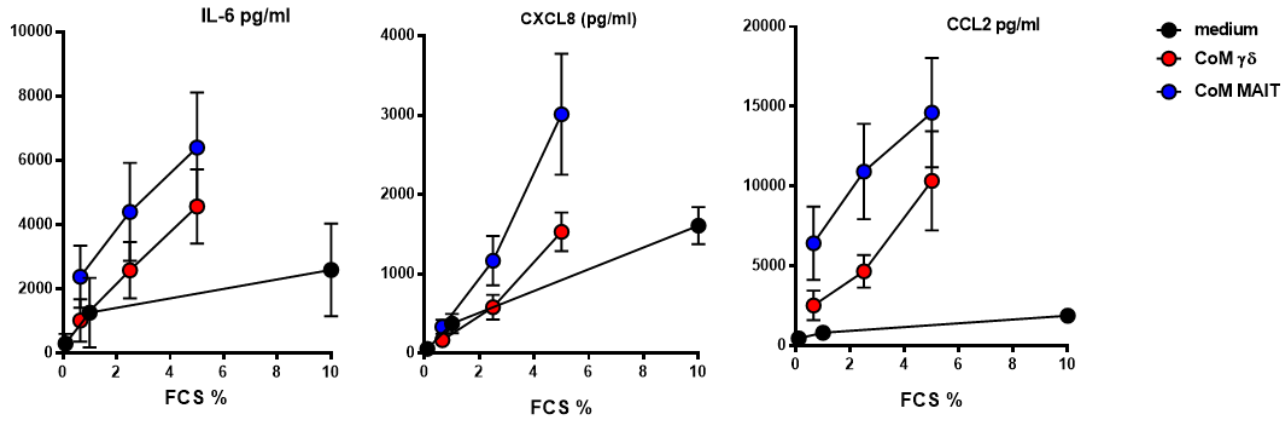

Figure 6.3. Effect of complete medium containing different percentages of FCS on CXCL8, CCL2 and IL-6 secretion by HPMC.
Quiescent cells were exposed to HPMC complete medium containing 0.1-10% FCS (black) for 24 hours and compared with the percentage of FCS contained in CoM derived from γδ T cells (red) or MAIT cells (blue). Means± SEM from 4-5 independent experiments.

6.3.2 HPMC and HPFB release pro-inflammatory cytokines and chemokines in response to IFN-γ and TNF-α

To investigate whether IFN-γ and TNF-α were responsible for the cytokine and chemokine release by HPMC and HPFB, I cultured them in the presence of increasing concentrations of the recombinant cytokines (Figure 6.4-5).

HPMC showed a dose-dependent increase of CCL2 release with IFN-γ or TNF-α (Figure 6.4A). As predicted (Robson et al., 2001), there was no induction of CXCL8 by IFN-γ , but TNF-α induced dose dependent CXCL8 release (Figure 6.4B). Similarly, production of IL-6 increased significantly in response to TNF-α, but not IFN-γ (Figure 6.4C).

HPFB treated with low concentrations of IFN-γ released IL-6 and CCL2 above background levels but this production did not increase at higher IFN-γ concentrations (Figure 6.5A-B). As seen before with HPMC, HPFB did not release relevant amounts of CXCL8 in response to IFN-γ (Figure 6.5C). TNF-α stimulation of HPFB resulted in a dose dependent production of IL-6, CCL2 and CXCL8 (Figure 6.5A-C).

To establish whether combined stimulation of HPMC and HPFB with both IFN-γ and TNF-α induced a synergistic production of chemokines and cytokines from these cells, I treated HPMC with a combination of a fixed concentration of IFN-γ (500 U/ml) plus different

concentrations of TNF-α (1-50 ng/ml). This resulted in significant synergistic up-regulation of CCL2 and IL-6, but not of CXCL8, by HPMC in comparison with the expected IFN-γ and TNF-α additive value (Figure 6.4A-C).

Similarly, treatment of HPFB with a combination of IFN-γ and TNF-α resulted in a synergistic upregulation of IL-6 and CCL2 (Figure 6.5A-B). By contrast, IFN-γ tended to inhibit significantly CXCL8 release by HPFB when combined with TNF-α, in comparison with the amount induced by TNF-α alone (Figure 6.5C). CXCL10 production by HPFB was barely detectable after treatment with either TNF-α or IFN-γ, whereas there was significant upregulation of this chemokine when both cytokines were added together (Figure 6.6D). In summary, these results confirmed that TNF-α and IFN-γ derived from unconventional T cells can induce HPMC and HPFB activation, as well as cytokine and chemokines release from these cells.

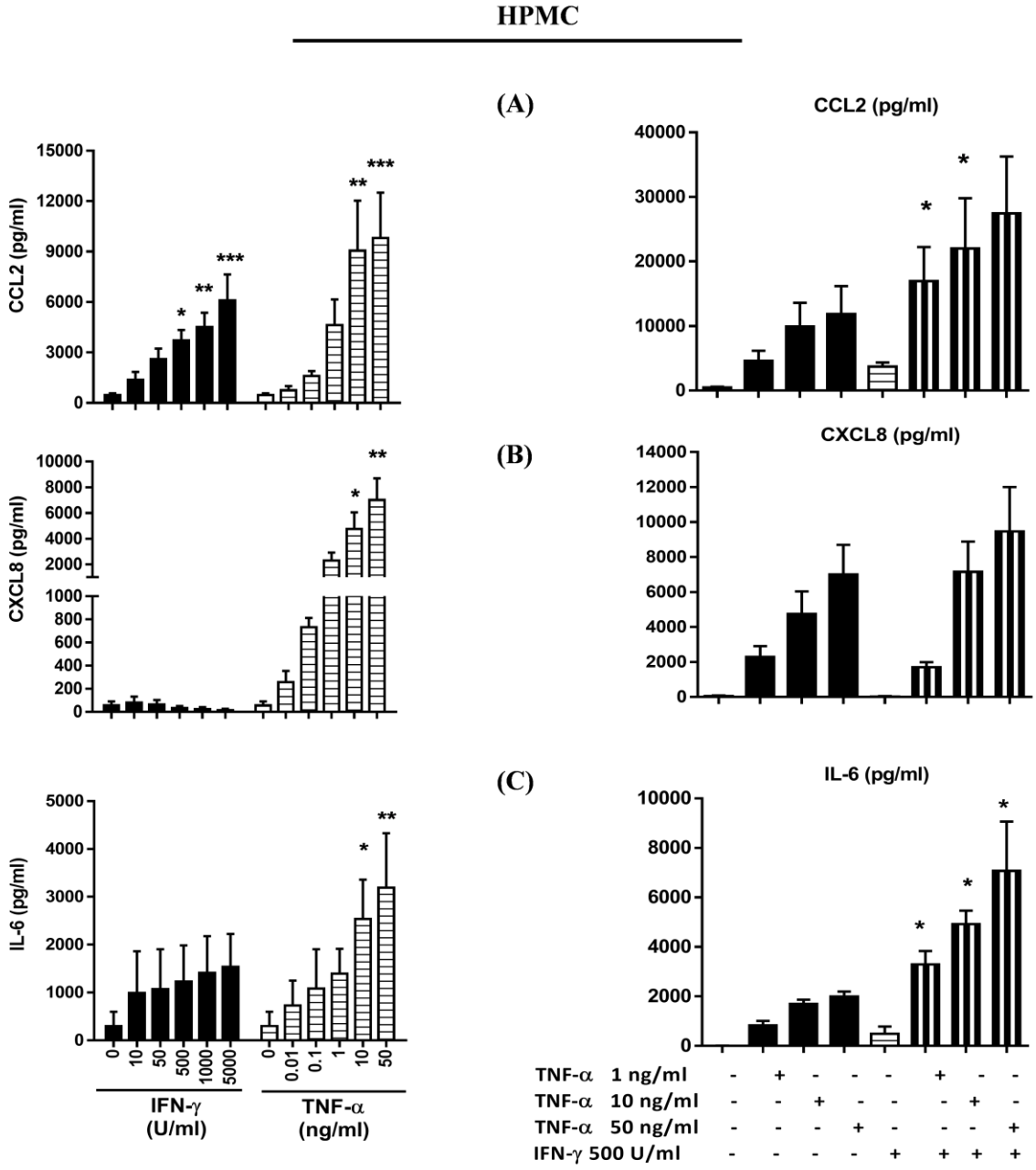

Figure 6.4. Dose-dependent effects of recombinant IFN-γ and TNF-α on CXCL8, CCL2 and IL-6 release by HPMC.

(A-C, *left*) Quiescent cells were treated with increasing doses of either IFN-γ or TNF-α for 24 hours before quantification of chemokines and cytokines in culture supernatants. (A-C, *right*) Effect of recombinant IFN-γ and TNF-α alone and in combination on CXCL8, CCL2 and IL-6 secretion by HPMC. Data were analysed by RM one-way ANOVA or Friedman test with Holm-Sidak or Dunn's post hoc test, respectively. Comparisons were made with control medium. Comparisons between additive and combined cytokine effects were made using the Kruskal-Wallis test with Dunn's post hoc test. Data are expressed as the mean ± SEM from 4 to 7 experiments with cells isolated from separate donors. Differences were considered significant as indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

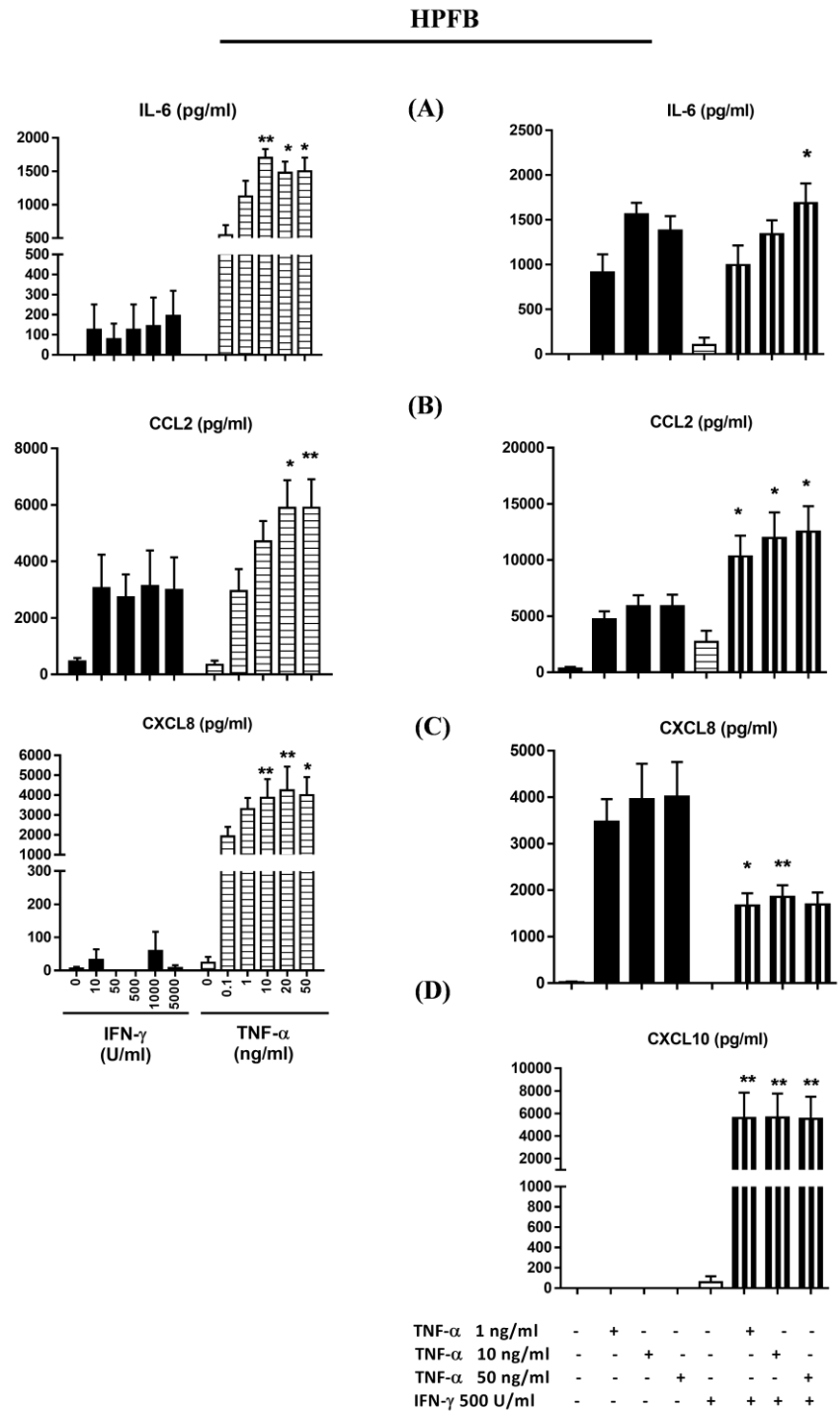

Figure 6.5. Dose-dependent effect of recombinant IFN-γ and TNF-α on IL-6, CCL2, CXCL8, CCL10 released by HPFB.

(A-C, *left*) Quiescent cells were treated with increasing doses of either IFN-γ (1-5000 U/ml) or TNF-α (0.1-50 ng/ml) for 24 hours before quantification of chemokines and cytokines in culture supernatants. (A-D, *right*) Effect of recombinant IFN-γ and TNF-α alone and in combination on IL-6, CCL2, CXCL8, and CXCL10 secretion by HPFB. Data were analysed by RM one-way ANOVA or Friedman test with Holm-Sidak or Dunn's post hoc test, respectively. Comparisons were made with control medium. Comparison between the additive and the combined effect when cytokines were added together were made by Kruskal-Wallis test with Dunn's post hoc test. Data are expressed as the mean ± SEM from 4 to 7 experiments with cells isolated from separate donors. Differences were considered significant as indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

6.3.3 Pre-treatment of unconventional T cells CoM with sTNFR and anti-IFN-γ antibodies abrogates HPMC and HPFB activation

To analyse activation of HPMC and HPFB by IFN-γ or TNF-α in unconventional T cell CoM, this last was pre-treated with anti-IFN-γ or sTNFR (etanercept), alone or in combination, before addition to HPMC and HPFB cultures.

In the presence of CoM from γδ T cells or MAIT cells that was pre-blocked with anti-IFN-γ, release of CCL2 and IL-6 by HPMC was reduced, but there was not effect on CXCL8 production (Figures 6.6 and 6.7). Blocking of TNF-α alone had the greatest effect on production of IL-6, followed by CCL2, compared to the positive control (Figure 6.6A and 6.7A). Although the measurement was not significant, the amount of CXCL8 was reduced between 4 and 10 fold in the presence of sTNFR (Figure 6.6B and 6.7B).

HPMC cultured in the presence of γδ T cell or MAIT cell CoM pre-blocked with both sTNFR and anti-IFN-γ released a decreased amount of CCL2, IL-6 and CXCL8 (Figure 6.6 and 6.7) in comparison to the positive control.

HPFB cultured in unconventional T cell CoM blocked with sTNFR alone or in combination with anti-IFN-γ produced decreased IL-6 and CXCL8 (Figure 6.8A-B and 6.9A-B), whereas CCL2 was significantly reduced only when the two cytokines were blocked together ($p<0.001$, Figure 6.8A and 6.9A).

CXCL10 was not detected when HPMC and HPFB were cultured in unconventional T cells CoM pre-treated with anti-IFN-γ alone or in combination with sTNFR (Figures 6.8B and 6.9B).

These data show that neutralisation of TNF-α and/or IFN-γ in γδ T and MAIT cell CoM potentially decrease the production of CCL2, CXCL8, CXCL10 and IL-6 by HPMC and HPFB, with CXCL8 and IL-6 secretion being particularly sensitive to inhibition of TNF-α, while CXCL10 secretion was mainly driven by IFN-γ.

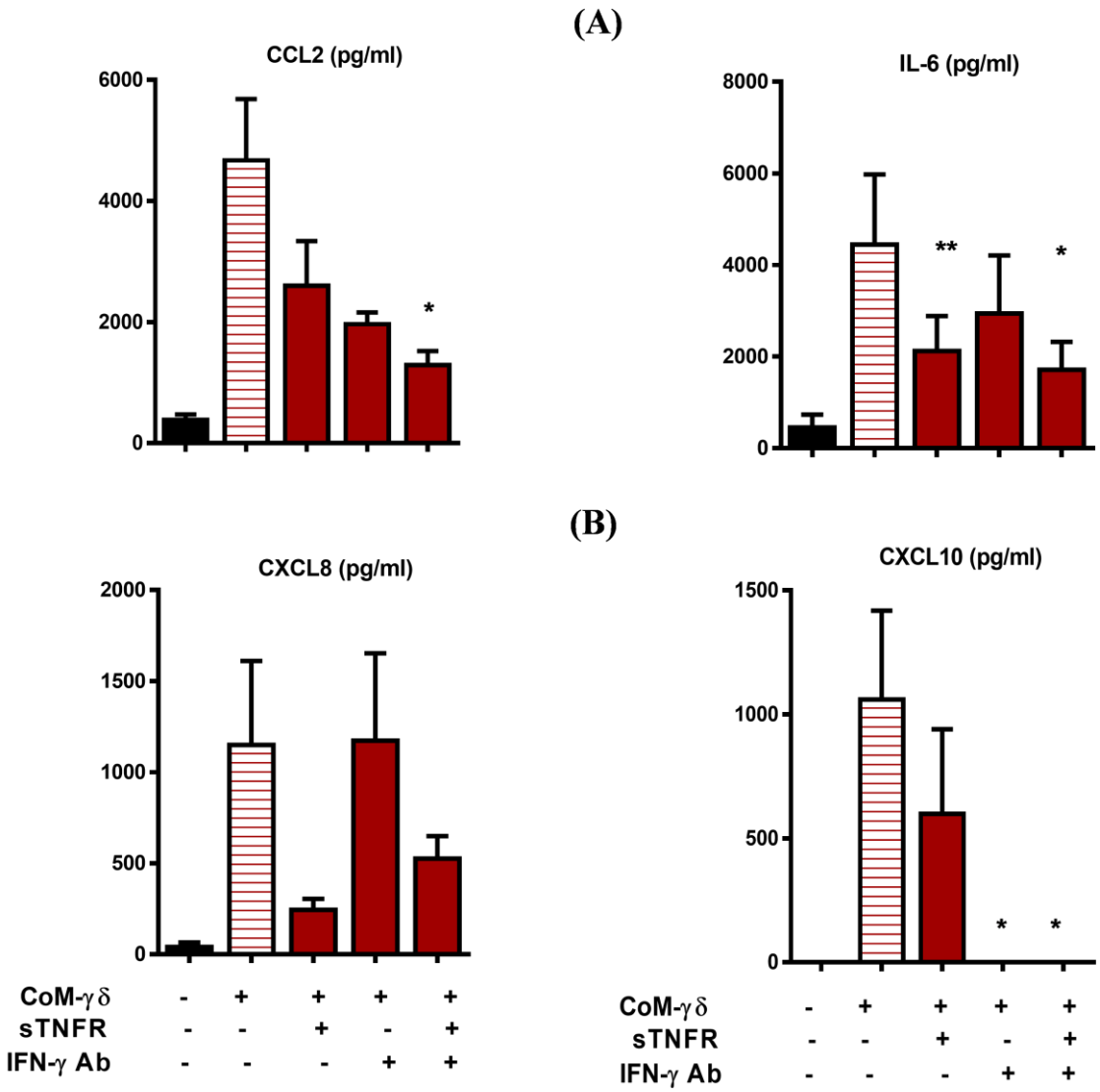

Figure 6.6. Effect of TNF-α and IFN-γ blockade on induction of HPMC cytokine and chemokine secretion in response to CoM-γδ.

CoM from FACS sorted Vγ9$^+$ T cells stimulated overnight in the presence of HMB-PP was diluted 4-fold with control medium and supplemented with antibody against IFN-γ or sTNFR alone or in combination (all at 10 µg/ml). HPMC were exposed to pre-treated CoM for 24 hours and the release of cytokines and chemokines was measured by ELISA. Data were analysed by RM one-way ANOVA or Friedman test with Holm-Sidak or Dunn's post hoc test, respectively. These data were compared with values detected in the absence of neutralising reagents. Data are expressed as the mean ± SEM from 4-7 experiments with HPMC isolated from separate donors. Differences were considered significant as indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

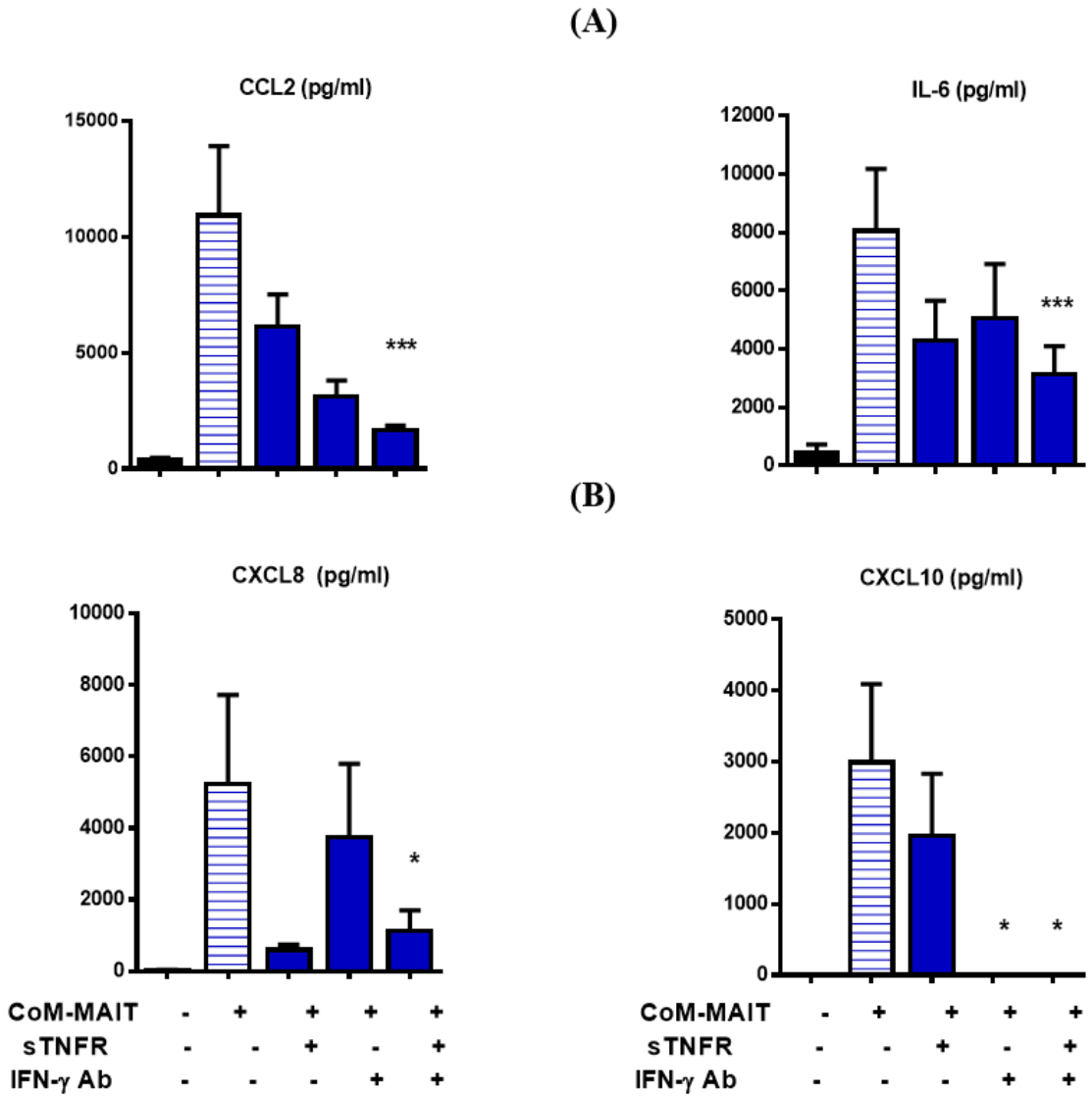

Figure 6.7. Effect of TNF-α and IFN-γ blockade on induction of HPMC cytokine and chemokine secretion in response to CoM-MAIT.

CoM from FACS sorted Vα7.2^{+} T-cells stimulated overnight in the presence of anti-CD3/CD28 beads was diluted 4-fold with control medium and supplemented with antibody against IFN-γ or sTNFR alone or in combination (all at 10 µg/ml). HPMC were exposed to the pre-treated CoM for 24 hours and the release of cytokines and chemokines was measured by ELISA. Data were analysed by RM one-way ANOVA or Friedman test with Holm-Sidak or Dunn's post hoc test, respectively. These data were compared with values detected in the absence of neutralising reagents. Data are expressed as the mean ± SEM from 4-7 experiments with HPMC isolated from separate donors. Differences were considered significant as indicated in the figure: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

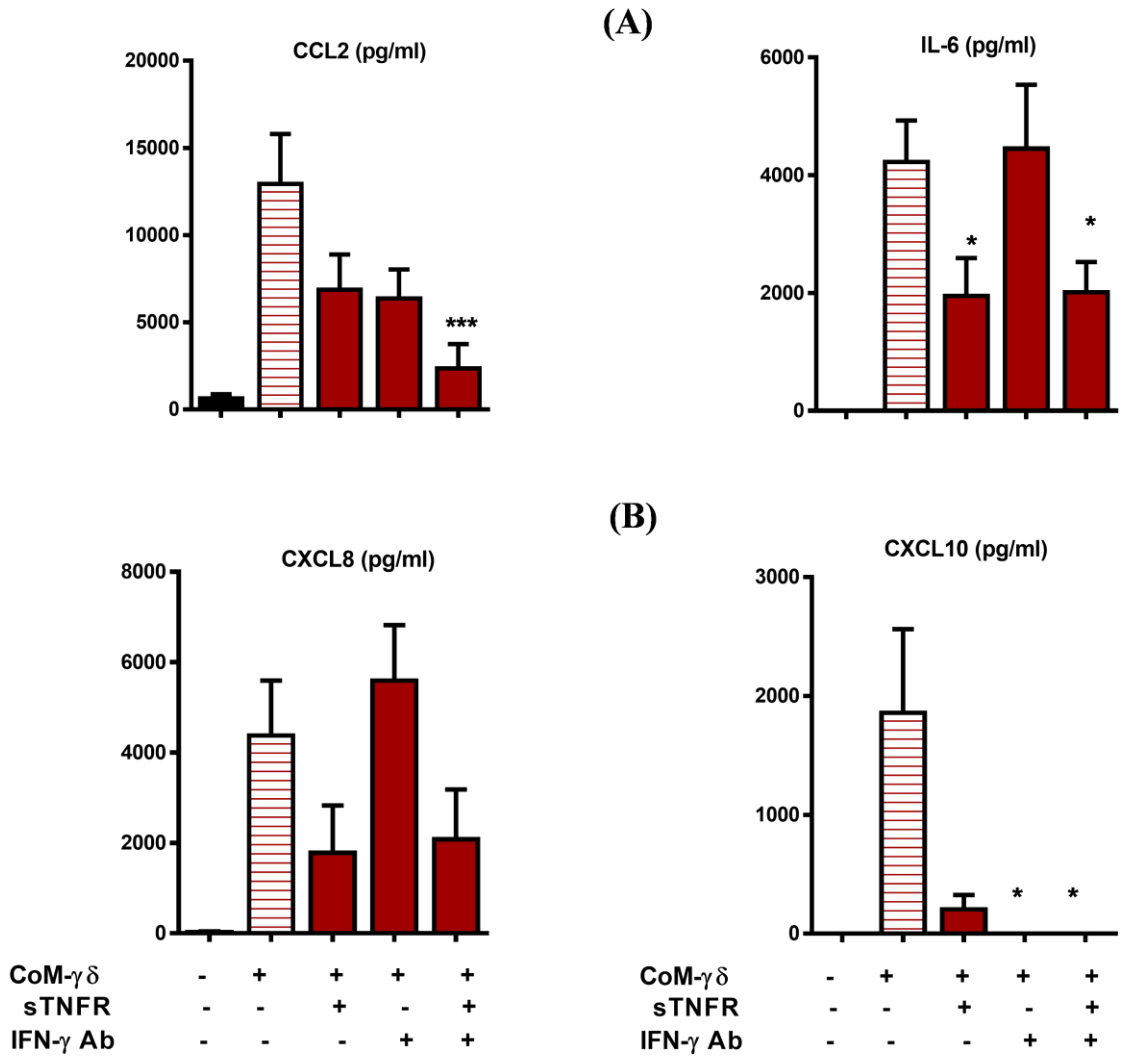

Figure 6.8. Effect of TNF-α and IFN-γ blockade on induction of HPFB cytokine and chemokine secretion in response to CoM-γδ.

CoM from FACS sorted Vγ9$^+$ T cells stimulated overnight in the presence of HMB-PP was diluted 4-fold with control medium and supplemented with antibody against IFN-γ or sTNFR alone or in combination (all at 10 µg/ml). HPFB were exposed to the pre-treated CoM for 24 hours and the release of cytokines and chemokines was measured by ELISA. Data were analysed by RM one-way ANOVA or Friedman test with Holm-Sidak or Dunn's post hoc test, respectively. These data were compared with value detected in the absence of neutralising reagents. Data are expressed as the mean ± SEM from 4-7 experiments with HPFB isolated from separate donors. Differences were considered significant as indicated in the figure: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

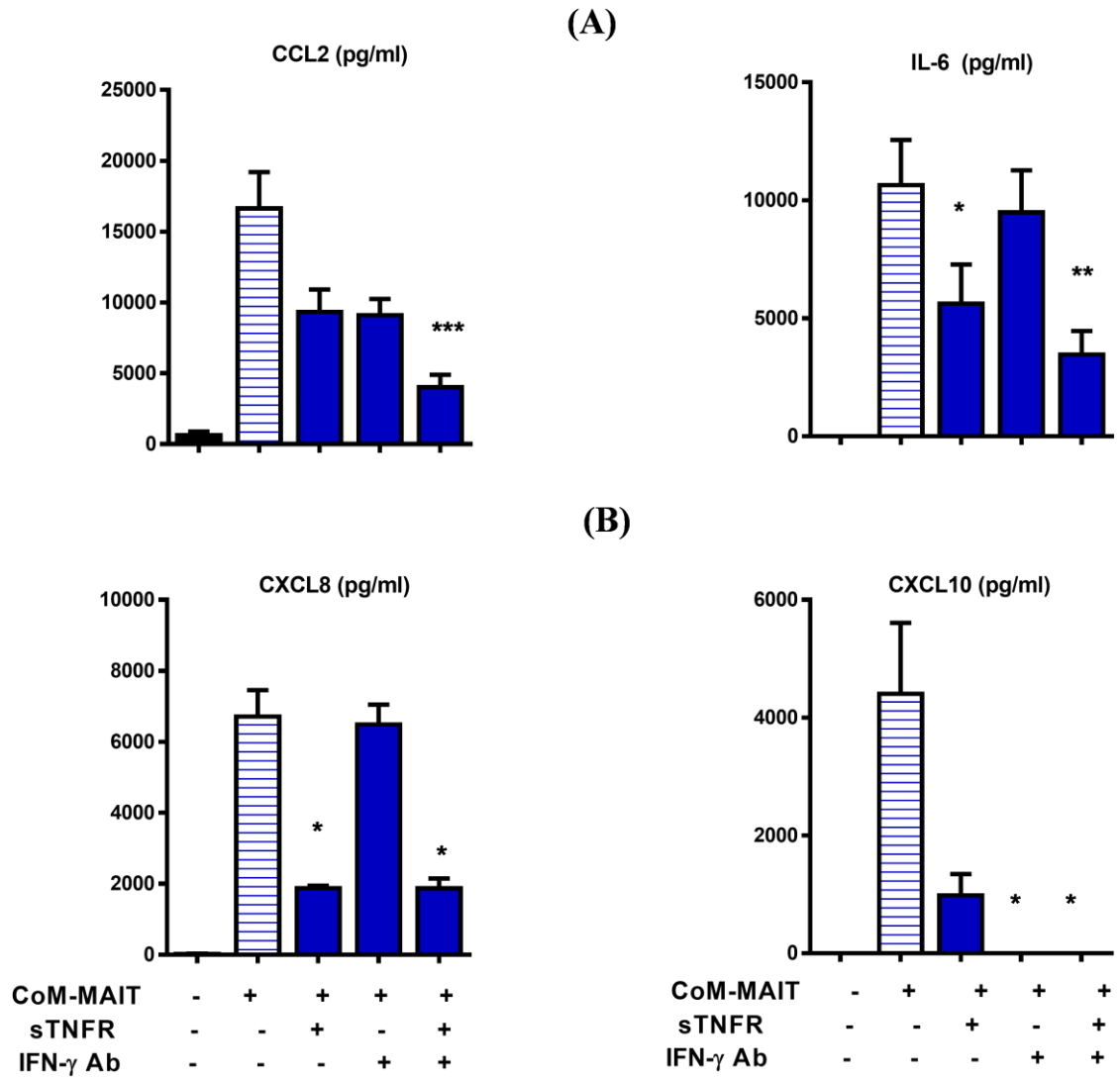

Figure 6.9. Effect of TNF-α and IFN-γ blockade on induction of HPFB cytokine and chemokine secretion in response to CoM-MAIT.

CoM from FACS sorted Vα7.2^{+} T-cells stimulated overnight in the presence of anti-CD3/CD28 beads was diluted 4-fold with control medium and supplemented with antibody against IFN-g or sTNFR alone or in combination (all at 10 µg/ml). HPFB were exposed to the pre-treated CoM for 24 hours and the release of cytokines and chemokines was measured by ELISA. Data were analysed by RM one-way ANOVA or Friedman test with Holm-Sidak or Dunn's post hoc test, respectively. These data were compared with value detected in the absence of neutralising reagents. Data are expressed as the mean ± SEM from 4-7 experiments with HPFB isolated from separate donors. Differences were considered significant as indicated in the figure: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

6.3.4 HPMC and HPFB activation by PDE of patients with Gram⁻ infections

As described in the previous Chapter, γδ T cells and MAIT cell numbers increase in PD associated infection caused by HMB-PP$^+$ and vitamin B2$^+$ bacteria. Most of these bacteria are Gram⁻ bacteria (e.g. *E. coli*, *Acinetobacter*, *Enterobacter*) and are associated with poor clinical outcomes (Chapter 5). Gram⁻ infections in PD are characterized by increased numbers of neutrophils and monocytes in the peritoneal cavity, and elevated levels of cytokines such as IL-1β and TNF-α (Lin et al., 2013). TNF-α is produced by unconventional T cells *in vitro* in response to both synthetic ligands and Gram⁻ bacterial extract (Chapter 3). To determine if increased presence of pro-inflammatory cytokines during Gram⁻ infection contributes to chemokine production by peritoneal HPMC and HPFB, I cultured these cells in the presence of PDE derived from stable patients or patients presenting with acute Gram⁻ peritonitis (Table 6.1).

When I stimulated HPMC with PDE from infected patients, production of CCL2 and CXCL8 was significantly induced compared to control cultures treated with PDE from stable patients (Figure 6.10A).

Of note, the maximum amounts of CCL2 and CXCL8 were released after treatment of HPMC with PDE from patient P1 who presented with relatively high levels of TNF-α, IL-1β and IFN-γ (Table 6.1). In contrast, I observed lower amounts of CXCL8 after treatment of HPMC with PDE from patient P2, who presented with relatively low levels of TNF-α, IL-1β and IFN-γ.

I next sought to investigate which cytokines in the PDE were responsible for the induction of CCL2 and CXCL8 secretion by HPMC. To achieve this, PDE was pre-treated with sTNFR, anti-IFN-γ and anti-IL-1β, alone or in different combinations. As shown in Figure 6.10B, I could significantly inhibit the secretion of CXCL8 and CCL2 (up to 34.7% ± 9.8 and 25.4% ± 13, respectively), by a combined blocking of TNF-α and IL-1β. Other treatments showed various degrees of inhibition yet not significantly. Together these results highlight the importance of IL-1β and TNF-α in the acute inflammatory response to Gram⁻ bacteria.

Table 6.1. Characteristics of PDE from patients with Gram⁻ infections.

Patients with peritonitis			
	P1	**P2**	**P3**
Causative organism	*Enterobacter*	*E. coli*	*Acinetobacter*
PDE cytokines/chemokines (pg/ml)			
CCL2	5027	8288	51
CXCL8	65	2665	111
IFN-γ	167	63	151
TNF-α	1790	355	1154
IL-1β	161	40	26

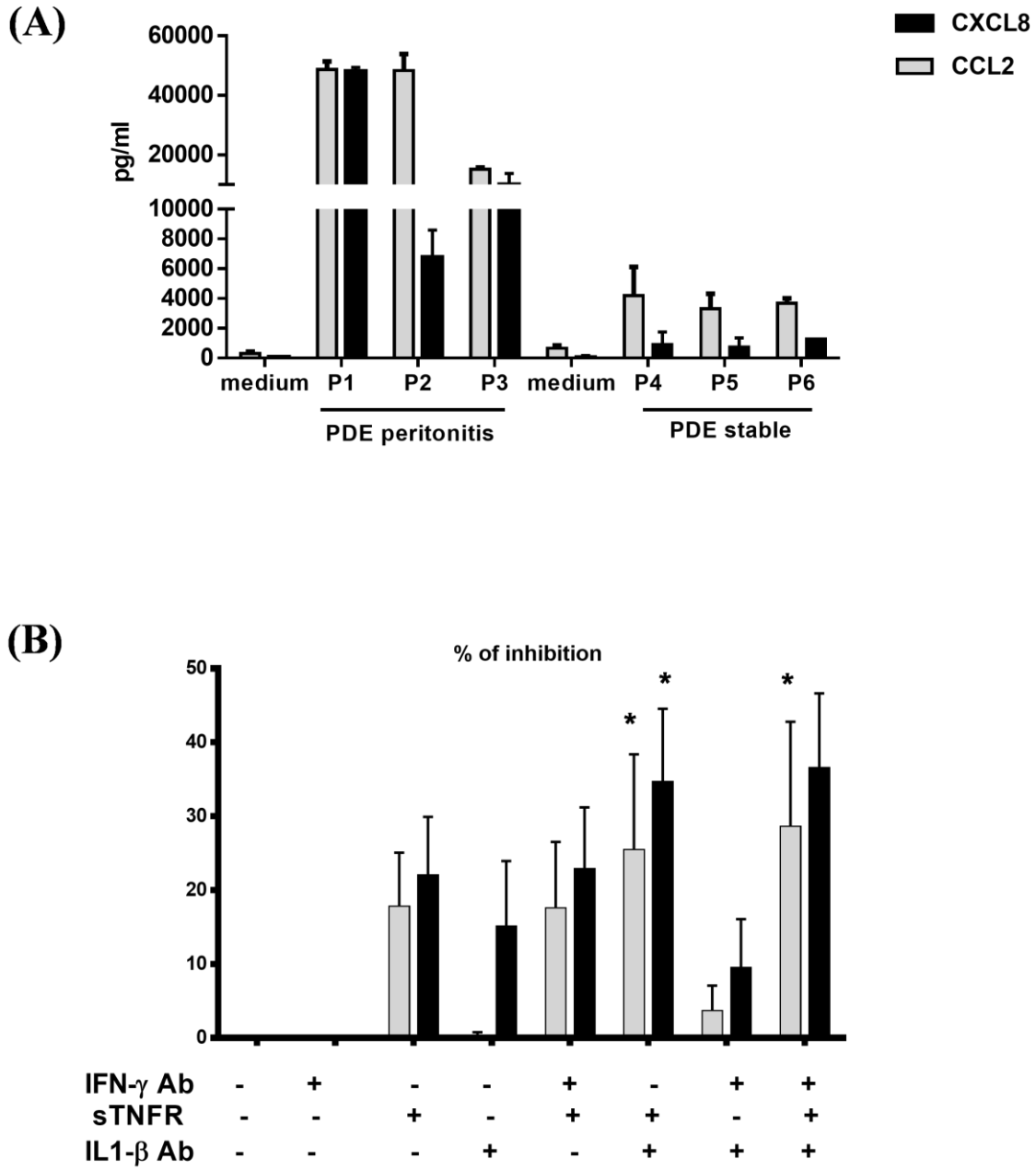

Figure 6.10. Effect of PDE on chemokine secretion by HPMC.
(A) PDE from three infected patients (P1-P3) and three stable patients (P4-P6) was diluted 1:4 with culture medium and added to HPMC cultures for 24 hours. CCL2 and CXCL8 were measure by ELISA and results are adjusted by the dilution factor. (B) PDE from infected patients was supplemented with anti-IFN-γ, IL1-β or sTNFR alone or in different combinations. Resting HPMC were then exposed to pre-blocked PDE for 24 hours. Data are expressed as the mean ± SEM from 3-4 experiments with cells isolated from separate donors. Data were analysed by Friedman test with Dunn's post hoc test. Comparisons were made with values detected in absence of antibodies. Differences were considered significant as indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

6.3.5 HPMC release cytokines and chemokines in response to Gram$^+$ and Gram$^-$ bacteria

HPMC are the first cells to encounter microbes during episodes of PD associated infections. These infections are caused most frequently by Gram$^+$ *Staphylococcus* species, and cells respond to *S. epidermidis* by releasing chemokines and cytokines (Colmont et al., 2011). Infections caused by Gram$^-$ bacteria, even if less frequent, are more severe. However, the role of HPMC in response to Gram$^-$ bacteria has received comparatively little attention, despite the fact that Gram$^-$ infections are generally associated with poorer clinical outcomes.

To define the potential impact of bacterial infections on local tissue, I here studied HPMC responses to a range of Gram$^-$ (*E. coli, K. pneumoniae, P. aeruginosa*) and Gram$^+$ (*E. faecalis, C. striatum, S. aureus*) bacteria. HPMC produced IL-6, CXCL8 and CCL2 following treatment with either Gram$^+$ or Gram$^-$ extracts (Figure 6.11). This response was significant when cells were cultured in the presence of *E. coli* and *P. aeruginosa* extracts, in comparison with other organisms (Figure 6.11).

As shown in Figure 6.12, similar results were obtained when using HPFB as responder cells. Taken together, these results suggest that both types of bacteria contribute to activation of peritoneal epithelial and stromal cells, and that Gram$^-$ bacteria are able to trigger a more severe response compared to Gram$^+$ microbes, as evidenced by more pronounced secretion of inflammatory chemokines and cytokines.

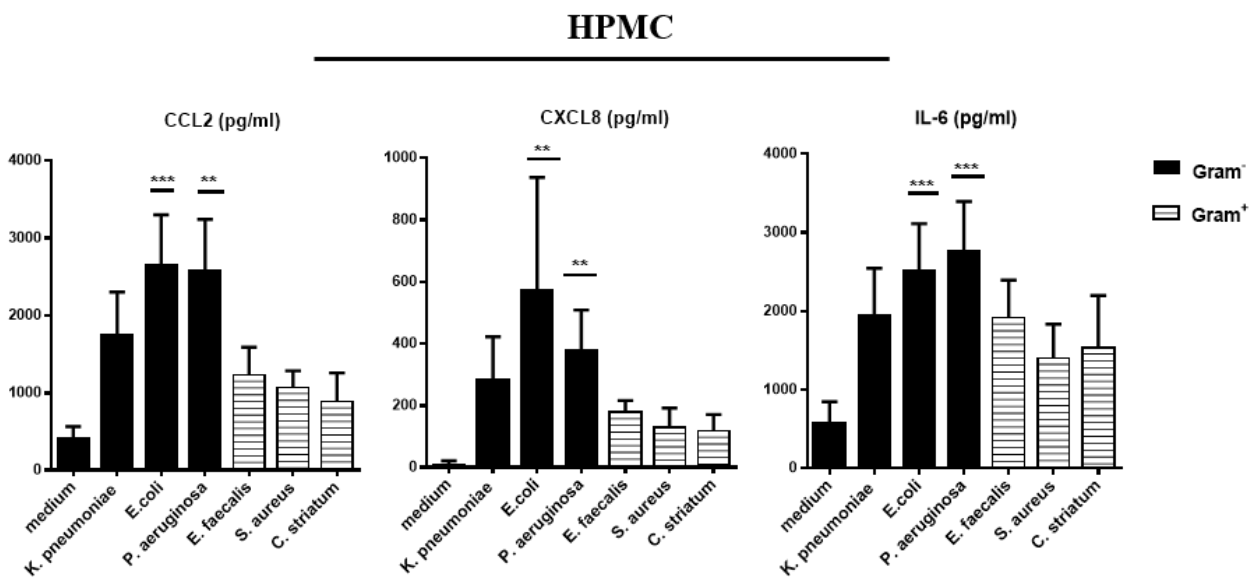

Figure 6.11. Effect of Gram$^+$ and Gram$^-$ bacterial extracts on CXCL8, CCL2 and IL-6 release by HPMC.

Quiescent HPMC were exposed to Gram$^-$ (*P. aeruginosa, K. pneumoniae, E. coli*) or Gram$^+$ (*S. aureus, E. faecalis, C. striatum*) bacterial extracts at a protein concentration of 100 µg/ml for 24 hours. ELISA was performed after 24 hours. Data are expressed as the mean ± SEM from 4 to 5 experiments with cells isolated from separate donors. Data were analysed by Friedman test with Dunn's post hoc test. Differences were considered significant as indicated: *, p<0.05; **, p<0.01; ***, p<0.001.

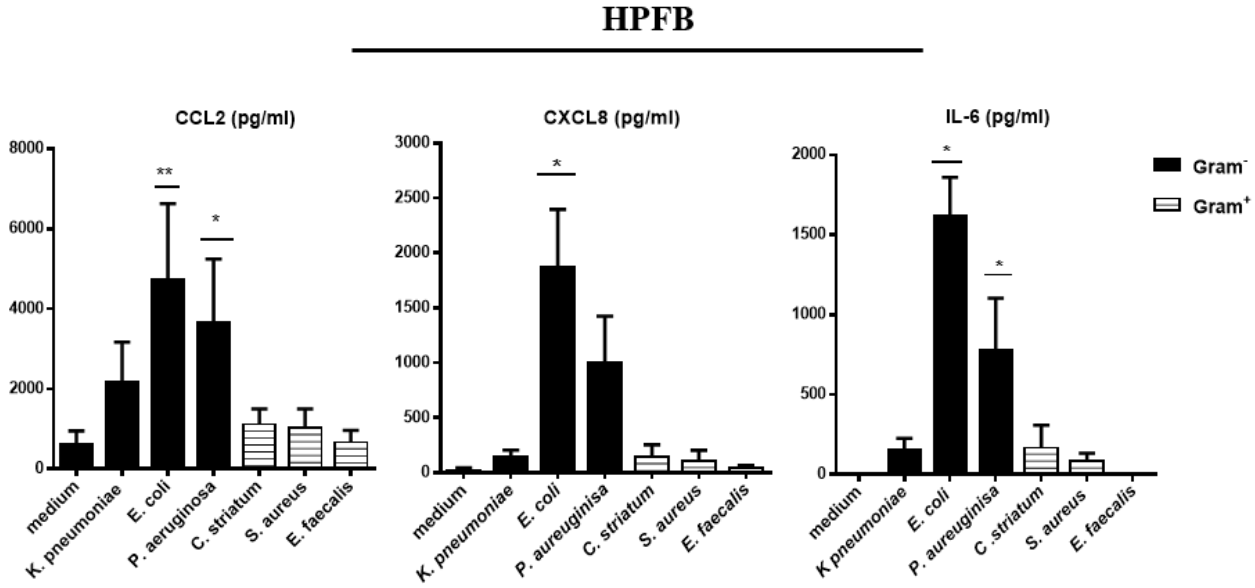

Figure 6.12 Effect of different Gram$^+$ and Gram$^-$ bacterial extracts on CXCL8, CCL2 and IL-6 release by HPFB.

Quiescent HPFB were exposed to Gram$^-$ (*P. aeruginosa, K. pneumoniae, E. coli*) or Gram$^+$ (*S. aureus, E. faecalis, C. striatum*) bacterial extracts at a protein concentration of 100 µg/ml for 24h. ELISA was performed after 24h. Data are expressed as the mean ± SEM from 4 to 5 experiments with cells isolated from separate donors. Data were analysed by one-way ANOVA, comparisons were made with medium controls. Differences were considered significant as indicated: *, p<0.05; **, p<0.01; ***, p<0.001.

6.4 Effect of unconventional T cell derived cytokines on epithelial and mesenchymal marker expression by HPMC

6.4.1 Unconventional T cell induced morphological changes in HPMC

Repeated episodes of peritonitis together with a long term PD effluent exposure can result in peritoneal fibrosis and ultrafiltration dysfunction. TNF-α is one of the cytokines implicated, together with TGF-β, IL-1β and IL-6, in mesothelial to mesenchymal transition of HPMC, which can lead to long term peritoneal membrane damage (Aroeira et al., 2007; López-Cabrera, 2014). In the experiments above, when HPMC were co-cultured with CoM derived from γδ T cells or MAIT cells, they underwent striking changes from an epithelial-like appearance to a spindled fibroblastic shape within 24 hours (Figure 6.13A). This effect was similar to the one observed when HPMC were cultured in the presence of IFN-γ and TNF-α (Figure 6.14). These morphological changes were diminished when TNF-α and/or IFN-γ were neutralized in unconventional T cell CoM prior to addition to HPMC (Figure 6.13B). These findings imply that TNF-α and IFN-γ derived from activated unconventional T cells might at least contribute to induction of morphological changes in the peritoneal membrane during PD associated infection.

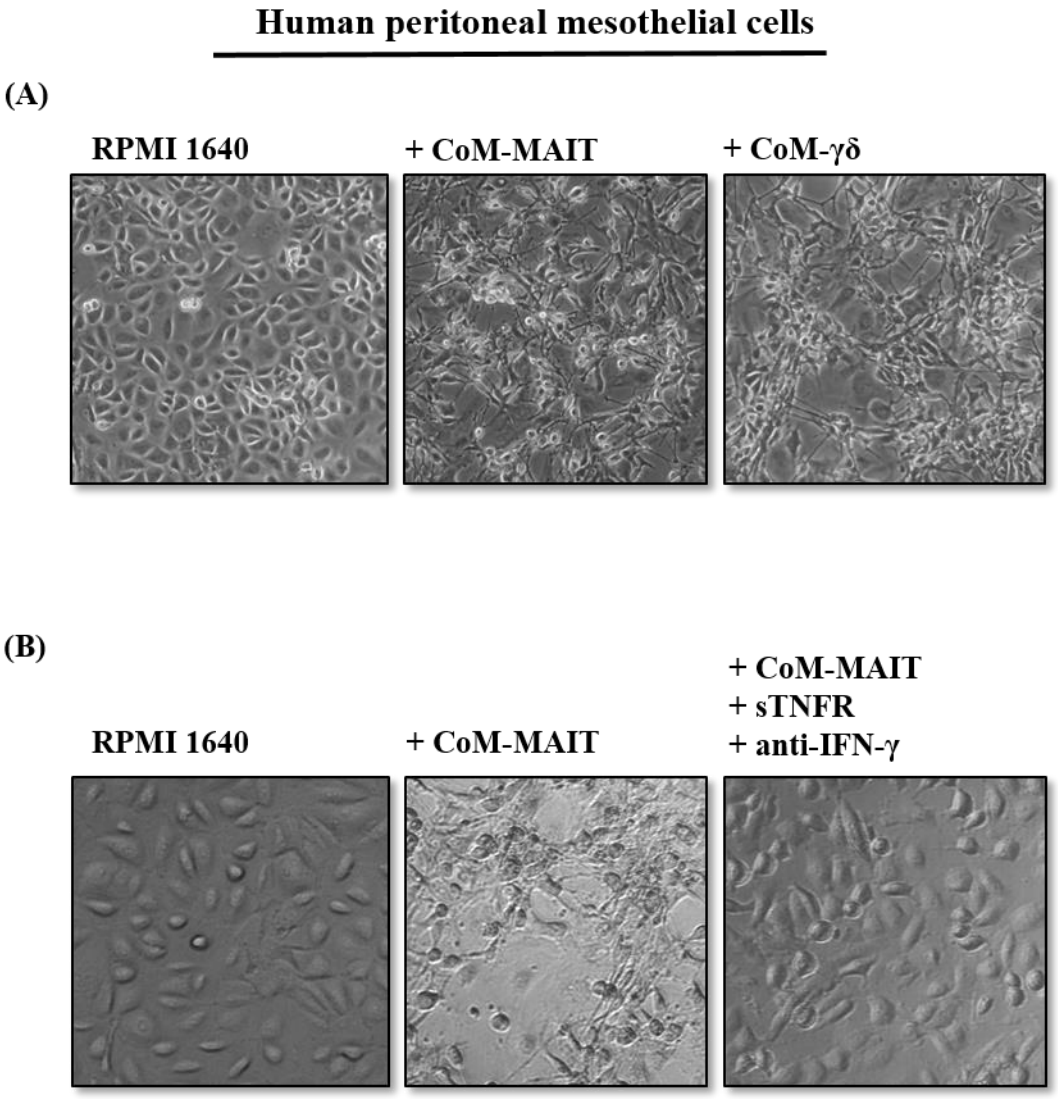

Figure 6.13. Morphologic HPMC changes in the presence of unconventional T cell CoM.
(A) Images were captured using a light microscope with a ×20 objective lens following 24 hours of culture in RPMI-1640 medium or conditioned medium derived from MAIT cells or γδ T cells. (B) CoM derived from MAIT cells was +/- pre-treated with sTNFR and anti-IFN-γ blocking antibody and added to HPMC cultures for 24 hours. Representative of 3 experiments.

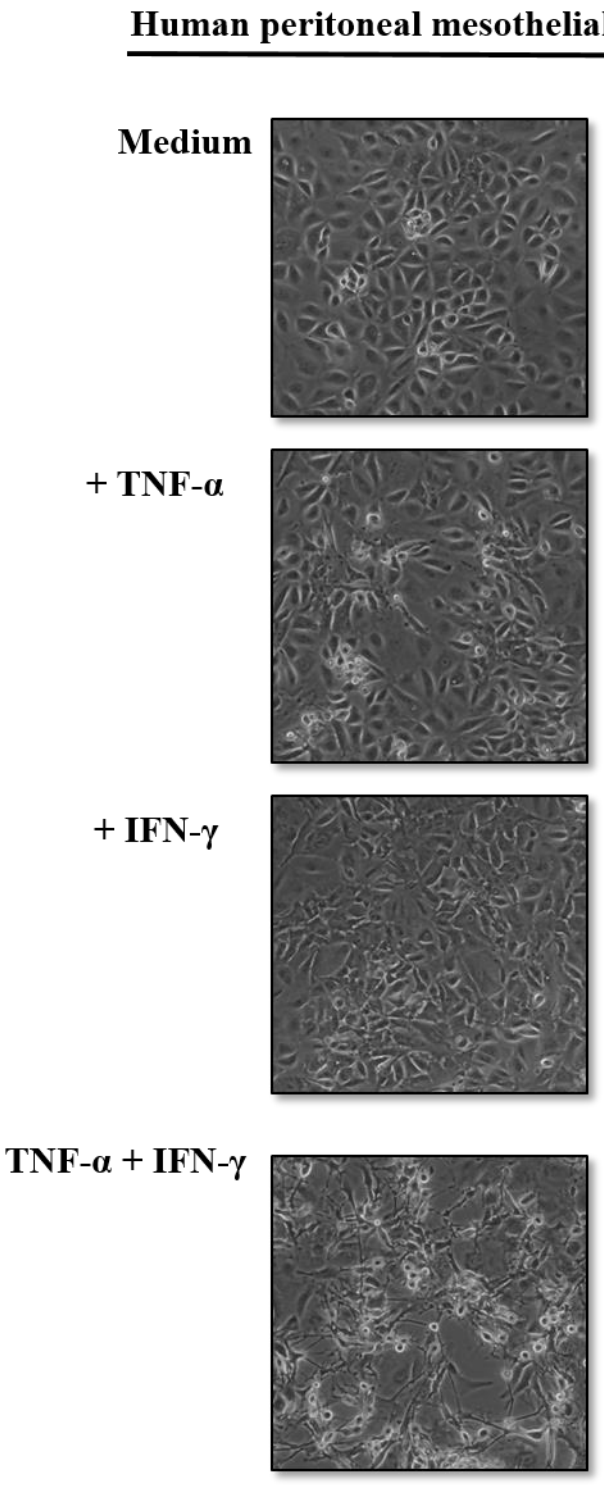

Figure 6.14. Morphologic HPMC changes induced by TNF-α and IFN-γ.
Images were captured using a light microscope with a ×20 objective lens following 24 hours of culture in the presence of 5 ng/ml of TNF-α and IFN-γ, alone or in combination, in the absence of FCS. Representative of 3 experiments.

6.4.2 TNF-α induced significant changes in HPMC epithelial markers

To study the effects of TNF-α on the modulation of epithelial and mesenchymal markers associated with peritoneal membrane failure I firstly cultured HPMC with increasing concentrations of TNF-α. I then examined expression of epithelial and mesenchymal markers that are associated with mesothelial to mesenchymal transition (MMT) at the mRNA level. As shown in Figure 6.15, I found a decrease in mRNA expression of epithelial markers implicated in cell-cell junctions such as E-cadherin, ZO-1 and occludin. Among the mesenchymal markers, I observed a significant downregulation of collagen-1 expression but not of the others marker, including fibronectin, α-SMA and SNAIL (Figure 6.15).

SNAIL is implicated in the switch between the epithelial and the mesothelial phenotype and usually affects the expression of E-cadherin by TNF-α through the protein kinase B (Wu and Zhou, 2010). To check if an early upregulation of SNAIL was also responsible for the downregulation of E-cadherin in my system, I incubated HPMC with 5 ng/ml of TNF-α and measured the expression of mesenchymal markers at different time points. Figure 6.16 shows that there were no changes in the expression of SNAIL at early time points (less than 24 hours). Similarly, no changes were observed for α-SMA, whereas collagen-1 significantly increased after 6 hours and then decreased again after 48 hours. Although not significantly, fibronectin expression also showed a tendency to increase after 12 and 48 hours in the presence of TNF-α (Figure 6.16). In summary, these data show a possible contribution of TNF-α to the modulation of expression levels of epithelial markers implicated in the MMT process. However, the same effect was less evident with regard to key mesenchymal markers.

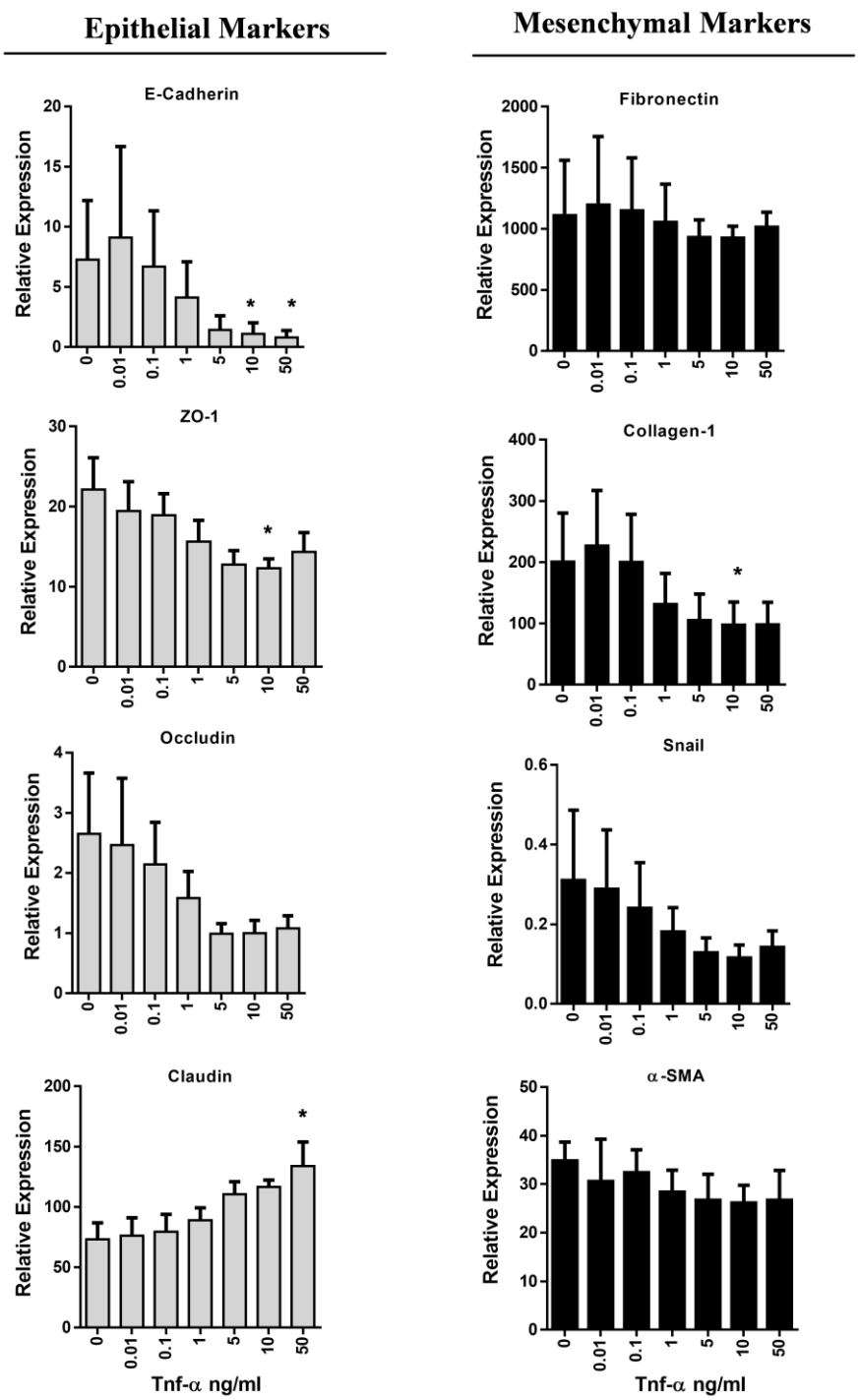

Figure 6.15. Relative expression of epithelial and mesenchymal HPMC markers in response to TNF-α. HPMC expression of epithelial markers E-cadherin, ZO-1, occludin, claudin-1 (left panels), and mesenchymal markers fibronectin, collagen-1, SNAIL and α-SMA (right panels) was detected by RT-qPCR following exposure to TNF-α for 48 hours at the concentrations shown. Data were expressed as expression levels relative to 1,000 copies of GAPDH, and are shown as mean ± SEM of 3 independent experiments. Data were analysed by RM one-way ANOVA or Friedman test with Holm-Sidak or Dunn's post hoc test, respectively. Comparison were made with control medium. Differences were considered significant as indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ****$p<0.0001$.

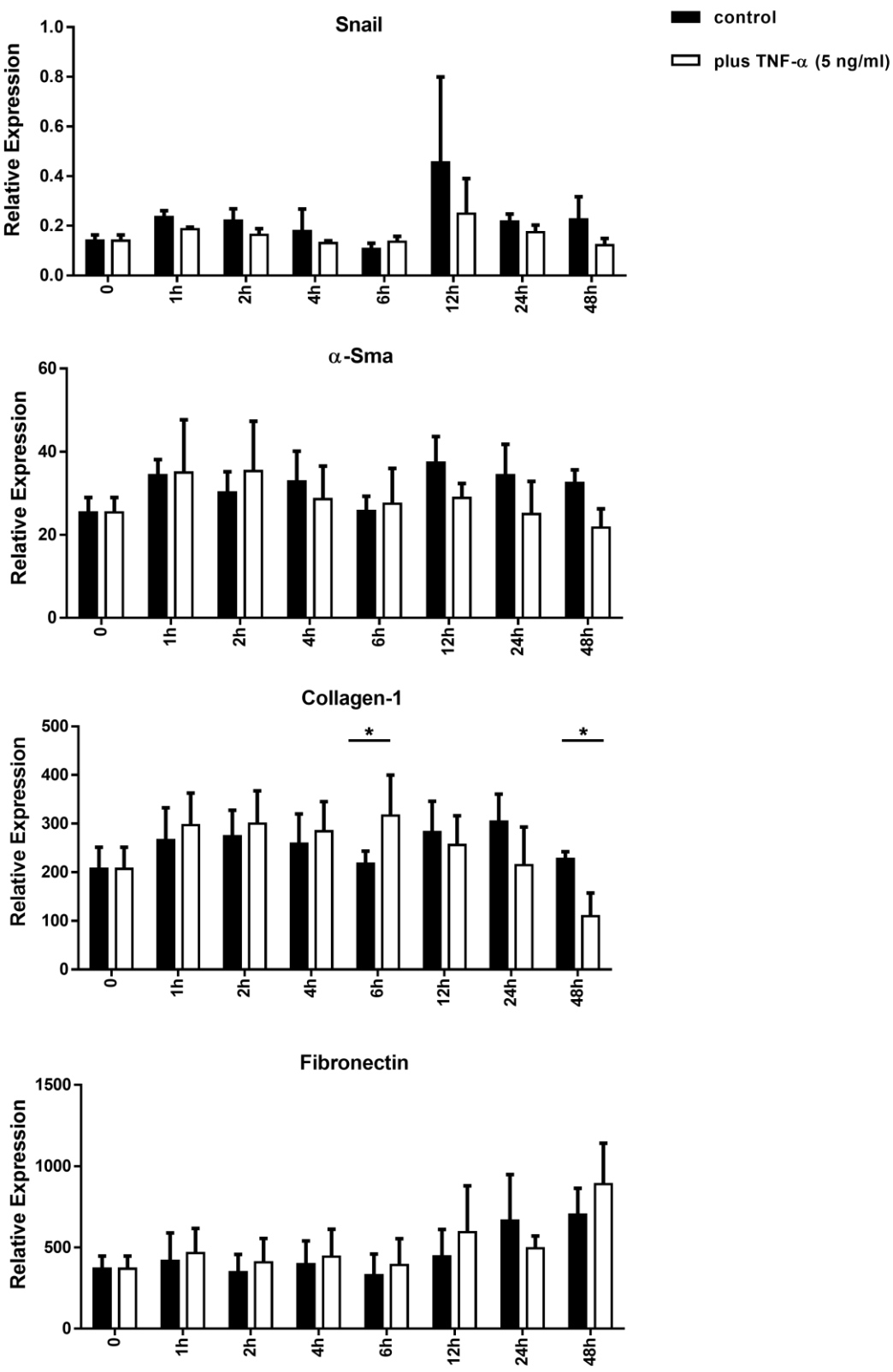

Figure 6.16. Relative expression of HPMC mesenchymal markers in response to TNF-α over 48 hours. Expression of mesenchymal markers fibronectin, collagen-1, SNAIL and α-SMA by HPMC were detected by RT-qPCR relative to GAPDH, after co-culturing HPMC with 5 ng/ml of TNF-α at the time points shown. Data are expressed as mean ± SEM of 3 independent experiments. Data were analysed by two-way ANOVA with Sidak post-hoc test, comparisons were made with control medium at each time point. Differences were considered significant as indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ****$p<0.0001$.

6.4.3 Synergistic modulation of epithelial and mesenchymal markers by TNF-α and IFN-γ

Since CoM from activated unconventional T cells contained between 5 and 10 ng/ml each of TNF-α and IFN-γ, I next analysed expression of epithelial and mesenchymal markers in HPMC cultured with 5 ng/ml of TNF-α and IFN-γ either alone or in combination.

As shown in Figure 6.17, E-cadherin was downregulated by TNF-α, and this effect was more pronounced when TNF-α was combined with IFN-γ (Figure 6.17). The same effect was observed for the expression of ZO-1 and claudin-1, and was more pronounced for occludin (Figure 6.17). Of the mesenchymal markers, a significant downregulation of collagen-1, α-SMA and SNAIL was observed when TNF-α and IFN-γ were added together (Figure 6.17).

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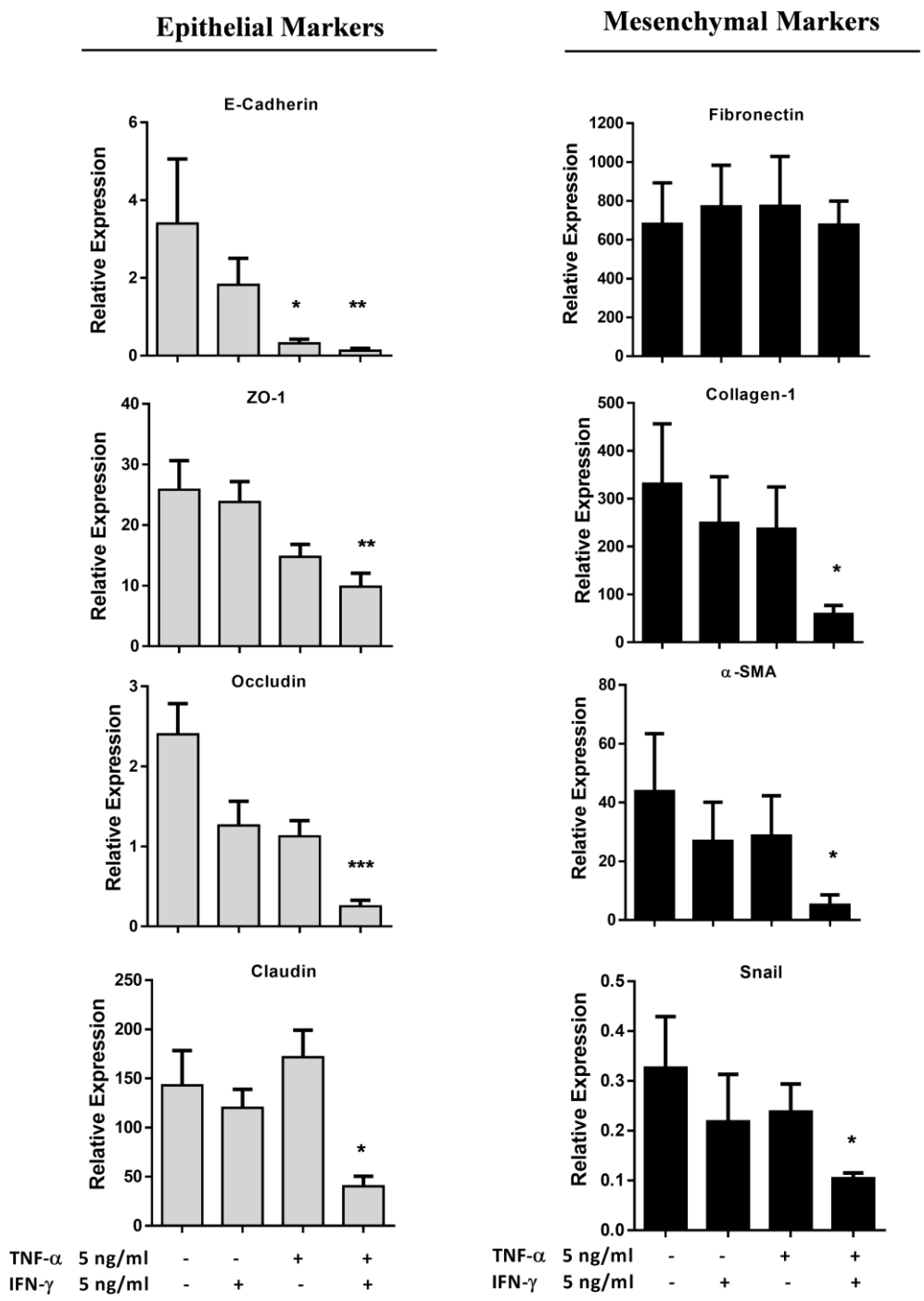

Figure 6.17. Relative expression of epithelial and mesenchymal markers by HPMC in the presence of TNF-α and IFN-γ.

Expression of the epithelial markers E-cadherin, ZO-1, occludin, claudin-1 (left panel), and the mesenchymal markers fibronectin, collagen-1, α-SMA and SNAIL (right panel) by HPMC was detected by RT-qPCR relative to GAPDH after 24 hours following exposure to the indicated concentrations of TNF-α and IFN-γ, alone or combination. The results are expressed as the mean ± SEM of 6 independent experiments. Data were analysed by RM one-way ANOVA or Friedman test with Holm-Sidak or Dunn's post hoc test, respectively. Comparison were made with the condition in absence of blocking antibody. Differences were considered significant as indicated in the Figures: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ****$p<0.0001$.

Next, HPMC were cultured for 24 hours in the presence of unconventional T cell CoM to test the effect of unconventional T cell-released cytokines on expression of epithelial and mesenchymal markers. As shown in Figure 6.18, E-cadherin and occludin were significantly downregulated in the presence of CoM derived from MAIT cells, whereas no one of the epithelial marker were significantly decreased in the presence of CoM derived from γδ T cells (Figure 6.18), maybe due to the relatively small number of experiments performed.

Under the same conditions, both γδ T cell and MAIT cell CoM induced a significant upregulation of fibronectin alone amongst mesenchymal markers (Figure 6.19).

Given the profound production of IL-6 by HPMC in response to TNF-α and IFN-γ, the mRNA level of IL-6 was used as a positive control in these cultures. As shown in Figure 6.20A, the expression of IL-6 was increased 20-fold compared to the untreated control when TNF-α and IFN-γ were combined. A similar level of IL-6 was detected when HPMC were cultured in the presence of CoM derived from unconventional T cells (Figure 6.20B).

Collectively, these results define a potential mechanism by which the presence of TNF-α and IFN-γ released by unconventional T cells during acute inflammatory responses might contribute to peritoneal membrane damage during PD associated infections.

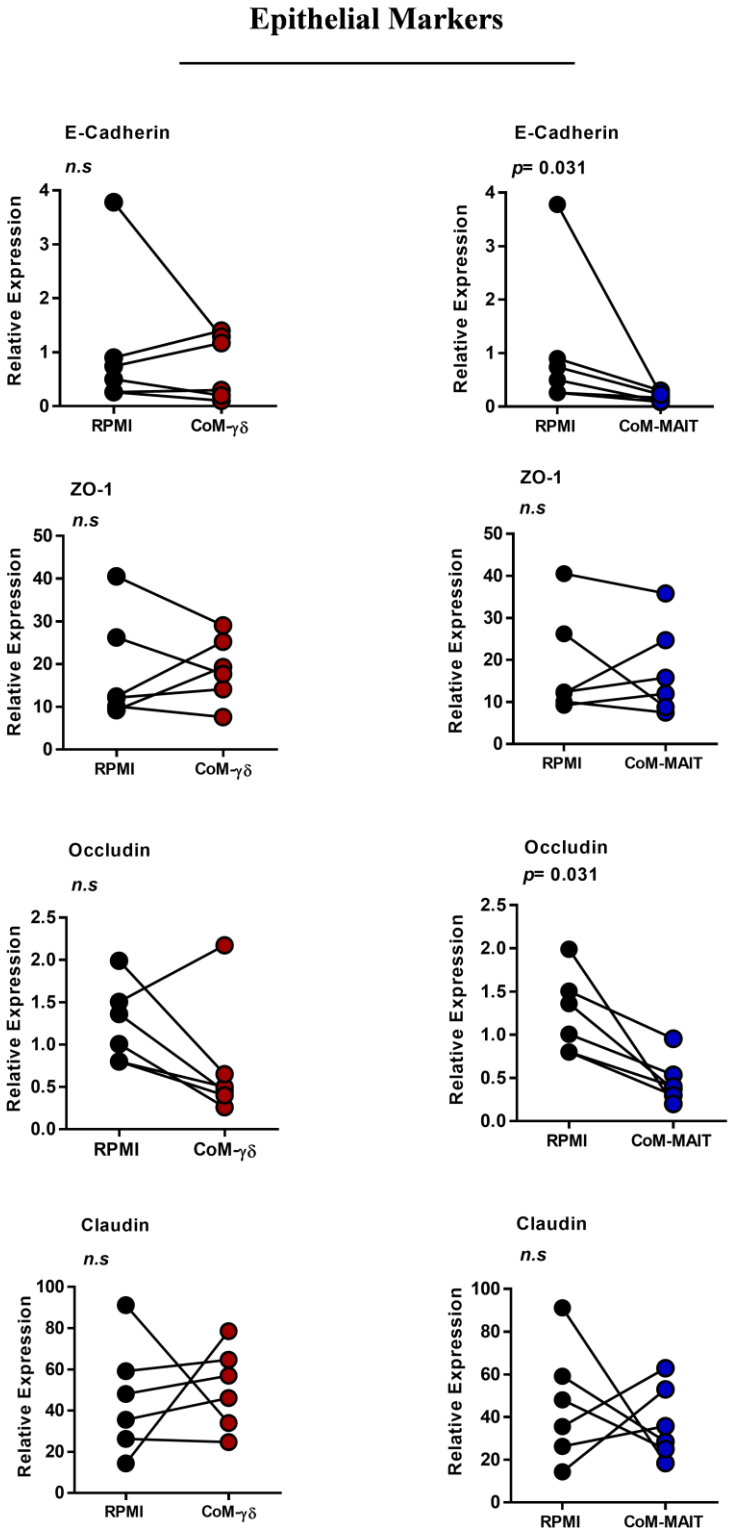

Figure 6.18. Relative expression of epithelial markers in HPMC in the presence of CoM derived from unconventional T cells.

Expression of the epithelial markers E-cadherin, ZO-1, occludin, claudin-1 after 24 hours of co-culture with conditioned medium derived from γδ T cells (left panel) or MAIT cells (right panel). Expression levels were detected by RT-qPCR relative to GAPDH. Each data point represents an individual. Data were analysed by Wilcoxon matched pairs tests. Differences were considered significant as indicated in the figure: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ****$p<0.0001$.

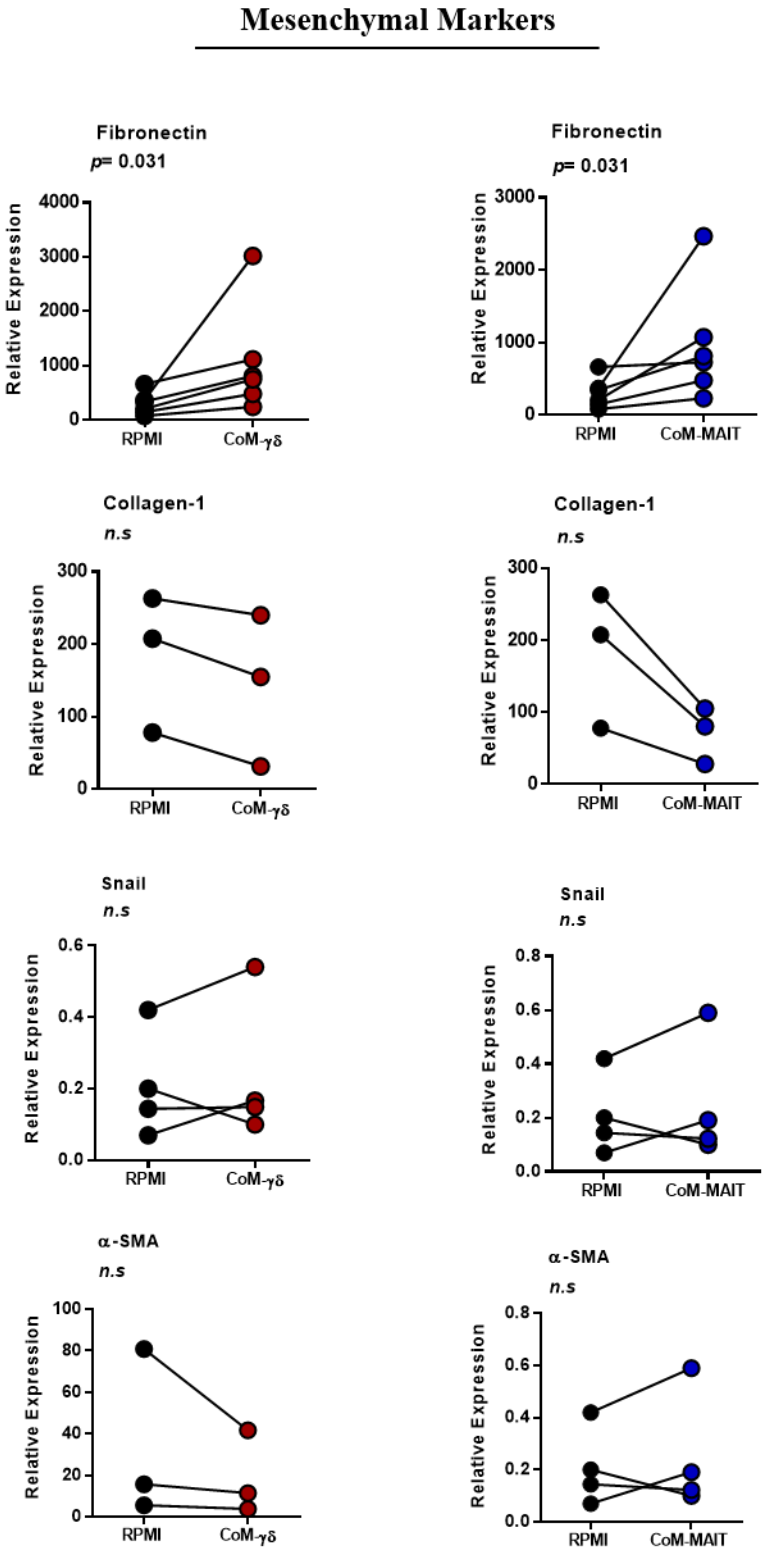

Figure 6.19. Relative expression of mesenchymal markers in HPMC in the presence CoM derived from unconventional T cells.

Expression of mesenchymal markers fibronectin, collagen-1, SNAIL and α-SMA, relative to GAPDH in HPMC were detected by RT-qPCR, after 24 hours of co-culture with conditioned medium derived from γδ T cells (left panel) or MAIT cells (right panel). Data were analysed by Wilcoxon matched pairs test. Differences were considered significant as indicated in the figure: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ****$p<0.0001$.

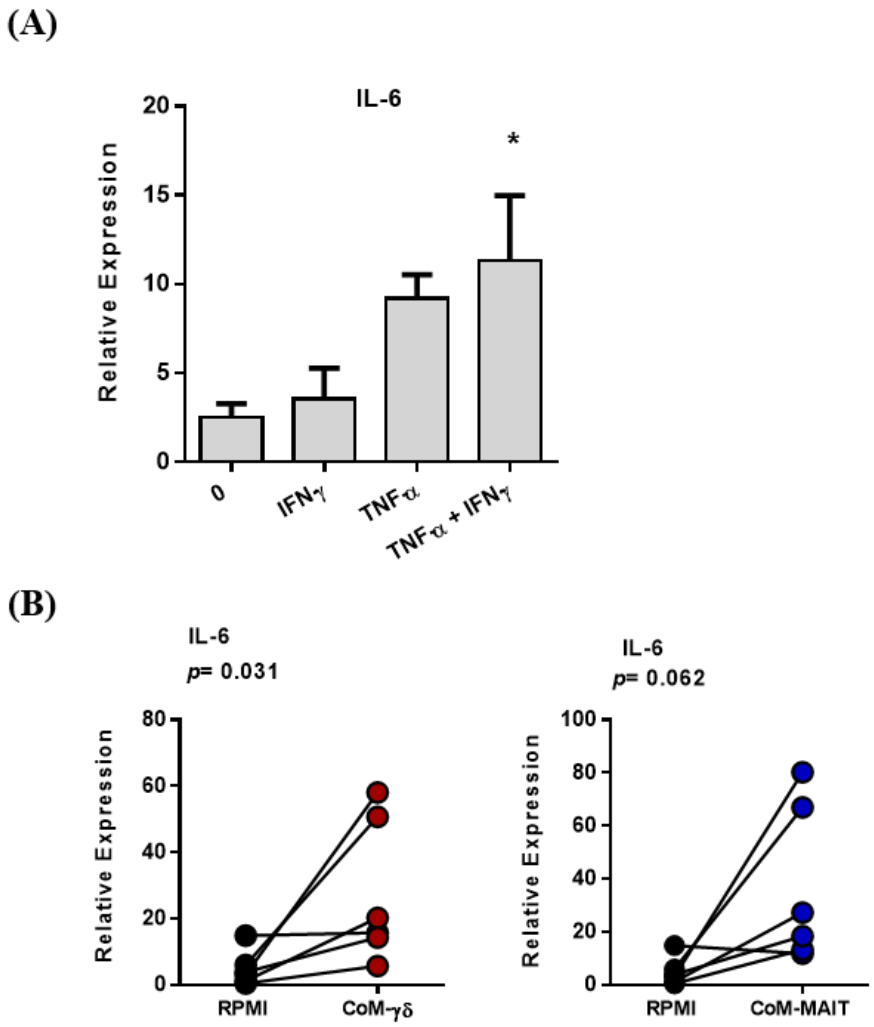

Figure 6.20. Relative expression of IL-6 mRNA in HPMC in presence of TNF-α, IFN-γ or CoM derived from unconventional T cells.

Expression IL-6 mRNA relative to GAPDH in HPMC was detected by RT-qPCR, following 24 h of co-culture with TNF-α and/or IFN-γ (A) or with conditioned medium derived from γδ T cells or MAIT cells (B). The results are expressed as the mean ± SEM of 6 independent experiments. Data were analysed by RM one-way ANOVA with Holm-Sidak post-hoc test. Comparisons were made with control medium. For CoM data were analysed by Wilcoxon matched pairs test. Differences were considered significant as indicated in the Figures: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ****$p<0.0001$.

6.5 Discussion

In this Chapter I showed for the first time that activated unconventional T cells not only interact with other immune cells during episodes of infection (Davey et al., 2014; Eberl and Moser, 2009) but also contribute to the production of pro-inflammatory mediators by peritoneal tissue cells and induce phenotypic and morphological alterations in primary HPMC.

The balance in the production of chemokines CXCL8, CCL2 and CXCL10 is important to fight infection at its onset and subsequently to resolve inflammation. Neutrophils are the first cells to migrate to the peritoneal cavity during infection, normally their recruitment is regulated by CXCL8 (Luster, 2002). Here I showed that CoM derived from both γδ and MAIT cells induced production of CXCL8 by both HPMC and HPFB via a mechanism regulated by TNF-α but not IFN-γ. These results concur with previous studies showing IFN-γ regulated the switch from PMN to MNC recruitment, downregulating CXCL8 induced by TNF-α (Robson et al. 2001; Proost et al. 2006; Topley, et al. 1993).

IL-6 increases with PD treatment and PD associated infection (Lambie et al., 2013; Topley et al., 1993a) and has pro- and anti-inflammatory effects in combination with IFN-γ (Fielding et al., 2014; Jones, 2005). In this study, IL-6 was produced by HPMC and HPFB following treatment with unconventional T cell CoM in a primarily TNF-α dependent process. It is likely that IFN-γ sensitises peritoneal cells to the subsequent action of TNF-α, for example by upregulating TNF-α's receptors, and this could explain the synergistic effect induced by IFN-γ and TNF-α when combined together (Kawka et al., 2014; Topley et al., 1993a).

Induction of IL-6 production by unconventional T cells can in turn, together with sIL-6R released by recruited neutrophils, have a positive feedback on chemokines produced by HPMC. Furthermore, together with IFN-γ, IL-6 can modulate the switch from recruitment of polymorphonuclear to mononuclear cells to the site of infection (Hurst et al., 2001; McLoughlin et al., 2003). The presence of IFN-γ is also responsible for resident tissue cell secretion of CXCL10, a chemokine responsible for leukocyte migration to the infection site (Luster, 2002). Here, CXCL10 was not induced by IFN-γ alone, possibly due to the suboptimal concentration used in these experiments, but was released in the presence of CoM derived from both unconventional T cells. Blocking experiments confirmed that this effect was dependent on the presence of IFN-γ in the supernatant. These results agree with

those from an earlier study where IL1-β and TNF-α induced CXCL10 by HPMC only when combined with IFN-γ (Visser et al., 1998).

As described in Chapter 4, CCL2 increases in the peritoneal cavity during episodes of acute infection. CCL2 is generally responsible for mononuclear cell recruitment to the site of infection and is produced mainly by tissue cells such as mesothelial cells and fibroblasts following IL-1β and IFN-γ activation (Topley et al., 1996; Witowski et al., 2001, 2009). I here observed, that activated unconventional T cells can trigger CCL2 from tissue stromal cells and this effect is caused by both IFN-γ and TNF-α present in the CoM (Glik and Douvdevani, 2006; Visser et al., 1998). Unconventional T cells express CCR2, these findings therefore suggest that these cells (*i*) may be recruited to the site of infection in response to locally secreted CCL2; (*ii*) once at the site, become activated by microorganisms producing the corresponding ligands and release IFN-γ and TNF-α at the site of infection, and (*iii*) induce a positive feedback loop enhancing the production of further chemokines by local tissue cells.

Next, I observed that PDE effluents from patients with $Gram^-$ infection triggered significantly higher CXCL8 and CCL2 production, and that this effect was caused in part by IL-1β and TNF-α in the effluent. This is in agreement with a previous study in the host laboratory (Lin et al., 2013), where in contrast to $Gram^+$ results, $Gram^-$ infection were characterized by an increased influx of neutrophils to the peritoneal cavity and increased PDE concentrations of IL-1β and TNF-α. The release of IL-1β and TNF-α is mainly induced by the endotoxin LPS, present on the outer cells wall of $Gram^-$ bacteria, following binding of TLR on resident macrophages during PD associated infection (Kumar et al., 2009). Indeed, simultaneous secretion of IL-1β and TNF-α is responsible for synergistic upregulation of inflammatory chemokines (*e.g.* CXCL8, CCL2 and RANTES) by both mesothelial cells and fibroblasts (Loghmani et al., 2002; Topley et al., 1993b; Visser et al., 1998), confirming involvement of these chemokines in recruitment of leukocytes to the site of infection.

During episodes of peritoneal infection, it is likely that mesothelial cells are amongst the first cells to encounter pathogens. PD associated infections most commonly caused by $Gram^+$ organisms such as *S. epidermidis*, *S. aureus*, *Streptococcus* and *Enterococcus*, and less frequently by $Gram^-$ bacteria, the latter being associated with poorer outcomes (Chapter 5). In this study I showed that HPMC not only participate in the peritoneal inflammation in

the presence of Gram^{+} bacterial extract (Colmont et al., 2011), but also become activated in the presence of Gram^{-} microbial extracts. In particular, when Gram^{+} and Gram^{-} bacteria were compared, I observed increased release of pro-inflammatory chemokines and cytokines in the presence of *E. coli* and *P. aeruginosa.* It has been shown that HPMC release inflammatory mediators like IL-6 after treatment with TLR2 agonists and cell free supernatant from *S. epidermidis*, but that they do not express the receptor for the Gram^{-} associated pathogen molecule LPS (TLR4) nor response to LPS *in vitro* (Colmont et al., 2011). However, the molecular compositions of LPS may vary between genera such that LPS derived from *E. coli* and *Klebsiella* might bind not only to TLR4, but also to TLR2, triggering a different modality of infection resolution (Oliveira-Nascimento et al., 2012). Colmont et al. showed that HPMC cells respond to flagellin (Colmont et al., 2011), a component present in flagellated bacteria such as *E. coli* and *P. aeruginosa*, and a potent agonist of TLR5 (Hayashi et al., 2001). This may explain the profound induction of HPMC pro-inflammatory chemokines by these organisms, and their association with technique failure in PD associated infection (Feng et al., 2014; Siva et al., 2009).

I observed a similar pattern of activation by HPFB. HPFB activation was more evident in the presence of Gram^{-} bacterial extracts than Gram^{+} extracts. This activation may was caused by the presence of TLR2 and TLR4 on HPFB (Abe et al., 2014). Gram^{-} bacteria may trigger stronger inflammatory response by HPFB thanks to the presence of LPS and porins (Loghmani et al., 2002; Perfetto et al., 2003). Indeed, these PAMPs activate transcription factor NF-κB, which induces an array of genes implicated in inflammation (Medzhitov, 2001). Moreover, fibroblast activation by Gram^{-} bacteria such as *P. aeruginosa* might be induced independently by TLR activation. For instance, it has been demonstrated that elastase induces secretion of CXCL8 from dermal fibroblasts by binding to the epidermal growth factor receptor (Azghani et al., 2014).

Finally, the present findings demonstrate that TNF-α and IFN-γ produced by activated unconventional T cells induced morphological and phenotypical changes in mesothelial cells that are usually associated with EMT. This process starts with the dissociation of the intercellular junctions as a result of downregulation of epithelial markers E-cadherin, claudins, occludins, ZO-1 and upregulation of mesenchymal markers α-SMA, collagen-1, fibronectin and the transcription factor SNAIL (Thiery and Sleeman, 2006). E-cadherin expression is fundamental to maintain the integrity of the peritoneal membrane. E-cadherin secures adhesion between cells by binding β-catenin and p120 catenin in the cytoplasm,

these molecules are also associated with the actin cytoskeleton via α-catenin (Lamouille et al., 2014). Destabilization of E-cadherin results in the β-catenin degradation or translocation to the nucleus, where it acts as transcription factor. These processes are regulated by SNAIL, which is upregulated via the ERK/NF-κB pathway in both cancer and peritoneal membrane associated EMT (Strippoli et al., 2008; Wu and Zhou, 2010).

In the present study, TNF-α induced downregulation of epithelial markers such as E-cadherin and occludin, an effect increased in conjunction with IFN-γ. This concurs with previous studies in which epithelial cells lost apical-basolateral polarity and acquired a migratory phenotype following downregulation of E-cadherin and upregulation vimentin and N-cadherin (Hanada et al., 2003; Lv et al., 2015). Synergistic downregulation of these epithelial markers in the presence of TNF-α and IFN-γ might result from crosstalk between signalling pathways activated by these two cytokines such as NF-κB by TNF-α, and JAK/STAT by IFN-γ (Ohmori et al., 1997; Wang et al., 2013).

E-cadherin expression is repressed by SNAIL, which can be induced by pathways like WNT and PI3K-AKT acting on GSK3β or phosphorylation by transcription factors Notch and NF-κB (Lamouille et al., 2014), and TNF-α can induce SNAIL dependent E-cadherin upregulation (Strippoli et al., 2008; Wu and Zhou, 2010; Yanez-Mo et al., 2003). This contrasts with my findings where I did not observe an upregulation of SNAIL induced by TNF-α. It may be the case that TNF-α was able to regulate other transcriptional factor responsible of EMT process such as TWIST, ZEB-1 and ZEB-2 but not SNAIL (Wang, 2013). This may also explain why I observed downregulation of most mesenchymal markers after 24 hours and upregulation of fibronectin only with conditioned medium from unconventional T cells. Indeed, fibronectin can be induced by transcription factors such as SNAIL and TWIST. The downregulation of mesenchymal markers could also be explained by the anti-fibrotic activity of TNF-α and IFN-γ in the regulation of tissue gene expression. It has been shown that these two cytokines inhibit expression of collagen and fibronectin on epithelial fibroblasts, opposing the effects of TGF-β-SMAD3 pathways (Ghosh et al., 2001; Hatamochi et al., 1994). TGF-β, the most potent pro-fibrotic factor, is induced in the presence of glucose degradation products from PD solutions and in episodes of peritonitis, and is able to drive EMT (Aroeira et al., 2007; LEASK, 2004; Selgas et al., 2006). It has been shown that TNF-α accentuates the ability of TGF-β to suppress epithelial markers and increase MMP secretion (Borthwick et al., 2012). However, TNF-α did not change or reduce the expression of mesenchymal markers when combined with TGF-β (Borthwick et al.,

2012). Therefore, increased level of TNF-α at the site of infection, together with elevated levels of TGF-β and IL-6, could drive EMT. This means that is fundamental the balance between these molecules for infection resolution and tissue regeneration during PD associate infection.

Overall, these findings identify unconventional T cell-derived TNF-α and IFN-γ as major stimulators of peritoneal tissue cells, likely to enhance local inflammation and contribute to the recruitment of further monocytes, neutrophils and lymphocytes to the site of infection. Moreover, the magnitude of inflammatory chemokines produced by peritoneal tissue cells in the presence of $Gram^-$ bacteria might help explain the difference in clinical outcome between $Gram^+$ and $Gram^-$ bacterial infection.

Chapter 7. General discussion and future work

7.1 General discussion

Novel methods for the early diagnosis of peritonitis in PD are urgently needed. Ideally, with such diagnostic tools, patients at risk of technique failure could be identified earlier thus helping improve patient outcomes. Infection and associated inflammation remain the major reasons for treatment failure and death of PD patients (Piraino et al., 2011). Diagnosis of infection by microbiological culture results is inefficient, slow and often unclear, leading to a widespread use of broad-spectrum antibiotics to cover both $Gram^+$ and $Gram^-$ infections. This may contribute to prolonged treatments, chronic/recurrent infections, catheter removal and mortality. Moreover, inappropriate antibiotic prescription may contribute to the emergence of anti-microbial resistant "superbugs", which are spreading at an alarmingly high rate (Arias and Murray, 2009).

During infections, the immune system interacts with microbe-associated molecules at various levels leaving a pathogen-specific signature of immunological biomarkers (Lin et al., 2013). Such "immune fingerprints" could be exploited to discriminate between different types of pathogens, which might consequently help design novel tests for infection diagnosis. For instance, pattern associated molecules (HMB-PP, vitamin B2 derivatives) can be recognised by Vγ9/Vδ2 T cells and MAIT cells via the non-polymorphic presenting molecules BTN3 and MR1, respectively (Liuzzi et al., 2015). In this thesis, it was shown that Vγ9/Vδ2 T cells and MAIT cells are able to respond specifically to HMB-PP^+ and/or vitamin $B2^+$ organisms by the release of pro-inflammatory cytokines. Moreover, it was observed that high levels of Vγ9/Vδ2 T cells and MAIT cells were associated with HMB-PP^+ and vitamin $B2^+$ infections in the peritoneal cavity at day 1 of acute peritonitis. This is particularly important given that infections caused by HMB-PP^+ and/or vitamin $B2^+$ pathogens (*e.g. E. coli*, *Pseudomonas*, *Mycobacterium*) are responsible for poor outcomes in PD. In particular, an assessment of the frequency of these unconventional T cells combined with the proportions of other immune cells (*e.g.* neutrophils, monocytes and $CD4^+$ T cells) and concentrations of soluble mediators (*e.g.* TNF-α, IL-1β, IFN-γ, CXCL-10, IL-22) might help design a diagnostic test that is able to identify the causative pathogen at day 1 of the infection (Lin et al., 2013; Liuzzi et al., 2015). Such a combination would not only help discriminate between $Gram^+$ and $Gram^-$ infections but also between certain subgroups such as $Gram^+$ HMB-PP^+ vitamin $B2^+$ (*e.g. Mycobacterium*, *Corynebacterium*), $Gram^+$

HMB-PP$^-$ vitamin B2$^+$ (*e.g. Staphylococcus*) and Gram$^+$ HMB-PP$^-$ vitamin B2$^-$ species (*e.g. Streptococcus, Enterococcus*). Ultimately, the use of such a diagnostic tool would help prescribe antibiotic more prudently and hence lead to better patient outcomes.

Previous work in the Eberl laboratory showed that the presence of Vγ9/Vδ2 T cells at the site of infection is not only able to predict Gram$^-$ infection but also to identify patients at high risk of technique failure within the first 3 months after infection. Here, the analysis of peritonitis outcomes from the ANZDATA registry demonstrated the association of HMB-PP and vitamin B2 positive infections with poor outcomes. This finding suggests that, if the infection is not resolved in time, both Vγ9/Vδ2 T cells and MAIT cells may contribute to the amplification of local inflammation and long-term membrane damage.

In order to better understand the role of unconventional T cells during inflammation and long-term membrane damage, the activation of Vγ9$^+$ T cells and MAIT cells in the presence of HMB-PP and vitamin B2 positive and negative bacterial extracts was studied. Specifically, in this study it was demonstrated that *(i)* γδ T cells and MAIT cells together account for up to 50% of peritoneal T cells releasing TNF-α in response to *E.coli* bacterial extract; *(ii)* under the same conditions, peritoneal Vγ9/Vδ2 T cells alone contribute to up to 60% of IFN-γ production; and *(iii)* unconventional T cells derived TNF-α and IFN-γ are potent stimulators of peritoneal mesothelial cells and fibroblasts. The stimulation of peritoneal γδ T cells and MAIT cells results in the induction of the pro-fibrotic cytokine IL-6 as well as chemokines such as CCL2, CXCL8 and CXCL10 and triggers phenotypical changes in mesothelial cells (Figure 7.1). TNF-α, actually tends to increase at the site of infection and is associated with technique failure and mortality in PD patients (Davey et al., 2011a).

Of note, the crosstalk between unconventional T cells and peritoneal immune and stromal cells in response to HMB-PP and/or vitamin B2 producing bacteria can be manipulated experimentally at various check points, by *(i)* blocking antibodies against the TCR, thus preventing recognition of HMB-PP by Vγ9/Vδ2 T cells and vitamin B2 by MAIT cells; *(ii)* adding antibodies to block the accessory molecules MR1 for MAIT cells and BTN3 for Vγ9/Vδ2 T cells; and *(iii)* neutralisation of soluble TNF-α, which is important for γδ T cell proliferation. It is also released by neutrophil and monocytes in the presence of LPS, and acts as survival factor for all three types of cells (Figure 7.1) (Davey et al., 2011a, 2014).

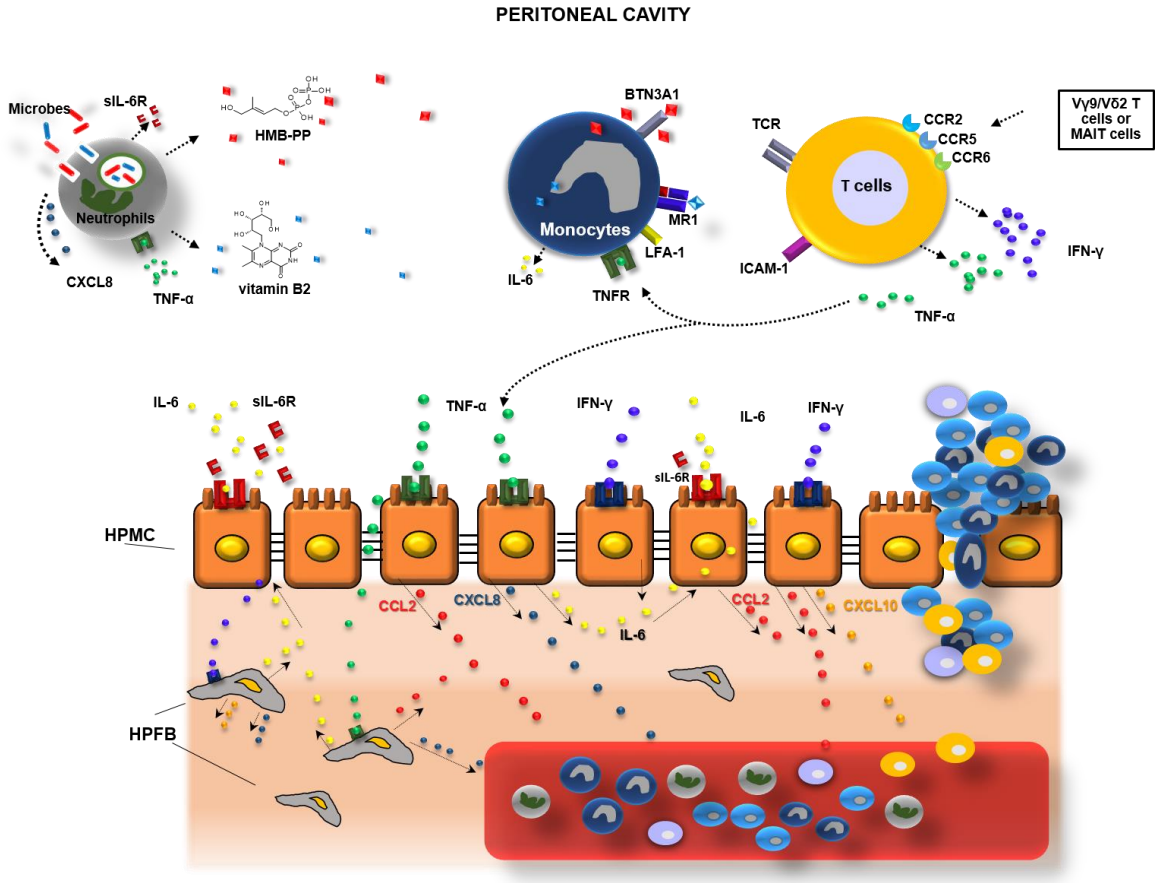

Figure 7.1. Amplification of inflammation by peritoneal γδ T cells and MAIT cells.
Neutrophils are able to engulf invading microbes and release microbial metabolites such as HMB-PP and vitamin B2 into the microenvironment, which become visible to Vγ9/Vδ2 T cells in the context of BTN3A1 and to MAIT cells in the context of MR1. At the same time, monocytes provide contact-dependent signals necessary for unconventional T cells activation (e.g. LFA-1-ICAM-1 interaction). Once activated at the site of infection, recruited or resident Vγ9/Vδ2 T cells and MAIT cells release the pro-inflammatory cytokines TNF-α and IFN-γ. In turn, TNF-α supports local γδ T cell expansion and acts as a monocyte and neutrophil survival signal. TNF-α also induces the release of the chemokines such CCL2 and CXCL8 from human peritoneal mesothelial cells (HPMC) and human peritoneal fibroblasts (HPFB). CCL2 is also induced in the presence of IFN-γ at the site of infection. IFN-γ but not TNF-α trigger the release of CXCL10 from both HPMC and HPFB. Once released, CCL2 and CXCL10 are responsible for the recruitment of mononuclear cells such as monocytes and T cells to the site of infections. CCL2 is also responsible for the recruitment of peripheral Vγ9/Vδ2 T cells and MAIT cells to the peritoneal site. CXCL8 is responsible for the recruitment of neutrophils to the site of infection and is also released by neutrophils themselves after activation by microbial metabolites (e.g. LPS). Under the same conditions, neutrophils release the soluble form of the interleukin-6 receptor (sIL-6R). The pro-inflammatory cytokine IL-6 is released by activated monocytes and macrophages as well as HPMCs and HPFBs which may have recruited sIL-6R. In this way the IL-6/IL-6R complex signals will suppress the production of CXC chemokines and promote the production of mononuclear chemokines such as CCL2 and CCL5.

The assessment of Vγ9/Vδ2 T cells and MAIT cells as cellular biomarkers in PD may also give an indication on appropriate antimicrobial treatments to control infections caused by "superbugs". Indeed, the spreading of antibiotic resistance limits the treatment choice for PD patients at day 1 of the infection. Vancomycin-resistant *E. faecium* (VRE) and methicillin-resistant *S. aureus* (MRSA) bacteria present a therapeutic challenge among the Gram positive bacteria (Arias and Murray, 2009; Reynolds, 2009). Similarly, $Gram^-$ bacteria can become resistant to almost all β-lactamase due to the spread of enzymes such as New Delhi metallo-β-lactamase (NDM) among these species (Jacoby and Munoz-Price, 2005). It has been found that up to 36% of *E. coli* peritonitis were caused by β-lactamase resisting strains and were associated with poor response to single antibiotic treatment (Feng et al., 2014). In addition, with the rise of new β-lactamases the detection of resistant bacteria by microbiological culture can be suboptimal (Jacoby and Munoz-Price, 2005). This means that new effective antibiotics are needed and available agents need to be used more prudently. In this respect, it is noteworthy that most $Gram^-$ bacteria are also HMB-PP positive (Liuzzi et al., 2015). The production of this metabolite can be abrogated by the use of the antibiotic fosmidomycin (Jomaa, 1999). Most importantly, fosmidomycin was found to be effective against $Gram^-$ bacteria that are β-lactamase resistant (Davey et al., 2011c). Of note, a possible future use of this antimicrobial in PD might not only help inhibit the growth of HMB-PP positive bacteria but also possess anti-inflammatory properties by abrogation of the HMB-PP dependent Vγ9/Vδ2 T activation.

Many pathogenic bacteria are dependent on the biosynthesis of riboflavin (vitamin B2) (Mack and Grill, 2006). The enzymes catalysing the last two steps in the riboflavin pathway (lumazine and riboflavin synthases, respectively) have been studied in detail and found to be conserved among all organisms (Morgunova et al., 2010). Since a number of inhibitors of these two enzymes have already been identified (Mack and Grill, 2006), the riboflavin pathway represents a promising target to design novel anti-infective agents and to inhibit the vitamin B2 dependent MAIT cell activation *in vivo*. Taken together, the presence of increased levels of Vγ9/Vδ2 T and MAIT cells at day 1 of acute infections may thus give both an indication on the severity of the infection and directions on the choice of treatment.

In summary, the findings in this thesis elucidate a potential mechanism for the amplification of acute inflammatory response by unconventional T cells in PD. Vγ9/Vδ2 T cells and MAIT cells are recruited specifically to the site of infection in response to $HMB\text{-}PP^+$ and $vitamin\ B2^+$ bacteria, respectively. Once at the site of infection, they may contribute to the

amplification of peritoneal inflammation by releasing IFN-γ and TNF-α and orchestrating antimicrobial responses. However, in the case of an unresolved infection, unconventional T cells might also be pathogenic, affecting long-term peritoneal membrane damage and consequently technique survival. This suggests that a diagnostic test that would be able to identify unconventional T cells at day 1 of the infection would not only give information as to the type of causative organism present in the peritoneal cavity but would also be able to tell whether the patient is at increased risk of subsequent technique failure.

This clinical model offers a number of opportunities to study the immune response into acute infections caused by $Gram^+$ and $Gram^-$ bacteria as well as fungi. Most other acute diseases, for instance respiratory or urinary tract infections, do not show such a wide spectrum of organisms. Moreover, access to anatomical locations other than blood is usually limited, especially during acute disease, and the collection of samples from the site of inflammation is rarely possible. In contrast, in PD patients peritoneal effluent can be sampled repeatedly and non-invasively providing unrestricted access to the site of infection. The immunological profile of stable PD patients allows to establish pre-infection baseline parameters in individuals prone to develop peritonitis later on. Collectively, this study demonstrated that PD can be used as model for investigations into local immune responses and monitoring individuals before, during and after defined microbial infections.

7.2 Future work

The aim of my research was to study how unconventional T cells contribute to the amplification of inflammation during PD associated infection. In particular, it was studied how activated unconventional T cells influence the activation of peritoneal mesothelial cells and fibroblasts. However, further work is necessary to clarify whether peritoneal mesothelial cells and fibroblasts have an impact on unconventional T cell activation in the presence of microbial metabolites such as HMB-PP and vitamin B2, or bacterial extracts in general. For instance, it might be that peritoneal mesothelial cells, equally as other epithelial cells, express antigen presenting molecules (*e.g.* BTN3 and MR1) and have properties of antigen uptake followed by unconventional T cell stimulation and activation (Hausmann et al., 2000; Jeffery et al., 2016).

Another open question is as to where exactly unconventional T cells are localised in the peritoneal cavity. Are they in contact with mesothelial cells? Do they migrate to the connective tissue? Future studies are necessary to try to identify these cells in sections of omentum and peritoneum tissue of patients that were either stable or had a history of peritonitis. Such a study would not only clarify the exact localisation of these cells but also confirm the presence of unconventional T cells in the peritoneal cavity depending on the causative pathogen.

It remains to be investigated as to how much these findings on the use of unconventional T cells as diagnostic tool in PD can be applied to monitoring the course of the disease and the response to treatment. Many questions remain in this regard: do the frequency of unconventional T cells remain stable over time in the absence of infection? Is the presence of unconventional T cells during the stable phase affected by the previous history of infections? Does the composition of peritoneal immune cells return to pre-infections baseline levels?

Moreover, future studies are needed to define the mechanisms that underline microbial pattern recognition by unconventional T cells. This raises the questions: how do unconventional T cells' TCRs interact with non-polymorphic presenting molecules? Which molecules are involved in antigen uptake and intracellular trafficking allowing antigen presentation? Do unconventional T cells use different TCRs to recognise different pathogens?

In the future, the use of immune fingerprints in PD may be extended to other local or systemic infections such as sepsis at the time of patient care. Uunconventional T-cells play a pivotal role in the orchestration of early inflammatory responses, and emerging mechanistic insights have started to unlock the secrets of innate-like recognition encoded by specific portions of the TCR repertoire. The highly constrained genetic and microbial elements inherent within each of these various systems therefore offer unique molecular targets for the development of novel and universally applicable diagnostics and therapeutics.

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Appendix

Publications during my PhD studies

- **Liuzzi AR**, McLaren JE, Price DA, Eberl M. 2015. Early innate responses to pathogens: pattern recognition by unconventional human T-cells. Current Opinion in Immunology 36:31-37.
- Davey MS, Morgan MP, **Liuzzi AR**, Tyler CJ, Khan MWA, Szakmany T, Hall JE, Moser B, Eberl M. 2014. Microbe-specific unconventional T cells induce human neutrophil differentiation into antigen cross-presenting cells. Journal of Immunology 193(7):3704-3716.
- Eberl M, Friberg IM, **Liuzzi AR**, Morgan MP, Topley N. 2014. Pathogen-specific immune fingerprints during acute infection: the diagnostic potential of human γδ T-cells. Frontiers in Immunology 5: 469.
- **Anna Rita Liuzzi**, Ann Kift-Morgan, Melisa López Antón, Amy C. Brook, Ida M. Friberg, Jingjing Zhang, Gareth W. Roberts, Kieron L. Donovan, Chantal S. Colmont, Mark A. Toleman, Timothy Bowen, David W. Johnson, Nicholas Topley, Bernhard Moser, Donald J. Fraser, and Matthias Eberl. Human γδ T-cells and MAIT cells expand at the site of microbial infection, amplify inflammatory responses and induce local tissue remodelling. Manuscript in preparation.

Presentations during my PhD studies

- Oral presentation, European Training & Research in Peritoneal Dialysis (EuTRIPD), Summerschool, Amsterdam and Zandvoort, Netherland, July 2012. Title "Evaluating the role of Toll-like receptor 2 (TLR2), C3a and the crosstalk between TLR2 and C3a receptor in peritoneal dialysis-associated peritoneal fibrosis".
- Oral presentation, EuTRIPD, Winterschool, Amsterdam, December 2012, Netherland.
  Title "Evaluating the role of Toll-like receptor 2 (TLR2), C3a and the crosstalk between TLR2 and C3a receptor in peritoneal dialysis-associated peritoneal fibrosis".
- Oral presentation, EuTRiPD, Summerschool, Vienna, Austria, June 2013.

Title “Evaluating the role of Toll-like receptor 2 (TLR2), C3a and the crosstalk between TLR2 and C3a receptor in peritoneal dialysis-associated peritoneal fibrosis”.

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  Title “Pathogen-specific microbial sensing in early infection: implications for diagnosis and therapy of peritoneal dialysis patients”.

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  Title “Pathogen-specific microbial sensing in early infection: implications for diagnosis and therapy of peritoneal dialysis patients”.

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Pathogen-specific immune fingerprints during acute infection: the diagnostic potential of human γδ T-cells

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APOCALYPSE NOW: THE END OF MODERN MEDICINE AS WE KNOW IT

> Gentlemen, it is the microbes who will have the last word. [Messieurs, c'est les microbes qui auront le dernier mot]. – Louis Pasteur, 1822–1895

The last 200 years have seen a dramatic reduction in the prevalence and severity of microbial infections, due to the implementation of groundbreaking measures ranging from improved sanitation and hygiene and the introduction of aseptic techniques to the development of successful vaccines and the discovery of effective antibiotics. Devastating infections that were common until the late nineteenth century such as cholera, diphtheria, plague, syphilis, tuberculosis, and typhoid came into the reach of effective control, at least in developed countries, and with a minimized risk of wound infections surgical procedures began to revolutionize modern medicine. Antibiotics, in particular, radically transformed the treatment and prevention of microbial infections and have saved millions of lives since their introduction (1). However, antibiotic usage is invariably linked to the selective pressure it exerts on the target organism to develop escape strategies (2).

We are at present witnessing how the pendulum begins to swing backwards, with anti-microbial resistances developing on an unprecedented global scale. New classes of Gram-positive and Gram-negative "superbugs" are emerging and spreading at an alarming rate, some of which are virtually insusceptible to all available drugs (3–5). The once apocalyptic vision of a "post-antibiotic era" where common infections and minor injuries may result untreatable and eventually fatal is rapidly becoming a real possibility (1, 2, 6, 7), heralding what Margaret Chan, Director-General of the WHO, in 2012 called "the end of modern medicine as we know it." The appearance of multidrug-resistant bacteria has been identified by the WHO, the Centers for Disease Control and Prevention (CDC) in the USA and their European counterpart, the ECDC, as one of the major global health challenges humankind is facing in the twenty-first century (8–10). According to Sally Davies, the UK Chief Medical Officer, "there are few public health issues of greater importance than anti-microbial resistance in terms of impact on society" (11).

There is now an urgent call for anti-microbial stewardship programs that aim to prescribe antibiotics more prudently, and to tailor their use to defined patient groups who will benefit most. The fact that the prevalence of resistance appears to correlate directly with antibiotic consumption across different countries (12) argues in favor of the immediate effectiveness of such tightly controlled programs. As highlighted in a recent Outlook issue in *Nature*, "the potential to save lives with faster and more targeted diagnoses, decrease unnecessary and often incorrect prescriptions, and even help identify early on where bacterial resistance could occur, will have a drastic effect on the way patients are treated" (13).

MISSION IMPOSSIBLE: THE FUNDAMENTAL FLAWS OF CONVENTIONAL DIAGNOSIS

> When it concerns the search for pathogenic organisms suspected in the diseased body, in the first instance bacteria, then during conventional microscopic examination carried out without special preparations and artifices one encounters the most substantial, at times virtually insurmountable, obstacles. [Wenn es sich nun darum handelt, die im erkrankten Körper vermutheten pathogenen Organismen, zunächst Bacterien, aufzusuchen, so begegnet man bei der gewöhnlichen ohne besondere Vorbereitungen und Kunstgriffe ausgeführten mikroskopischen Untersuchung den erheblichsten, stellenweise geradezu unübersteiglichen Hindernissen]. – Robert Koch, 1843–1910 (14)

More than a century after Robert Koch's landmark discovery of the causative agents of anthrax, cholera, and tuberculosis, the diagnosis of suspected infections still depends largely on the definitive identification of the likely pathogen in biological samples. However, standard microbiological culture is inefficient and slow (typically >1–2 days, for a confirmed diagnosis of tuberculosis >4 weeks), and in many cases no organism can be grown despite

Abbreviation: HMB-PP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate.

clinical signs of infection, indicating that conventional diagnostic methods are not specific and/or rapid enough to target therapy (15–17). Early management of patients with acute symptoms who require immediate medical intervention, including virtually all hospital-based infections, thus remains largely empirical. As direct consequence, the fundamental uncertainty about the real cause underlying the clinical signs observed leads to inappropriate and unnecessary treatments exposing patients to drug-related side effects; raising the risk of opportunistic, chronic, or recurrent infections; and contributing to the emergence and spread of multidrug resistance (1–7). This dilemma eventually results in potentially avoidable patient morbidity/mortality, and imposes a considerable burden on health care systems and societies (8–11). There remains an unmet clinical need for rapid and accurate diagnostic tests for patients with acute infections. According to Kessel and Sharland (18), "new technology focusing on rapid diagnosis of specific bacteria and resistance genes, along with combination biomarkers indicating bacterial or viral infections, especially if adapted to near patient testing, could have a major impact on targeting appropriate antibiotic treatment."

In order to circumvent the almost insurmountable obstacles of a rapid and accurate identification of the causative pathogen by traditional microbiological techniques, efforts are being made to utilize state-of-the-art molecular methods. Approaches based on the detection of microbial nucleic acids, cell wall constituents, or other unique features of distinct pathogens by PCR, chromatography, or mass spectrometry certainly complement culture-based tests and speed up microbial identification, yet they require considerable resources and may not be applicable to primary care or home settings (19–23). Moreover, they do not provide information about the pathogenicity of the identified species and its interaction with the host. Of note, neither microbiological nor molecular methods discriminate between pathogens causing disease, asymptomatic carriage, and sample contaminants, and thus even positive test results require extensive interpretation by the treating physician (24–26).

There is a plethora of disease-related markers that are commonly assessed by clinicians to aid a correct diagnosis, ranging from basic blood and urine parameters to indicators of tissue damage, tumor progression and autoimmunity, among others. However, there is a conspicuous paucity of biomarkers for accurate diagnosis of microbial disease. Current biomarkers of inflammation such as C-reactive protein (CRP) or procalcitonin (PCT) are often not sensitive or specific enough and are only poor surrogates for acute infections (22, 27, 28). The vast majority of research on novel diagnostics has so far focused on identifying individual factors and assessing their performance in isolation. Yet, it may come as no surprise that none of these proposed parameters have reached sufficient discriminatory power on their own, given the complex and multifactorial processes underlying local and systemic inflammatory responses to a broad range of pathogens (29, 30). As a result, neither the direct identification of the causative pathogen nor the measurement of currently used biomarkers of inflammation is sufficiently accurate or rapid for a reliable point-of-care diagnosis of acute microbial infection.

QUANTUM OF SOLACE: EXPLOITATION OF PATHOGEN-SPECIFIC HOST RESPONSES FOR NOVEL DIAGNOSTICS

> The immune system appears to have originated as a set of effector cells having multiple distinct receptors that discriminate self from infectious non-self by recognition of patterns found exclusively on microorganisms. – Charles A. Janeway, Jr., 1943–2003 (31)

Key to developing better and stratified approaches to treating infection is a detailed understanding of the intricate host–pathogen relationships in disease, in order to exploit the unique sophistication of the human immune system for diagnostic and therapeutic purposes (32, 33). In a radical departure from current practice, our research is based upon the premise that each type of infection evokes a distinct pathogen-specific host response – what we refer to as "immune fingerprint." A patient's early anti-microbial response itself is likely to provide far more detailed insight into the true cause and severity of acute infections than conventional methods, independently of the subsequent clinical course of the disease (34). The human immune system is a highly complex network of interdependent cellular and humoral players that has evolved over millions of years in order to survey the body for potentially hazardous structures and initiate an appropriate defense. The communication with invading micro-organisms thus occurs at multiple levels, giving rise to a plethora of biomarkers of potential relevance for diagnostic purposes. Different pathogens interact uniquely with different components of the innate immune system due to the efficient self/non-self discrimination based on conserved microbial signals such as non-methylated bacterial DNA, bacterial flagella, and cell wall constituents. These structures are typically recognized by members of the Toll-like receptor family and/or other pattern recognition receptors expressed by sentinel cells (35–37). However, there is also emerging evidence that certain types of innate or "unconventional" T-cells such as γδ T-cells and mucosal-associated invariant T (MAIT) cells are able to detect common microbial metabolites through their T-cell receptors, by sensing intermediates of the non-mevalonate and riboflavin biosynthesis pathways that are unique to certain types of microorganisms (38, 39).

Vγ9/Vδ2 T-cells represent a unique subpopulation of human T-cells (40, 41) that appears to have a particularly crucial role in contributing to immune fingerprints of diagnostic relevance (34). This is due to their exquisite responsiveness to the microbial isoprenoid precursor (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) that is produced by the majority of Gram-negative pathogens and a large proportion of Gram-positive species such as *Clostridium difficile*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis*, while it is not found in other bacteria including staphylococci and streptococci as well as fungi (42–44). The rapid and sensitive response of Vγ9/Vδ2 T-cells to a broad range of pathogens evokes Janeway's criteria for a "pathogen-associated molecular pattern" in that HMB-PP is an invariant metabolite in many different species that is essential in the microbial physiology but absent from the human

host (43, 45). Bacterial extracts prepared from HMB-PP producing species typically activate Vγ9/Vδ2 T-cells much stronger than extracts prepared from HMB-PP deficient micro-organisms (42, 44, 46), and peripheral and/or local Vγ9/Vδ2 T-cell levels are often elevated in patients infected with defined HMB-PP producing pathogens (43, 47). Elegant proof of concept for this responsiveness comes from the demonstration that HMB-PP producing wildtype *L. monocytogenes* activate Vγ9/Vδ2 T-cells far better, both *in vitro* (48) and in primate models *in vivo* (49), than genetically engineered *L. monocytogenes* that are identical to the parental strain except for an inability to produce HMB-PP. Similarly, overexpression of HMB-PP synthase through genetic manipulation increases the stimulatory potential of bacteria such as *E. coli*, *L. monocytogenes*, *M. tuberculosis*, and *Salmonella enterica* on Vγ9/Vδ2 T-cells *in vitro* (42, 46, 48, 50, 51) and *in vivo* (52). Our own data demonstrate that even in heterogeneous patient cohorts infected with a whole spectrum of diverse bacteria, differences in Vγ9/Vδ2 T-cell frequencies between patients with microbiologically confirmed infections caused by HMB-PP producing and HMB-PP deficient species remain apparent. This is true both for peritoneal dialysis patients with acute peritonitis as an exemplar of localized immune responses restricted to the peritoneal cavity (34, 46, 53), as well as on a systemic level in the peripheral blood of critically ill patients with severe sepsis (54). Most importantly, studies in patients with acute peritonitis suggest that a diagnostic test measuring local Vγ9/Vδ2 T-cells on the first day of presentation with acute symptoms may not only indicate the presence of Gram-negative (predominantly HMB-PP producing) bacteria but also identify patients at an increased risk of inflammation-related downstream complications (34).

The exquisite responsiveness of Vγ9/Vδ2 T-cells and other unconventional T-cells to microbial metabolites shared by certain pathogens but not by others identifies these cell types as key constituent of diagnostically relevant immune fingerprints at the point of care. This is especially the case when Vγ9/Vδ2 T-cell levels are assessed locally and when they are combined with other powerful discriminators such as peritoneal proportions of neutrophils, monocytes, and $CD4^+$ T-cells in the inflammatory infiltrate as well as intraperitoneal concentrations of certain soluble immune mediators (34) (**Figure 1**). Such a combination with further parameters provides additional information as to the precise nature of the causative pathogen, for instance to distinguish between immune responses induced by Gram-negative (LPS producing) and Gram-positive (LPS deficient) bacteria, and is also likely to help increase sensitivity owing to the age and gender-dependent variability of Vγ9/Vδ2 T-cell levels (55). Pathogen-specific immune fingerprints that discriminate between certain subgroups of patients (e.g., with Gram-negative vs. Gram-positive bacterial infections) can be determined within hours of presentation with acute symptoms, long before traditional culture results become available, and by guiding early patient

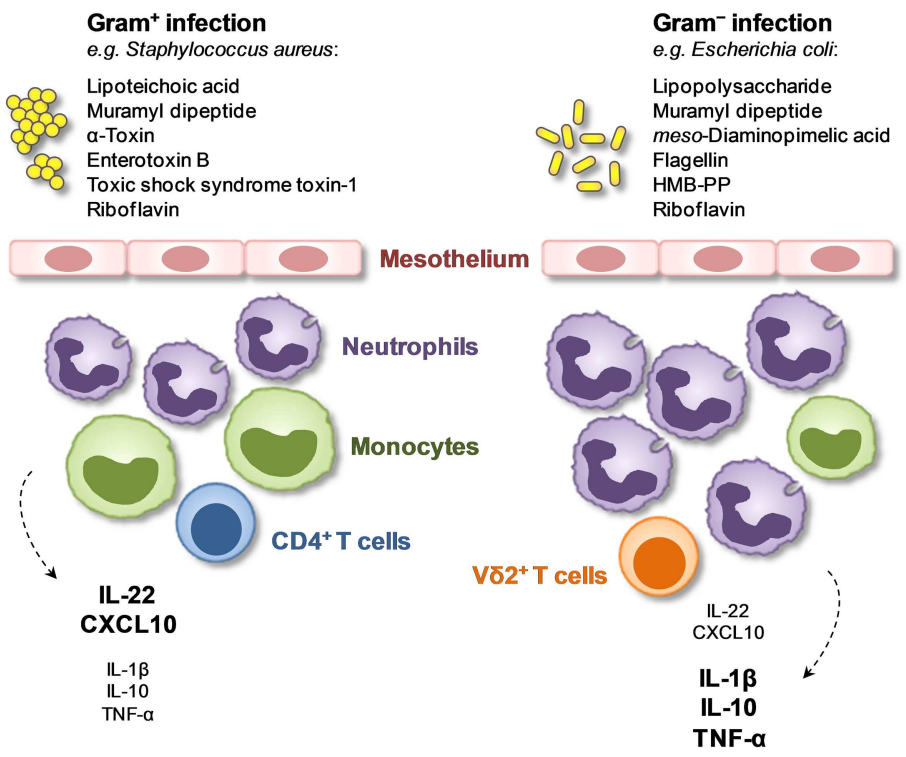

FIGURE 1 | Local immune fingerprints in peritoneal dialysis patients on the day of presentation with acute peritonitis. Shown are cellular and humoral biomarkers that are associated with the presence of Gram-positive or Gram-negative bacteria and that may be exploited for novel diagnostic tests (34).

management and optimizing targeted treatment will contribute to improving outcomes and advancing antibiotic stewardship. It remains to be investigated how much these findings on diagnostic immune fingerprints in peritoneal dialysis patients can be extended to other local or systemic scenarios to diagnose infections at the point of care, and whether they can also be applied to monitoring the course of the disease and the response to treatment.

Applied research on γδ T-cells has so far focused predominantly on their use for novel immunotherapies against different types of cancers (56–58). Thirty years after the unexpected cloning of the TCRγ chain (59, 60) and 20 years after the first description of microbial "phosphoantigens" as specific activators of human Vγ9/Vδ2 T-cells (61, 62), the diagnostic potential of γδ T-cells is only beginning to unfold (34, 47, 63, 64).

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Microbe-Specific Unconventional T Cells Induce Human Neutrophil Differentiation into Antigen Cross-Presenting Cells

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The early immune response to microbes is dominated by the recruitment of neutrophils whose primary function is to clear invading pathogens. However, there is emerging evidence that neutrophils play additional effector and regulatory roles. The present study demonstrates that human neutrophils assume Ag cross-presenting functions and suggests a plausible scenario for the local generation of APC-like neutrophils through the mobilization of unconventional T cells in response to microbial metabolites. Vγ9/Vδ2 T cells and mucosal-associated invariant T cells are abundant in blood, inflamed tissues, and mucosal barriers. In this study, both human cell types responded rapidly to neutrophils after phagocytosis of Gram-positive and Gram-negative bacteria producing the corresponding ligands, and in turn mediated the differentiation of neutrophils into APCs for both $CD4^+$ and $CD8^+$ T cells through secretion of GM-CSF, IFN-γ, and TNF-α. In patients with acute sepsis, circulating neutrophils displayed a similar APC-like phenotype and readily processed soluble proteins for cross-presentation of antigenic peptides to $CD8^+$ T cells, at a time when peripheral Vγ9/Vδ2 T cells were highly activated. Our findings indicate that unconventional T cells represent key controllers of neutrophil-driven innate and adaptive responses to a broad range of pathogens. ***The Journal of Immunology*****, 2014, 193: 3704–3716.**

Neutrophils are the first cells that are recruited to sites of microbial infection. Although classically viewed as terminally differentiated cells, there is emerging evidence that neutrophils represent key components of the effector and regulatory arms of the innate and adaptive immune system (1–3). As such, neutrophils regulate the recruitment and function of various cell types and interact with immune and nonimmune cells. Intriguingly, neutrophils directly affect Ag-specific responses by facilitating monocyte differentiation and dendritic cell maturation, and by interacting with T cells and B cells (4–10). Murine neutrophils have been shown to present Ags to both $CD4^+$ and $CD8^+$ T cells (11–13), and to differentiate into neutrophil–dendritic cell hybrids in vitro and in vivo (14, 15). In humans, neutrophils with a phenotype consistent with a possible APC function, including expression of MHC class II, have been found in diverse inflammatory and infectious conditions (16–22). This notwithstanding, direct Ag presentation by neutrophils has to date not been demonstrated in patients, especially with respect to an induction of Ag-specific $CD8^+$ T cell responses upon cross-presentation of exogenous proteins.

The physiological context underlying the differentiation of neutrophils into APCs and the implications for Ag-specific immune responses remain unclear. Unconventional T cells such as human γδ T cells, NKT cells, and mucosal-associated invariant T (MAIT) cells represent unique sentinel cells with a distinctive responsiveness to low m.w. compounds akin to pathogen and danger-associated molecular patterns (23–25). Such unconventional T cells represent a substantial proportion of all T cells in blood and mucosal epithelia, accumulate in inflamed tissues, and constitute an efficient immune surveillance network in inflammatory and infectious diseases as well as in tumorigenesis. Besides orchestrating local responses by engaging with other components of the inflammatory infiltrate (26–29), unconventional T cells are also ideally positioned in lymphoid tissues to interact with freshly recruited monocytes and neutrophils (30–32). We previously showed that human γδ T cells enhance the short-term survival of neutrophils but did not characterize these surviving neutrophils on a phenotypical and functional level (28). In this work, we studied the outcome of such a crosstalk of human neutrophils with both γδ T cells and MAIT cells in vitro and translated our findings to patients with severe sepsis. We demonstrate that neutrophils with APC-like features can be found in blood during acute infection,

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Abbreviations used in this article: DMRL, 6,7-dimethyl-8-D-ribityllumazine; HMB-PP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; MAIT, mucosal-associated invariant T; MR1, MHC-related protein 1; PPD, *Mycobacterium tuberculosis* purified protein derivate; SIRS, systemic inflammatory response syndrome; sTNFR, soluble TNFR; TSST-1, *Staphylococcus aureus* toxic shock syndrome toxin-1.

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and that the phenotype and ex vivo function of circulating sepsis neutrophils was replicated in vitro upon priming of neutrophils by human γδ T cells and MAIT cells. Our findings thus provide a possible physiological context and propose a cellular mechanism for the local generation of neutrophils with APC functions, including their potential to cross-present soluble Ags to $CD8^+$ T cells, in response to a broad range of microbial pathogens.

Materials and Methods

Subjects

This study was approved by the South East Wales Local Ethics Committee under reference numbers 08/WSE04/17 and 10/WSE04/21 and conducted according to the principles expressed in the Declaration of Helsinki and under local ethical guidelines. Sampling of adult patients with sterile systemic inflammatory response syndrome (SIRS) or with acute sepsis (defined as patients with SIRS in conjunction with a proven or suspected infection) was carried out within the United Kingdom Clinical Research Network under study portfolio UKCRN ID 11231, "Cellular and Biochemical Investigations in Sepsis." All study participants provided written informed consent for the collection of samples and their subsequent analysis. A waiver of consent system was used when patients were unable to provide prospective informed consent due to the nature of their critical illness or therapeutic sedation at the time of recruitment. In all cases, retrospective informed consent was sought as soon as the patient recovered and regained capacity. In cases in which a patient died before regaining capacity, the initial consultee's approval would stand.

Sepsis patients had a proven infection as confirmed by positive culture of at least one relevant sample according to the local microbiology laboratory overseen by Public Health Wales, and developed at least three of the four following SIRS criteria over the previous 36 h: 1) temperature from any site >38˚C or core <36˚C; 2) heart rate of >90 beats/min (unless individual had a medical condition or was receiving treatment preventing tachycardia); 3) respiratory rate of >20 breaths/min, arterial $PaCO_2$ <32 mmHg, or mechanical ventilation for an acute process; and 4) total WBC >12,000 cells/mm^3 or <4,000 cells/mm^3 or differential WBC count showing >10% immature (band) forms (n = 37; age range 35–82 y, median 63 y; 51% female). Patients with sterile SIRS developed at least three of the four SIRS criteria but had no suspected or proven microbial infection (n = 14; age range 26–70 y, median 48 y; 21% female). All patients with sepsis or SIRS had at least one documented organ failure on recruitment to the study and were either mechanically ventilated, on inotropic support, or received acute renal replacement therapy. Healthy donors served as controls for the patient cohorts (n = 10; age range 31–68 y, median 59 y; 20% female). Individuals were excluded from the study if pregnant or breastfeeding; suffering from documented severe immune deficiency or severe liver failure; admitted postcardiac arrest; treated with high-dose steroids or immunosuppressant drugs for the last 6 mo; or unlikely to survive for the duration of the study period regardless of treatment.

Media, reagent, and Abs

Culture medium was RPMI 1640 medium supplemented with 2 mM L-glutamine, 1% sodium pyruvate, 50 μg/ml penicillin/streptomycin, and 10% FCS (Invitrogen). Synthetic (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) was provided by H. Jomaa (Justus-Liebig University Giessen); synthetic 6,7-dimethyl-8-D-ribityllumazine (DMRL) was provided by B. Illarionov (Hamburg School of Food Science). *Staphylococcus aureus* toxic shock syndrome toxin-1 (TSST-1) was purchased from Toxin Technology; *Mycobacterium tuberculosis* purified protein derivate (PPD) was purchased from Statens Serum Institut (Copenhagen, Denmark). *Salmonella abortus equi* LPS, brefeldin A, and BSA-FITC were purchased from Sigma-Aldrich. Recombinant IFN-γ, TNF-α, and GM-CSF was purchased from Miltenyi Biotec. Human T-activator CD3/CD28 Dynabeads, CFSE, and 10-kDa dextran-FITC were purchased from Life Technologies.

The following mAbs were used for surface labeling: anti-CD3 (UCHT1, SK7, HIT3a), anti-CD4 (SK3, RPA-T4), anti-CD8 (SK1, HIT8a, RPA-T8), anti-CD11c (S-HCL-3), anti-CD14 (M5E2, MOP9), anti-CD15 (HI98), anti-CD16 (3G8), anti-CD25 (M-A251), anti-CD27 (M-T271), anti-CD31 (WM-59), anti-CD32 (FLI8.26), anti-CD45RO (UCHL1), anti-CD49d (9F10), anti-CD50 (TU41), anti-CD54 (HA58), anti-CD56 (B159), anti-CD62L (DREG-56), anti-CD64 (10.1), anti-CD69 (FN50), anti-CD70 (Ki24), anti-CD71 (M-A712), anti-CD72 (J4-112), anti-CD83 (HB15e), anti-CD86 (2331), anti-CD209 (DCN46), anti–HLA-DR (L243), anti–TCR-Vδ2 (B6.1), anti-CCR4 (1G1), anti-CCR5 (2D7), anti-CCR7 (3D12), and anti-CXCR3 (1C6) from BD Biosciences; anti–TCR-Vβ2 (MPB2D5), anti–TCR-Vγ9 (Immu360), and anti-CD40 (mAB89) from Beckman Coulter; anti-CD11a (HI111), anti-CD66b (G10F5), anti-CD154 (24-31), anti-CD161 (HP-3G10), anti–HLA-ABC (w6/32), and anti–TCR-Vα7.2 (3C10) from BioLegend; anti-CD11b (ICRF44), anti-CD14 (61D3), anti-CD19 (SJ25C1), anti-CD25 (BC96), anti-CD45RA (HI100), and anti-CD80 (2D10.4) from eBioscience; anti–HLA-A2 (BB7.2) from Serotec; and anti-CCR9 (248601) and anti-CCR10 (314305) from R&D Systems; together with appropriate isotype controls. Intracellular cytokines were detected using anti–IFN-γ (B27, BD Biosciences; 4S.B3, eBioscience) and anti–TNF-α (6401.1111, BD Biosciences; 188, Beckman Coulter). Blocking reagents used included anti-Vα7.2 (3C10; BioLegend); anti–TCR-Vγ9 (Immu360; Beckman Coulter); anti-TLR4 (HTA125; eBioscience); anti-CD277 (103.2; D. Olive, Université de la Méditerranée, Marseille, France); anti–MHC-related protein 1 (MR1) (26.5; T. Hansen, Washington University School of Medicine, St. Louis, MO); anti–IFN-γ (25718) and anti–GM-CSF (3209) (BioLegend); and soluble TNFR (sTNFR) p75-IgG1 fusion protein (etanercept/Enbrel; Amgen).

Cells

Total leukocytes from healthy donors and patients were isolated from heparinized blood by mixing with HetaSep (StemCell Technologies), followed by sedimentation of RBCs (Supplemental Fig. 1A). Neutrophils were purified from whole blood or Lymphoprep (Axis-Shield) separated granulocytes by HetaSep sedimentation, followed by negative selection using the EasySep neutrophil enrichment kit (StemCell Technologies) (33), resulting in purities of >99.2% $CD14^-CD66b^+CD15^+$ and <0.1% contaminating monocytes (Supplemental Fig. 1B). Total $CD3^+$ T cells (>98%) were isolated from PBMC using the pan T cell isolation kit II (Miltenyi Biotec); $CD4^+$ and $CD8^+$ T cells (>98%) were obtained using the corresponding EasySep kits (StemCell Technologies). $Vγ9^+$ T cells (>98%) were purified using anti–Vγ9-PE-Cy5 (Immu360; Beckman Coulter) and anti-PE microbeads (Miltenyi Biotec); $Vα7.2^+$ T cells (>98%) were purified using anti–Vα7.2-PE (3C10; BioLegend) and anti-PE microbeads. Alternatively, $Vγ9^+$ $CD3^+$ γδ T cells or $Vα7.2^+$ $CD161^+$ $CD3^+$ MAIT cells were sorted to purities >99% using a FACSAria II (BD Biosciences).

Bacteria

Clinical isolates of *Enterobacter cloacae*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, and *S. aureus* (28) were grown in liquid Luria-Bertani broth and on solid Columbia blood agar (Oxoid). The distribution of the non-mevalonate and riboflavin pathways across microbial species was determined based on the absence or presence of the enzymes HMB-PP synthase (EC 1.17.7.1) and DMRL synthase (EC 2.5.1.78) in the corresponding genomes, according to the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg).

T cell culture

PBMC were stimulated with 0.1–100 nM HMB-PP or 0.1–100 μM DMRL. $Vγ9^+$ T cells or $Vα7.2^+$ T cells were cocultured with autologous monocytes at a ratio of 1:1, in the presence of 25% (v/v) cell-free supernatants from neutrophils that had phagocytosed live bacteria, as described previously (28). For blocking experiments, anti–TCR-Vα7.2, anti–TCR-Vγ9, anti-CD277, and anti-MR1 were used at 1–20 μg/ml.

Neutrophil culture

Freshly isolated neutrophils were cultured for up to 48 h in the absence or presence of autologous Vγ9/Vδ2 T cells or MAIT cells at a ratio of 10:1, and 10 nM HMB-PP or anti-CD3/CD28 dynabeads (1 bead per T cell). Alternatively, neutrophils were cultured with 25–50% (v/v) conditioned medium obtained from purified Vγ9/Vδ2 T cells or MAIT cells stimulated for 24 h with anti-CD3/CD28 dynabeads (1 bead per cell) or 100 nM HMB-PP. Other stimuli included 100 ng/ml LPS and 100 U/ml recombinant IFN-γ, TNF-α, and/or GM-CSF. sTNFR p75-IgG1 fusion protein, anti–IFN-γ, and anti–GM-CSF were used at 10 μg/ml. Neutrophil survival and activation were assessed by flow cytometry, after gating on $CD15^+$ cells and exclusion of $Vγ9^+$ or $Vα7.2^+$ cells where appropriate. For morphological analyses, neutrophils were centrifuged onto cytospin slides, stained with May-Grünwald-Giemsa solution, and analyzed by light microscopy.

Functional assays

Endocytosis and APC functions were assessed as before (34–38). Freshly purified neutrophils and neutrophils cultured for 24 h in the presence or absence of unconventional T cell–conditioned medium were incubated with 500 μg/ml 10-kDa dextran-FITC or BSA-FITC for up to 60 min at 4˚C or 37˚C. Endocytic uptake was measured immediately by flow cytom-

etry; the specific uptake of each reagent was calculated by subtracting the background MFI at 4°C from the MFI obtained at 37°C.

For MHC class II–restricted presentation of Ags, activated neutrophils were generated by 48-h culture with a combination of IFN-γ, GM-CSF, and/or TNF-α, or with unconventional T cell–conditioned medium. Neutrophils were pulsed with 10 ng/ml TSST-1 for 1 h. After extensive washing, neutrophils were mixed with autologous $CD4^+$ T cells at a ratio of 1:1; 1 h later 10 μg/ml brefeldin A was added and cultures were incubated for an additional 4 h. Activation of TSST-1–responsive $V\beta2^+$ $CD4^+$ T cells was assessed by intracellular cytokine staining and analysis by flow cytometry (35). To assess $CD4^+$ and $CD8^+$ T cell responses to complex Ag preparations, neutrophils were pulsed with 1–10 μg/ml PPD for the last 18 h of the 48-h culture phase. After extensive washing, neutrophils were mixed with CFSE-labeled autologous $CD3^+$ T cells at a ratio of 1:1 and incubated for 7 d. CFSE dilution in the $CD4^+$ and $CD8^+$ T cell populations was assessed by flow cytometry, after exclusion of $CD66b^+$ cells.

For MHC class I–restricted Ag presentation, Ag-specific HLA-A2–restricted $CD8^+$ T cell lines were generated using the immunodominant peptide of influenza matrix protein, M1(p58–66) (GILGFVFTL), as described before (37, 38). M1(p58–66)-specific responder $CD8^+$ T cells used in APC assays were >95% pure, as confirmed by tetramer staining (data not shown). Activated neutrophils from HLA-$A2^+$ donors were generated as above, using unconventional T cell–conditioned medium or recombinant cytokines, and pulsed for 1 h with 0.1 μM peptide. For cross-presentation assays, 0.01–1 μM recombinant influenza M1 protein was added during the last 18 h of the 48-h neutrophil culture period. Fresh neutrophils from HLA-$A2^+$ sepsis patients were incubated with 0.01–1 μM recombinant influenza M1 protein for 18 h or cultured in medium for 17 h prior to addition of 0.1 μM M1(p58–66) peptide for an additional 1 h. In each case, following extensive washing, neutrophils were incubated with HLA-$A2^+$ peptide-specific $CD8^+$ T cells at a ratio of 1:1; after 1 h, 10 μg/ml brefeldin was added and cultures were incubated for an additional 4 h. Activation of $CD8^+$ T cells was assessed by intracellular cytokine staining and analyzed by flow cytometry, after exclusion of $CD66b^+$ cells.

Flow cytometry

Cells were acquired on an eight-color FACSCanto II (BD Biosciences) and analyzed with FlowJo (Tree Star). Single cells of interest were gated based on their appearance in side and forward scatter area/height, exclusion of live/dead staining (fixable Aqua; Invitrogen), and surface staining. Apoptotic cells were identified using annexin-V (BD Biosciences).

ELISA

Cell culture supernatants were analyzed on a Dynex MRX II reader, using ELISA kits for IL-17A (R&D Systems) as well as IFN-γ and TNF-α (eBioscience). Cell-free plasma samples and unconventional T cell–conditioned media were analyzed on a SECTOR Imager 6000 using the ultrasensitive human proinflammatory 9-plex kit (Meso Scale Discovery).

Statistics

Data were analyzed using two-tailed Student *t* tests for normally distributed data and Mann–Whitney tests for nonparametric data (GraphPad Prism). Differences between groups were analyzed using one-way ANOVA with Bonferroni's posttests or with Kruskal–Wallis and Dunn's posttests; two-way ANOVA was used when comparing groups with independent variables.

Results

Unconventional human T cells respond to neutrophil-released microbial metabolites

$V\gamma9/V\delta2^+$ γδ T cells recognize the isoprenoid precursor HMB-PP, which is produced via the nonmevalonate pathway by a broad range of Gram-negative and Gram-positive bacteria (27, 39). $V\alpha7.2^+$ $CD161^+$ MAIT cells show a very similar responsiveness to an overlapping, but distinct spectrum of microorganisms by sensing intermediates of the microbial vitamin B2 biosynthesis (Table I) (40–43). We therefore sought to investigate the antimicrobial responses of these two types of unconventional T cells side by side. In this study, Vγ9/Vδ2 T cells, but not MAIT cells, responded to HMB-PP, as judged by induction of CD69 expression (Fig. 1A). In contrast, the riboflavin precursor DMRL induced a dose-dependent activation of MAIT cells, but not Vγ9/Vδ2 T cells. Blocking experiments confirmed a requirement for butyrophilin 3A/CD277 for Vγ9/Vδ2 T cells and the MHC-related protein MR1 for MAIT cells (Fig. 1B), in support of current models of Ag recognition (41–45).

We previously identified a crucial role for neutrophils in facilitating access to HMB-PP by Vγ9/Vδ2 T cells (28). As control, purified $V\gamma9^+$ T cells readily responded to neutrophils after phagocytosis of clinically relevant bacteria, in accordance with the distribution of the nonmevalonate pathways across the different pathogens (Table I). Strikingly, purified $V\alpha7.2^+$ T cells showed very similar responses depending on the utilization of the riboflavin biosynthesis pathway by the phagocytosed species. Activated $V\gamma9^+$ T cells and $V\alpha7.2^+$ T cells upregulated CD69 (Fig. 1C) and secreted IFN-γ (Fig. 1D), but not IL-17A (data not shown). The response of $V\alpha7.2^+$ T cells to microbial compounds was confined to the $CD161^+$ bona fide MAIT cell population (Fig. 1C, 1D). Both Vγ9/Vδ2 T cells and MAIT cells failed to respond to neutrophil-released microbial compounds in the presence of anti-CD277 and anti-MR1, respectively (Fig. 1D), and in the absence of autologous monocytes (Fig. 1E), highlighting a requirement for presentation by accessory cells. These findings reveal a remarkable similarity in the responsiveness of Vγ9/Vδ2 T cells and MAIT cells to microbial metabolites.

Patients with acute sepsis caused by HMB-PP–producing pathogens display elevated levels of activated γδ T cells

To resolve the existence of APC-like neutrophils in human infectious disease and determine a possible link with antimicrobial unconventional T cell responses, we recruited adult patients with newly diagnosed severe sepsis and characterized their circulating leukocytes phenotypically and functionally. As proof of principle for the involvement of unconventional T cells in early inflammatory responses, patients with acute sepsis revealed a substantial activation of Vγ9/Vδ2 T cells, as judged by CD69 expression, but not SIRS patients who served as noninfected controls (Fig. 1F, Supplemental Fig. 1A). Of note, we found a significant increase in the absolute counts and the proportion of Vγ9/Vδ2 T cells among all circulating T cells between patients with microbiologically confirmed infections caused by HMB-PP–producing as opposed to HMB-PP–deficient species (Fig. 1F). These clinical findings evoke earlier studies in patients with acute peritonitis (28) and further support the notion of a differential responsiveness of unconventional T cells to defined pathogen groups that can be detected both locally at the site of infection (46) and systemically in blood (Fig. 1F).

Unconventional human T cells induce prolonged neutrophil survival and activation

We recently showed that Vγ9/Vδ2 T cells trigger short-term (<20 h) survival of autologous neutrophils (28). In this study, highly purified neutrophils cocultured for extended periods with activated Vγ9/Vδ2 T cells or MAIT cells displayed a prolonged survival, as judged by exclusion of amine reactive dyes and retention of surface CD16 (FcγRIII) for at least 48 h (Fig. 2A). A similar effect was observed when incubating purified neutrophils with Vγ9/Vδ2 T cell or MAIT cell–conditioned culture supernatants, indicating a significant contribution of soluble factors in mediating the observed effects (Fig. 2B). In contrast to the highly active metabolite HMB-PP as specific activator of Vγ9/Vδ2 T cells, the MAIT cell activator used in the current study, DMRL, only possesses a relatively modest bioactivity. The true MAIT cell activator is far more potent than DMRL and active at subnanomolar concentrations, but not commercially available and difficult to synthesize chemically (41, 43). Most stimulation experiments with purified MAIT cells were therefore conducted with anti-CD3/CD28–coated beads. Importantly, use of either anti-CD3/CD28 beads or HMB-PP to

Table I. Distribution across clinically relevant microbial pathogens of key biosynthetic pathways that produce metabolites targeted by human unconventional T cells

	Nonmevalonate Pathway (Vγ9/Vδ2 T Cell Activation)	Vitamin B2 Synthesis (MAIT Cell Activation)
Gram-negative bacteria		
Acinetobacter baumannii	+	+
Chryseobacterium gleum	−	+
Enterobacter cloacae	+	+
Escherichia coli	+	+
Haemophilus influenzae	+	+
Helicobacter pylori	+	+
Klebsiella pneumoniae	+	+
Legionella pneumophila	−	+
Neisseria meningitidis	+	+
Pseudomonas aeruginosa	+	+
Shigella dysenteriae	+	+
Gram-positive bacteria		
Bacillus anthracis	+	+
Clostridium difficile	+	+
Corynebacterium diphtheriae	+	+
Enterococcus faecalis	−	−
Listeria monocytogenes	+	−
Mycobacterium tuberculosis	+	+
Propionibacterium acnes	+	+
Staphylococcus aureus	−	+
Streptococcus pyogenes	−	−
Other bacteria		
Borrelia burgdorferi	−	−
Leptospira interrogans	+	+
Mycoplasma genitalium	−	−
Mycoplasma penetrans	+	−
Treponema pallidum	−	−
Yeasts, fungi		
Aspergillus fumigatus	−	+
Candida albicans	−	+
Crytococcus neoformans	−	+
Saccharomyces cerevisiae	−	+

activate Vγ9/Vδ2 T cells elicited identical neutrophil responses (Fig. 2A–C and data not shown). Surviving neutrophils possessed a highly activated morphology, as judged by the presence of hypersegmented nuclei (Fig. 2C). The antiapoptotic effect of unconventional T cells was confirmed by the preservation of the total number of neutrophils present after 48 h of culture and the lack of annexin-V binding (Fig. 2D). As confirmation of their activated status, surviving neutrophils showed pronounced upregulation of CD11b and CD66b expression and complete loss of CD62L (Fig. 2E).

Unconventional T cell–primed neutrophils have a unique APC-like phenotype

Circulating neutrophils in healthy people do not express CD40, CD64 (FcγRI), CD83, or HLA-DR, yet all these surface markers were found on unconventional T cell–primed neutrophils (Fig. 2F). Moreover, these neutrophils also showed a marked upregulation of CD54 (ICAM-1) and HLA-ABC (Fig. 2F), suggestive of a possible function of unconventional T cell–primed neutrophils as APCs for both $CD4^+$ and $CD8^+$ T cells.

The chemokine receptors CCR7, CCR9, and CCR10 remained undetectable under those culture conditions (data not shown), arguing against trafficking of APC-like neutrophils to noninflamed lymph nodes, the intestine, or the skin. In contrast, APC-like neutrophils displayed enhanced expression levels of CXCR3 and CCR4 (data not shown), indicative of an increased responsiveness to inflammatory chemokines and supporting a local role during acute inflammation.

Neutrophils stimulated with defined microbial compounds on their own, in the absence of Vγ9/Vδ2 T cells or MAIT cells, failed to acquire a similar phenotype. Most notably, neutrophils cultured for 48 h in the presence of LPS did not show increased levels of HLA-ABC, HLA-DR, CD40, CD64, or CD83 compared with neutrophils cultured in medium alone (data not shown), emphasizing the crucial and nonredundant contribution of unconventional T cells and their specific ligands to the acquisition of APC characteristics by neutrophils.

Circulating neutrophils in sepsis patients display an APC-like phenotype

To resolve the existence of APC-like neutrophils in human infectious disease, we characterized circulating leukocytes in sepsis patients as a means to access neutrophils that had recently been activated in different infected tissues. Sepsis neutrophils displayed a strikingly altered phenotype compared with neutrophils from healthy individuals and SIRS patients and were characterized by markedly higher expression of CD40, CD64, and CD86 (Fig. 3A). We also found increased surface levels of CD83 and HLA-DR on circulating neutrophils in some patients with sepsis, although this was not significant across the cohort as a whole. Of note, there was a correlation between the expression of CD64 and HLA-DR on sepsis neutrophils, supporting a link between neutrophil activation and APC phenotype (Fig. 3B). These findings indicate the presence of APC-like neutrophils in sepsis patients, despite the generally presumed immune suppression in those individuals, as judged by reduced HLA-DR expression levels on monocytes (data not shown) (47).

Neutrophil survival and APC marker expression are mediated via unconventional T cell–secreted cytokines

To identify the unconventional T cell–derived factor(s) exerting the observed effects on neutrophils, we quantified proinflammatory

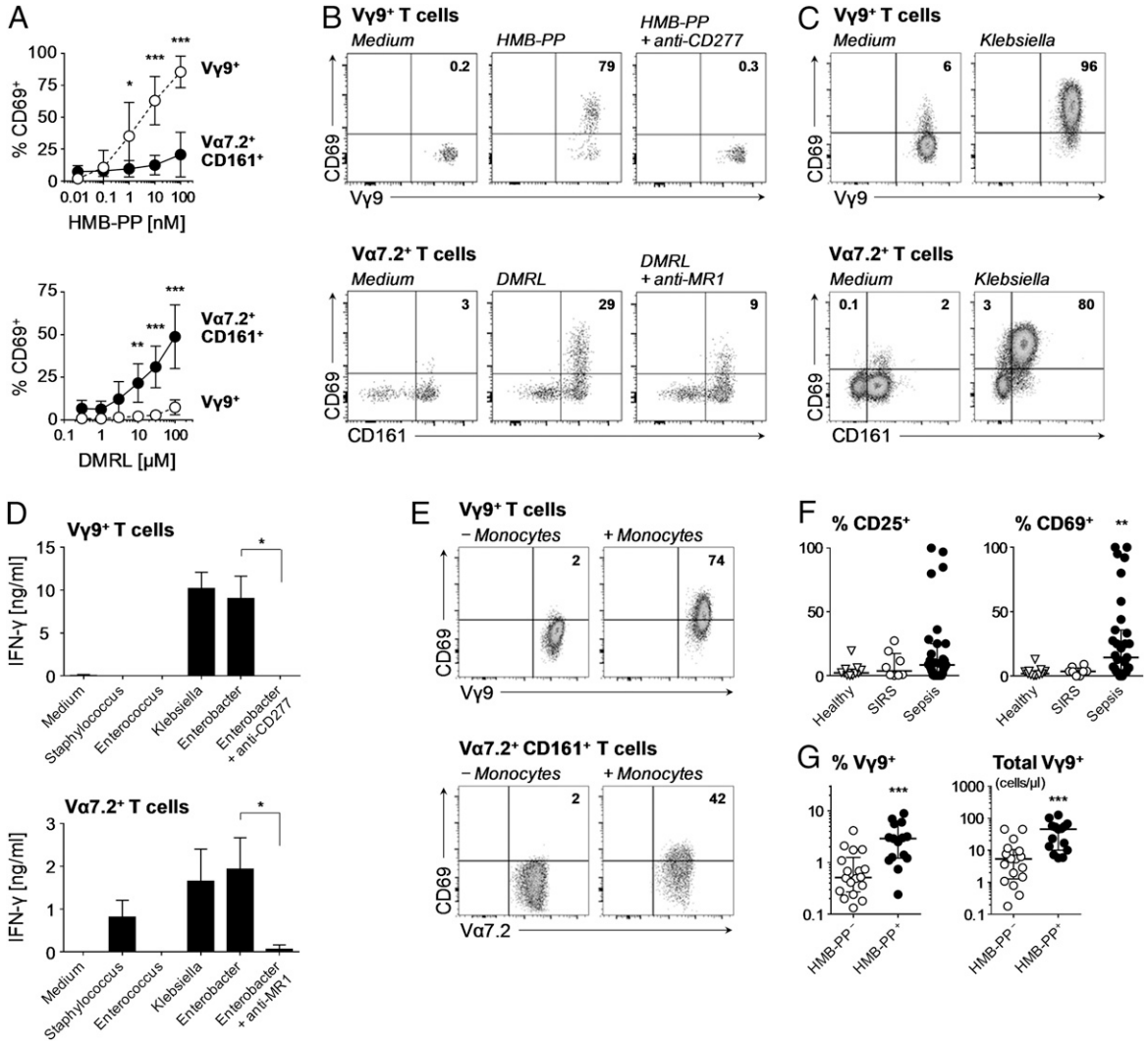

FIGURE 1. Unconventional human T cell responses to microbial metabolites in vitro and in vivo. (**A**) CD69 surface expression by Vγ9$^+$ T cells and Vα7.2$^+$ CD161$^+$ T cells in PBMC stimulated overnight with HMB-PP or DMRL (means ± SD, n = 5). Data were analyzed by two-way ANOVA with Bonferroni's post hoc tests. (**B**) Representative FACS plots of two donors showing CD69 expression by Vγ9$^+$ T cells and Vα7.2$^+$ T cells in PBMC stimulated overnight with 100 nM HMB-PP or 100 μM DMRL, in the absence or presence of anti-CD277 or anti-MR1 mAb. (**C**) CD69 expression by MACS-purified Vγ9$^+$ T cells or Vα7.2$^+$ T cells cocultured overnight with autologous monocytes in the presence of supernatants from neutrophils after phagocytosis of *Klebsiella pneumoniae* (representative of three donors). (**D**) IFN-γ secretion by MACS-purified Vγ9$^+$ T cells or Vα7.2$^+$ T cells cocultured overnight with autologous monocytes in the presence of supernatants from neutrophils after phagocytosis of different bacteria: HMB-PP$^-$ DMRL$^+$, *Staphylococcus aureus*; HMB-PP$^-$ DMRL$^-$, *Enterococcus faecalis*; and HMB-PP$^+$ DMRL$^+$, *Enterobacter cloacae* and *K. pneumoniae* (means ± SD, n = 3–4 donors). Differences between mAb-treated and untreated cultures were analyzed using Mann–Whitney tests. (**E**) CD69 expression by FACS-sorted Vγ9$^+$ T cells or Vα7.2$^+$ CD161$^+$ T cells cocultured overnight with or without autologous monocytes in the presence of supernatants from neutrophils after phagocytosis of *E. cloacae* (representative of two donors). (**F**) Surface expression by CD25 and CD69 on circulating Vγ9$^+$ T cells in healthy controls and in patients with SIRS or sepsis. Each data point represents an individual; lines and error bars depict medians and interquartile ranges. Data were analyzed using Kruskal–Wallis tests and Dunn's multiple comparison tests; comparisons were made with sepsis patients. (**G**) Proportion of Vγ9$^+$ T cells among all circulating T cells and absolute counts of circulating Vγ9$^+$ T cells (in cells/μl blood) in sepsis patients with microbiologically confirmed infections caused by HMB-PP–producing (*E. coli*, *Enterobacter aerogenes*, *Haemophilus influenzae*, *K. pneumonia*e, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, anaerobic Gram-negative bacilli, diphtheroid bacteria) or HMB-PP–deficient organisms (*Aspergillus fumigatus*, *Candida spp.*, *Staphylococcus spp.*, *Streptococcus pneumoniae*). Data were analyzed using Mann–Whitney tests. Differences were considered significant as indicated: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

mediators in the culture supernatants. These experiments revealed a dominant production (>1000 pg/ml on average) of GM-CSF, IFN-γ, and TNF-α by activated Vγ9/Vδ2 T cells and MAIT cells, but only very low levels (<25 pg/ml) of IL-1β, IL-6, and CXCL8, indicating that both unconventional T cell populations share a similar cytokine profile (Fig. 4A). Experiments using blocking reagents identified an involvement of GM-CSF, IFN-γ, and TNF-α in promoting neutrophil survival by both Vγ9/Vδ2 T cells and MAIT cells (Fig. 4B). Whereas neutralization of each individual cytokine on its own had a partial effect, combined blocking of GM-CSF and IFN-γ was most effective in inhibiting neutrophil survival, with blocking of TNF-α having little additive effect. In contrast, CD66b upregulation was mainly triggered by TNF-α (Fig. 4B). Of note, the effect of unconventional T cells on neutrophils could be mimicked in part by using recombinant GM-CSF, IFN-γ, and TNF-α. In this respect, only neutrophils cultured with a combination of all three cytokines exhibited a morphology characterized by hypersegmented nuclei (Fig. 4C). TNF-α was particularly important for the induction of CD40, CD54, CD66b, and MHC class I expression (Fig. 4D). Taken together,

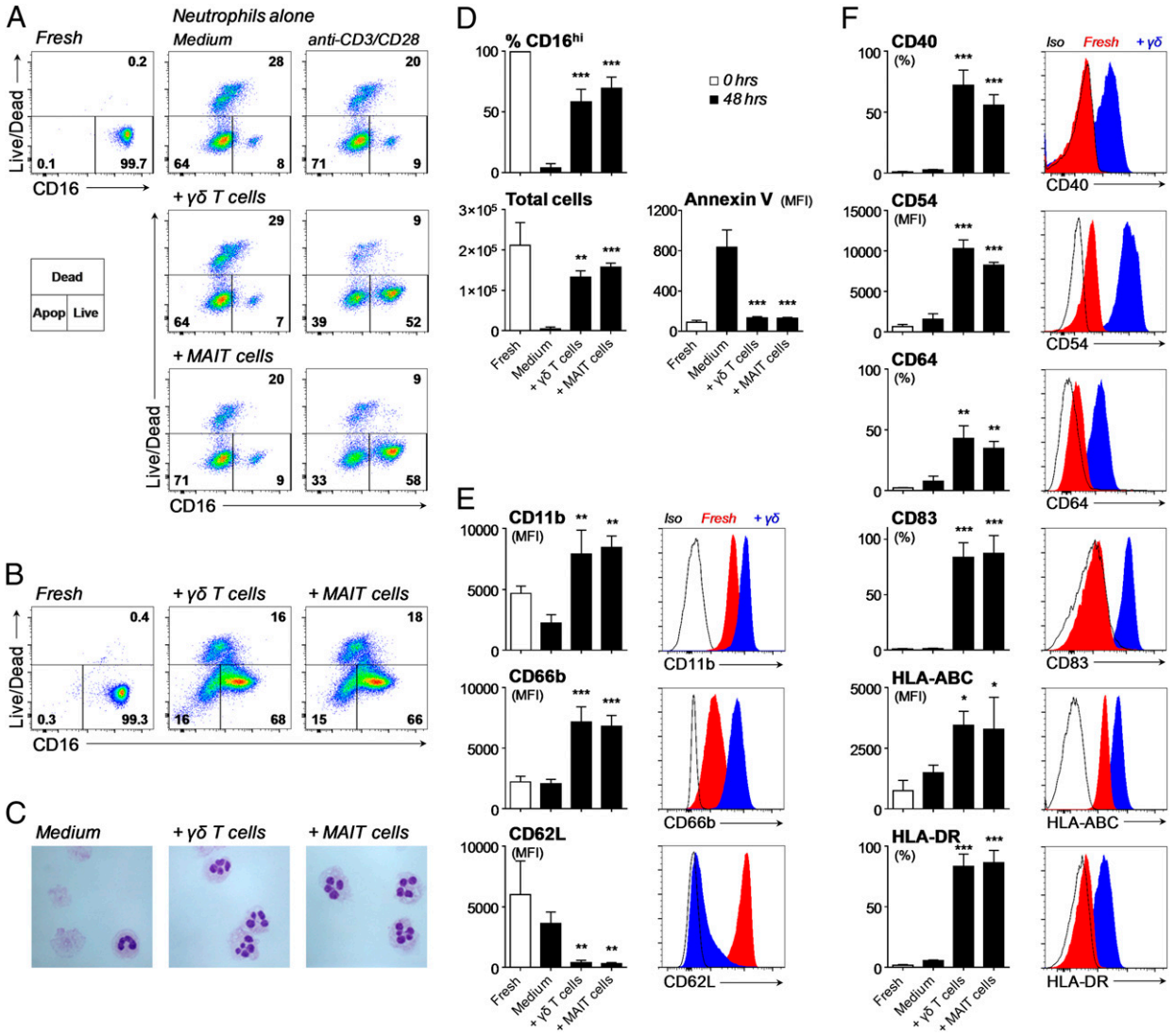

FIGURE 2. Survival, activation, and expression of APC markers by unconventional T cell–primed neutrophils. (**A**) Neutrophil survival judged by retention of CD16 expression and exclusion of live/dead staining after 48-h coculture with FACS-sorted Vγ9/Vδ2 T cells or MAIT cells, in the absence or presence of anti-CD3/CD28 beads. FACS plots are representative of three donors and depict total neutrophils after gating on $CD15^+$ $Vγ9^-$ or $CD15^+$ $Vα7.2^-$ cells. (**B**) Neutrophil survival after 48-h culture in the presence of HMB-PP–activated Vγ9/Vδ2 T cell or anti-CD3/CD28–activated MAIT cell–conditioned medium (representative of three donors). (**C**) Morphological analysis of surviving neutrophils after 48-h culture in the absence or presence of HMB-PP–activated Vγ9/Vδ2 T cell or anti-CD3/CD28–activated MAIT cell–conditioned medium (representative of two donors). Original magnification ×400. (**D**) Neutrophil survival after 48-h culture in the absence or presence of HMB-PP–activated Vγ9/Vδ2 T cell or anti-CD3/CD28–activated MAIT cell–conditioned medium. Shown are means ± SD for the proportion of $CD16^{high}$ cells (n = 9–10), the total number of neutrophils (n = 3), and annexin V staining on $CD16^{high}$ neutrophils (n = 3). Expression of (**E**) activation markers and (**F**) APC markers on freshly isolated neutrophils and $CD16^{high}$ neutrophils after 48-h culture in the absence or presence of HMB-PP–activated Vγ9/Vδ2 T cell– or anti-CD3/CD28–activated MAIT cell–conditioned medium. Data shown are means ± SD and representative histograms from three individual donors. Data were analyzed by one-way ANOVA with Bonferroni's post hoc tests; comparisons were made with medium controls. Differences were considered significant as indicated: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

these experiments identify microbe-responsive unconventional T cells as a rapid physiological source of GM-CSF, IFN-γ, and TNF-α and imply that the unique combination of cytokines secreted by unconventional T cells is key for the observed impact on neutrophils.

The particular requirement for TNF-α in the acquisition of the full APC phenotype is especially noteworthy when considering the cytokine/chemokine profiles in acutely infected patients. Plasma proteins that were highly elevated in sepsis patients included TNF-α as well as IL-6 and CXCL8 (Fig. 4E). Of note, there was a trend toward higher levels of TNF-α in patients with HMB-PP–positive infections (p = 0.09; data not shown). A proportion of individuals with sepsis also had increased plasma levels of GM-CSF, IFN-γ, and IL-1β, although this was not significant across the whole cohort (Fig. 4E). These findings confirm that the blood of sepsis patients contains proinflammatory mediators implicated in driving survival and activation of neutrophils, including their differentiation into APCs.

Unconventional T cell–primed neutrophils readily take up soluble Ags

We next tested the capacity of APC-like neutrophils to take up soluble Ags. Although freshly isolated neutrophils were not very efficient at endocytosing FITC-labeled BSA and dextran (10,000 Da) as model compounds, short-term exposure to Vγ9/Vδ2 T cell–conditioned medium led to a greatly enhanced uptake (Fig. 5A). With unconventional T cell–primed neutrophils kept in culture for 24 h before addition of BSA-FITC, Ag endocytosis was confined

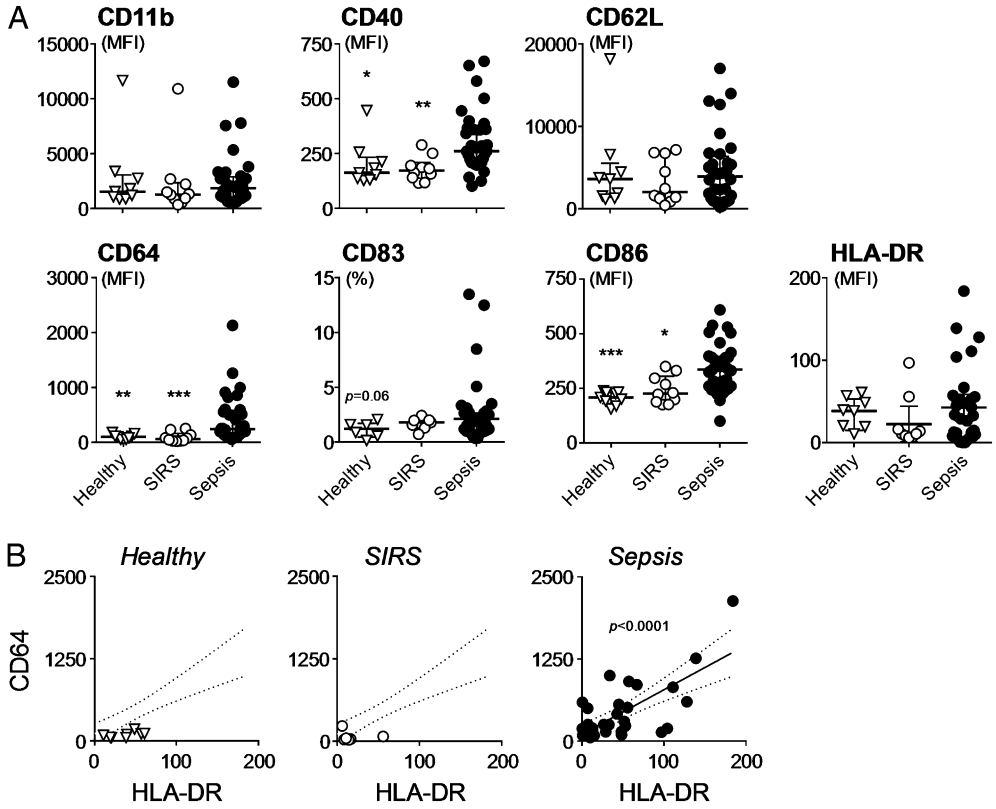

FIGURE 3. APC-like phenotype of circulating neutrophils during acute sepsis. (**A**) Surface expression of the indicated markers on circulating neutrophils in patients with SIRS (n = 14) or sepsis (n = 37) and in healthy controls (n = 10). Each data point represents an individual; lines and error bars depict medians and interquartile ranges. Data were analyzed using Kruskal–Wallis tests and Dunn's multiple comparison tests; comparisons were made with sepsis patients. (**B**) Correlation between surface expression of CD64 and HLA-DR on circulating neutrophils in healthy controls and in patients with SIRS or sepsis. Lines depict linear regression and 95% confidence bands as calculated for sepsis neutrophils. Differences were considered significant as indicated: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

to the $CD16^{high}$ APC-like population, whereas no such uptake was seen in the apoptotic $CD16^{low}$ population (Fig. 5B). In contrast, neutrophils cultured in medium alone showed no specific uptake of BSA in the $CD16^{high}$ population. These data indicate that unconventional T cells promote the uptake of exogenous Ags as a prerequisite for Ag processing and presentation by neutrophils.

Unconventional T cell–primed neutrophils are efficient APCs for $CD4^+$ and $CD8^+$ T cells

The functionality of cell surface–expressed HLA-DR on activated neutrophils was confirmed using the *S. aureus* superantigen, TSST-1, which cross-links MHC class II molecules with the TCR of $CD4^+$ T cells expressing a Vβ2 chain (35). Neutrophils exposed to Vγ9/Vδ2 T cell–conditioned medium or to a combination of GM-CSF, IFN-γ, and TNF-α were both capable of presenting TSST-1 to autologous $V\beta2^+$ $CD4^+$ T cells (Fig. 5C). When using the complex *M. tuberculosis* Ag, PPD, which requires intracellular processing, unconventional T cell–primed neutrophils displayed a striking capacity to trigger proliferation of both $CD4^+$ and $CD8^+$ T cells (Fig. 5D). Sequestration of TNF-α during the neutrophil-priming period by addition of sTNFR diminished both $CD4^+$ and $CD8^+$ T cell responses (Fig. 5E) as further confirmation of the key role for unconventional T cell–derived TNF-α in the acquisition of APC features by neutrophils.

Unconventional T cell–primed neutrophils cross-present Ags to $CD8^+$ T cells

Following up from the striking induction of PPD-specific $CD4^+$ and $CD8^+$ T cell responses, we assessed the potential of APC-like neutrophils to trigger $CD8^+$ T cell responses, by taking advantage of HLA-A2–restricted responder T cell lines specific for M1(p58–66), the immunodominant epitope of the influenza M1 protein (36–38). Using the M1(p58–66) peptide, which can be pulsed readily onto cell surface–associated MHC class I molecules for direct presentation to $CD8^+$ T cells, unconventional T cell–primed neutrophils showed a significantly improved Ag presentation, compared with freshly isolated neutrophils (Fig. 6A) and in agreement with the elevated levels of MHC class I molecules on APC-like neutrophils. Importantly, only unconventional T cell–primed neutrophils, but not freshly isolated neutrophils, were also able to induce robust responses by M1(p58–66)-specific responder $CD8^+$ T cells when utilizing the full-length M1 protein (Fig. 6A), a 251-aa–long Ag that requires uptake, processing, and loading of M1(p58–66) onto intracellular MHC class I molecules for cross-presentation to $CD8^+$ T cells (36–38). Control experiments supported the need for Ag uptake and processing, as recombinant M1 protein could not be pulsed directly onto neutrophils, demonstrating the absence of potential degradation products in the M1 protein preparation that might be able to bind directly to cell surface–associated MHC class I molecules on neutrophils or $CD8^+$ T cells (Fig. 6B). Neutrophils cultured for 48 h in the presence of GM-CSF and IFN-γ were also capable of enhanced presentation of M1(p58–66) peptide to M1-specific $CD8^+$ T cells. However, only neutrophils generated by incubation with a combination of GM-CSF, IFN-γ, and TNF-α readily processed the full-length M1 protein (Fig. 6A), demonstrating that TNF-α plays a pivotal role in the acquisition of a fully competent APC phenotype and function by neutrophils.

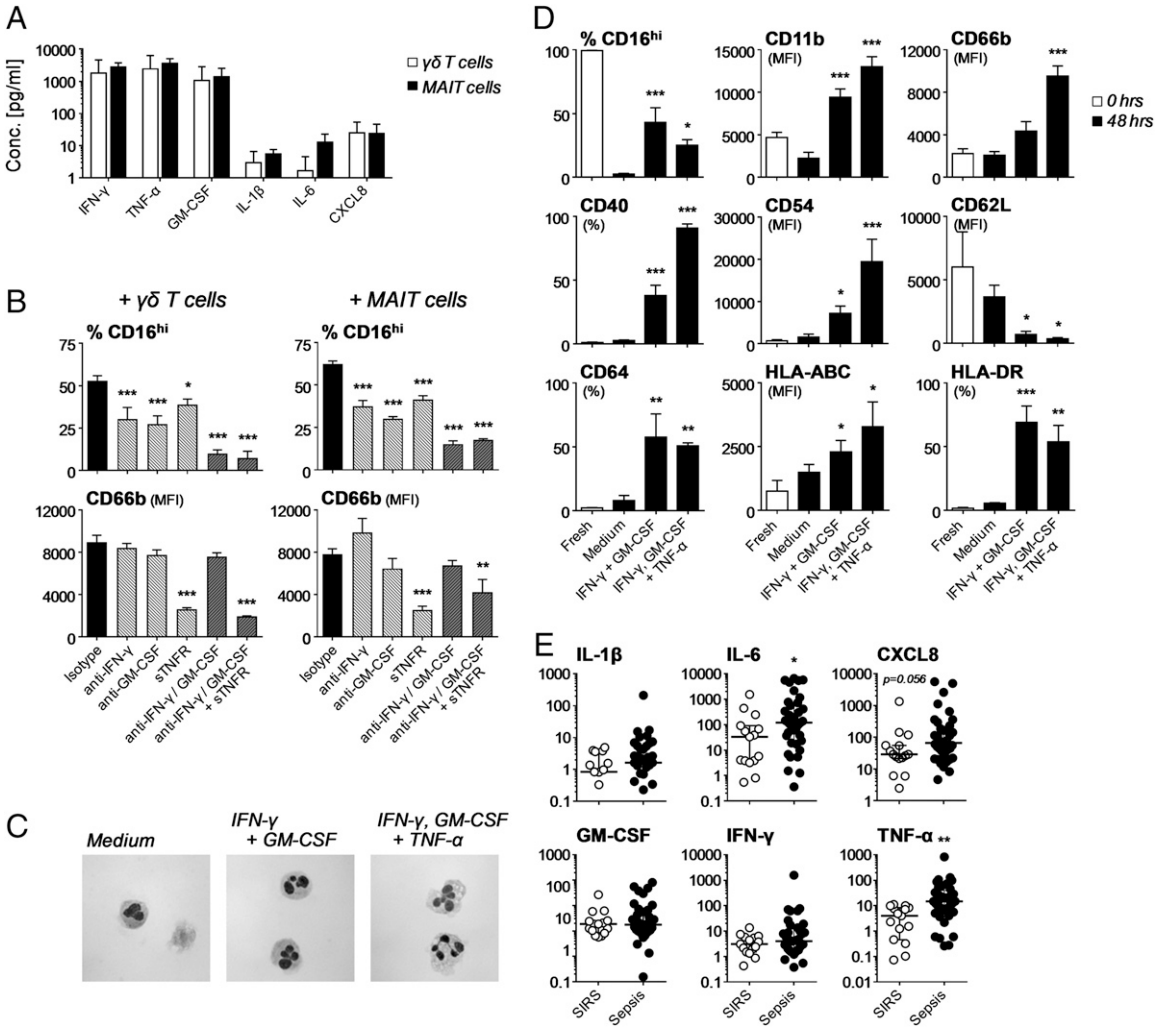

FIGURE 4. Effect of unconventional T cell–derived cytokines on neutrophil survival and APC marker expression. (**A**) Secretion of the indicated mediators into the culture supernatant by FACS-sorted Vγ9/Vδ2 T cells or MAIT cells stimulated overnight in the presence of HMB-PP or anti-CD3/CD28 beads, respectively, as detected using multiplex ELISA (means + SD, n = 2–3). (**B**) Neutrophil survival (as proportion of $CD16^{high}$ cells) and CD66b expression on $CD16^{high}$ neutrophils after 48-h culture in the presence of HMB-PP–activated Vγ9/Vδ2 T cell– or anti-CD3/CD28 MAIT cell–conditioned medium and neutralizing agents against GM-CSF, IFN-γ, and/or TNF-α (means + SD, n = 3). Data were analyzed by one-way ANOVA with Bonferroni's post hoc tests; comparisons were made with isotypes. (**C**) Morphological analysis of surviving neutrophils after 48-h culture in the absence or presence of GM-CSF, IFN-γ, and/or TNF-α (representative of two donors). Original magnification ×400. (**D**) Neutrophil survival and expression of the indicated markers on $CD16^{high}$ neutrophils after 48-h culture in the absence or presence of recombinant GM-CSF, IFN-γ, and/or TNF-α (means + SD, n = 3). Data were analyzed by one-way ANOVA with Bonferroni's post hoc tests; comparisons were made with medium controls. (**E**) Plasma levels of IL-1β, IL-6, CXCL8, GM-CSF, IFN-γ, and TNF-α in SIRS and sepsis patients (in pg/ml). Each data point represents an individual; lines and error bars depict medians and interquartile ranges. Differences between the two groups were analyzed using Mann–Whitney tests. Differences were considered significant as indicated: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Circulating neutrophils from sepsis patients are capable of cross-presenting Ags to $CD8^+$ T cells

It has not yet been established whether neutrophils are capable of triggering Ag-specific T cell responses in vivo. To translate our findings on APC-like neutrophils to the situation in acute infections, we isolated untouched neutrophils from sepsis patients to purities of 99.2–99.8%. Our experiments show that sepsis neutrophils and control neutrophils had a similar capacity to activate M1-specific responder $CD8^+$ T cells when pulsed with the peptide itself (Fig. 6C). Strikingly, only sepsis neutrophils, but not control neutrophils, were also able to take up the full-length M1 protein and cross-present the M1(p58–66) peptide to responder $CD8^+$ T cells (Fig. 6C, 6D), consistent with the differences in APC marker expression between patients and healthy individuals. These findings indicate that in acute sepsis neutrophils acquire an APC-like phenotype with the capacity to induce Ag-specific $CD8^+$ T cell responses that is reminiscent of neutrophils primed by unconventional T cells (Fig. 7).

Discussion

To our knowledge, the present study is the first demonstration that human neutrophils can assume Ag cross-presenting properties. Although our work does not formally demonstrate a causal link for the interaction of unconventional T cells and neutrophils in vivo, it does suggest a plausible scenario for the generation of APC-like neutrophils during acute infection. Our data support a model in which different types of unconventional T cells respond rapidly to neutrophils after phagocytosis of a broad range of bacteria at the site of infection, and in turn mediate the local differentiation of bystander neutrophils into APCs for both $CD4^+$ and $CD8^+$ T cells (Fig. 7). APC-like neutrophils may be particularly relevant for local responses by tissue-resident memory and/or freshly recruited

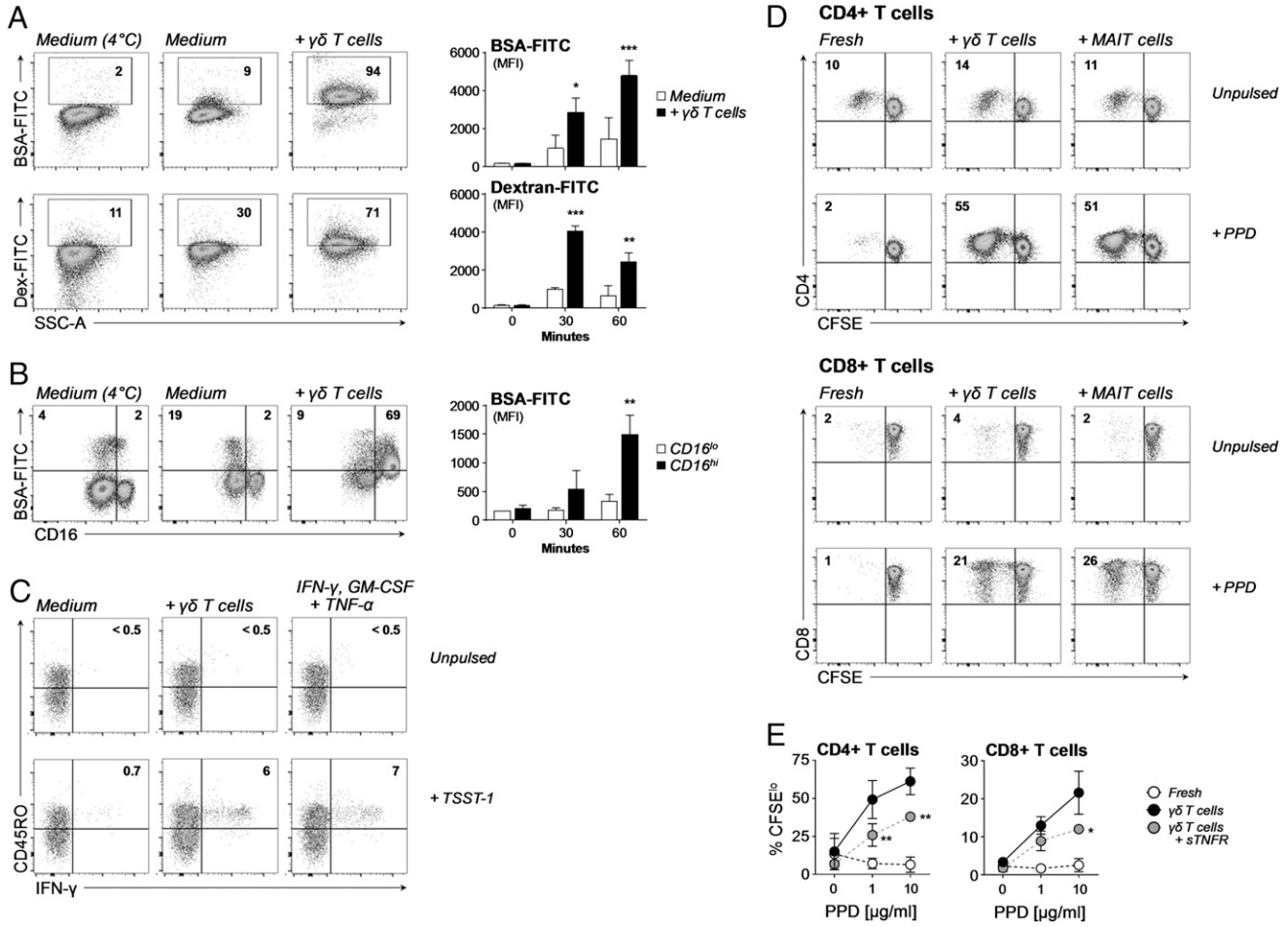

FIGURE 5. Efficient endocytosis of exogenous molecules and presentation of microbial Ags by unconventional T cell–primed neutrophils. (**A**) Endocytosis of FITC-labeled BSA and 10-kDa dextran by freshly isolated neutrophils incubated for 60 min at 4˚C or at 37˚C in the absence or presence of HMB-PP–activated γδ T cell supernatant. FACS plots are representative of two to three donors; specific uptake of FITC-labeled BSA and dextran by freshly isolated neutrophils was determined over 30 and 60 min (means + SD, $n = 2–3$). (**B**) Endocytosis of FITC-labeled BSA over 60 min by neutrophils that had been cultured overnight in the absence or presence of HMB-PP–activated γδ T cell supernatant. FACS plots are representative of three healthy donors; specific uptake of FITC-labeled BSA by γδ T cell–primed neutrophils was determined over 30 and 60 min (means + SD, $n = 2–3$). Data were analyzed by two-way ANOVA with Bonferroni's post hoc tests; comparisons were made with (A) medium controls or (B) $CD16^{low}$ cells. (**C**) IFN-γ production by superantigen-specific $CD4^+$ $V\beta2^+$ T cells in response to autologous neutrophils cultured for 48 h in medium or in the absence or presence of HMB-PP–activated Vγ9/Vδ2 T cell–conditioned medium or a combination of IFN-γ, GM-CSF, and TNF-α prior to pulsing with 10 ng/ml TSST-1 (representative of two donors). (**D**) Proliferation of $CD4^+$ and $CD8^+$ T cells in response to freshly isolated neutrophils and neutrophils cultured for 48 h in the presence of HMB-PP–activated Vγ9/Vδ2 T cell– or anti-CD3/CD28–activated MAIT cell–conditioned medium. Neutrophils were pulsed with 10 μg/ml PPD for 18 h prior to addition of CFSE-labeled bulk $CD3^+$ T cells; CFSE dilution of responder T cells was assessed after 7 d of coculture (representative of three donors). (**E**) Proliferation of CFSE-labeled $CD4^+$ and $CD8^+$ T cells in response to PPD-pulsed freshly isolated neutrophils and neutrophils cultured for 48 h in the presence of HMB-PP–activated Vγ9/Vδ2 T cell–conditioned medium with and without sTNFR. CFSE dilution of responder T cells was assessed after 7 d of coculture (means + SD, $n = 3$). Data were analyzed by one-way ANOVA with Bonferroni's post hoc tests; comparisons were made with Vγ9/Vδ2 T cell + sTNFR–treated neutrophils. Differences were considered significant as indicated: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

effector $CD4^+$ and $CD8^+$ T cells at the site of infection, rather than the priming of naive $CD4^+$ and $CD8^+$ T cells in secondary lymphoid tissues. Expression of the lymph node homing receptor CCR7 by activated neutrophils was reported before (30) but could not be confirmed in the current study (data not shown). Still, APC-like neutrophils may also gain access to inflamed draining lymph nodes through the action of inflammatory chemokines (7–10). Irrespective of the anatomical context, APC-like neutrophils may contribute to protective immune responses, by fighting the "first hit" infection as a result of inducing Ag-specific $CD4^+$ and $CD8^+$ T cells and by harnessing the T cell compartment against potential "second hit" infections. However, it is also thinkable that such an early induction of cytotoxic $CD8^+$ T cells may add to the systemic inflammatory response and ultimately lead to tissue damage and organ failure. Whereas the generation of APC-like neutrophils is likely to occur locally in the context of infected tissues, in severe inflammatory conditions, including sepsis, such APC-like neutrophils may eventually leak into the circulation and become detectable in blood. Larger stratified approaches are clearly needed to define the role of APC-like neutrophils in different infectious scenarios, locally and systemically, in clinically and microbiologically well-defined patient subgroups.

The presence of cross-presenting neutrophils in patients with sepsis is intriguing and may point to an essential role of APC-like neutrophils in acute disease. Sepsis patients who survive the primary infection often show signs of reduced surface expression of HLA-DR on monocytes and a relative tolerance of monocytes to LPS stimulation (47). As consequence of what is generally perceived as a loss of immune function, many patients are susceptible to subsequent nosocomial infections, including reactivation of

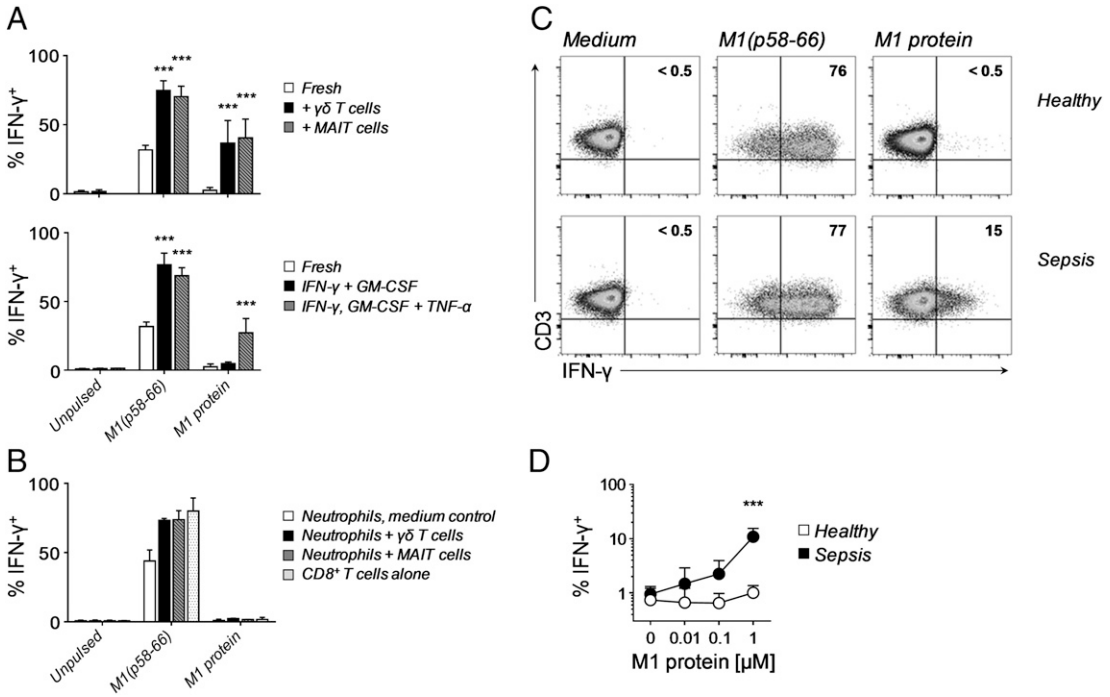

FIGURE 6. Cross-presentation of exogenous Ags by unconventional T cell–primed neutrophils and sepsis neutrophils. (**A**) IFN-γ production by Ag-specific $CD8^+$ T cells in response to neutrophils cultured for 48 h in the presence of HMB-PP–activated Vγ9/Vδ2 T cell– or anti-CD3/CD28–activated MAIT cell–conditioned medium (*top*), or neutrophils cultured for 48 h with the recombinant cytokines indicated (*bottom*). Neutrophils were pulsed for 1 h with 0.1 μM influenza M1(p58–66) peptide or for 18 h with recombinant M1 protein (means + SD, $n = 3$). Data were analyzed by two-way ANOVA with Bonferroni's post hoc tests; comparisons were made with freshly isolated neutrophils. (**B**) Failure of M1 protein to be pulsed directly onto neutrophils, as judged by IFN-γ production of Ag-specific $CD8^+$ T cells alone or in response to neutrophils cultured for 48 h in the absence or presence of HMB-PP–activated Vγ9/Vδ2 T cell– or anti-CD3/CD28–activated MAIT cell–conditioned medium. Neutrophils were pulsed for 1 h with either 0.1 μM influenza M1 (p58–66) peptide or 1 μM recombinant M1 protein; $CD8^+$ T cells were incubated directly with the peptide or M1 protein (means + SD, $n = 2$). (**C**) IFN-γ production by M1-specific $CD8^+$ T cells in response to freshly isolated neutrophils loaded with 0.1 μM synthetic M1(p58–66) peptide or 1 μM M1 protein. Data shown are representative of three $HLA\text{-}A2^+$ sepsis patients and three $HLA\text{-}A2^+$ healthy volunteers as controls. Sepsis patients recruited for these APC assays had confirmed infections as identified by positive culture results: *Escherichia coli* (urine), *Klebsiella pneumoniae* (respiratory culture), and *Staphylococcus epidermidis* (blood), respectively. (**D**) Summary of all stimulation assays conducted, shown as percentage of IFN-γ–positive $CD8^+$ T cells in response to freshly isolated neutrophils loaded with peptide or the indicated concentrations of M1 protein (means ± SD, $n = 3$). Data were analyzed by two-way ANOVA with Bonferroni's post hoc tests. Differences were considered significant as indicated: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

latent viruses that are associated with high mortality rates (48, 49). Trials specifically targeted at reversing this apparent monocyte deactivation have shown promising clinical results (50). However, our present findings suggest that HLA-DR expression by circulating monocytes is a poor surrogate marker for a systemic immune suppression and rather indicate that, contrary to the proposed general loss of function, certain cells such as neutrophils may actually assume APC properties under such conditions, as evidence of a gain of new function. Yet, with a complex and multilayered clinical phenomenon such as sepsis it is challenging to dissect the relevance of APC-like neutrophils for infection resolution and clinical outcome in vivo.

With their unique ability to recognize microbial metabolites in a non-MHC–restricted manner, unconventional T cells such as Vγ9/Vδ2 T cells and MAIT cells greatly outnumber Ag-specific conventional $CD4^+$ and $CD8^+$ T cells at the site of infection and represent early and abundant sources of proinflammatory cytokines (23, 27), among which GM-CSF, IFN-γ, and TNF-α each make key contributions. Although conventional T cells may produce a similar combination of cytokines and provide similar signals to neutrophils, preliminary findings in our laboratory indicate that Vγ9/Vδ2 T cells and MAIT cells represent together up to 50% of all TNF-α–producing T cells among peritoneal cells stimulated with bacterial extracts, suggesting that these two cell types are indeed major producers of proinflammatory cytokines in response to microbial stimulation (A. Liuzzi and M. Eberl, unpublished observations). Although we cannot rule out a further contribution of contact-dependent mechanisms, this observation builds upon earlier studies describing the generation of human neutrophils expressing MHC class II through the action of recombinant cytokines in vitro (22, 51–56) and in vivo (57–59). Previous investigations reported an upregulation of MHC class II on activated neutrophils under the control of GM-CSF and IFN-γ, albeit the physiological source of those mediators during acute infection was not defined. Most importantly, in this study, we describe a direct role for TNF-α in the efficient induction of MHC class I–restricted $CD8^+$ T cell responses by neutrophils. Of note, plasma from sepsis patients was previously shown to induce some (upregulation of CD64), but not other features (upregulation of CD11b, loss of CD62L) (60) that are characteristic for unconventional T cell–primed neutrophils, indicating that circulating cytokines alone do not confer APC properties. In support of local cell-mediated processes at the site of inflammation, our findings evoke earlier descriptions of APC-like neutrophils characterized by MHC class II expression in infectious and noninfectious inflammatory scenarios such as periodontitis (17) and tuberculous pleuritis (20), in which locally activated Vγ9/Vδ2 T cells were found (61–63). These associations lend further support to the existence of a peripheral immune surveillance network comprised of distinct types of unconventional T cells and their crosstalk with

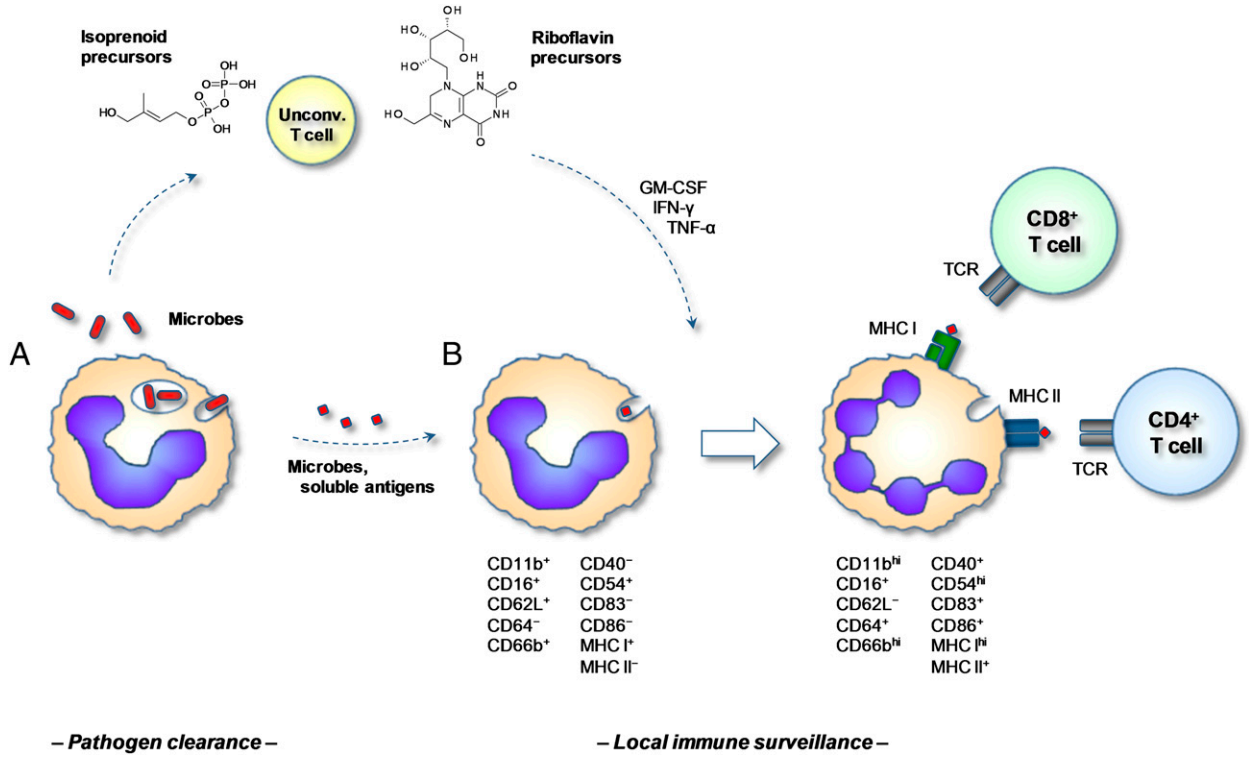

FIGURE 7. Proposed model for the local induction of APC-like neutrophils under the influence of microbe-responsive unconventional T cells. (**A**) Upon pathogen clearance, neutrophils release microbial metabolites into the microenvironment, where they stimulate local or freshly recruited unconventional T cells to release proinflammatory cytokines. (**B**) In the presence of unconventional T cell–derived mediators such as GM-CSF, IFN-γ, and TNF-α, bystander neutrophils acquire the capacity to act as APCs for tissue-resident and/or newly arriving effector and memory $CD4^+$ and $CD8^+$ T cells. Activated neutrophils may also gain access to inflamed draining lymph nodes and prime T cell responses in secondary lymphoid tissues (not depicted).

local immune and nonimmune cells. In the absence of unconventional T cell–derived signals, such as during sterile inflammation induced by LPS administration (64), neutrophils may not become fully primed, in accordance with our failure to induce APC-like neutrophils using LPS alone. Of note, a possible feedback regulation may require the activation of unconventional T cells to reach a certain threshold to overcome the inhibitory effect of bystander neutrophils (65–67).

Our present data demonstrate that both isoprenoid and riboflavin precursors are released by human neutrophils upon phagocytosis of live bacteria and depend on uptake by monocytes and loading onto butyrophilin 3A and MR1, respectively. The surprising similarities between Vγ9/Vδ2 T cells and MAIT cells illustrate their overlapping, yet distinct roles. Given the broad distribution of the nonmevalonate and riboflavin pathways across pathogenic, opportunistic, and commensal species, the vast majority of invading microbes is likely to be detected by either Vγ9/Vδ2 T cells or MAIT cells, or both. Our analysis of sepsis patients identified a systemic mobilization of Vγ9/Vδ2 T cells in response to HMB-PP–producing species, in support of their differential responsiveness to distinct groups of bacteria (28, 46). Because the present clinical study was conceived before information about the responsiveness of MAIT cells for riboflavin metabolites became available in the literature, we did not conduct a differential analysis for MAIT cells during acute sepsis. Of note, except for two cases of streptococcal infections, all bacterial and fungal pathogens identified in this patient cohort in fact possessed the riboflavin pathway, that is, were theoretically capable of stimulating MAIT cells. Intriguingly, Grimaldi et al. (68) recently reported a specific depletion of peripheral MAIT cells in sepsis patients with nonstreptococcal (i.e., riboflavin-producing) bacteria compared with infections caused by riboflavin-deficient species, which may indicate differences in the recruitment and retention of different types of unconventional T cells at sites of infection, depending on the nature of the causative pathogen and the underlying pathology (69–72). The contribution of tissue-resident and freshly recruited unconventional T cells to acute inflammatory responses has implications for clinical outcome and for the development of novel diagnostics and therapeutic interventions (46).

Taken together, our present study provides evidence 1) that Vγ9/Vδ2 T cells and MAIT cells respond similarly to microbial pathogens that produce the corresponding ligands when phagocytosed by human neutrophils, 2) that, once activated, both types of unconventional T cells trigger longer-term survival and differentiation of neutrophils into APC-like cells, 3) that unconventional T cell–primed neutrophils readily process exogenous Ags and prime both $CD4^+$ and $CD8^+$ T cells, and 4) that circulating neutrophils from patients with acute sepsis possess a similar APC-like phenotype and are capable of cross-presenting soluble proteins to Ag-specific $CD8^+$ T cells ex vivo. These findings define a possible physiological context for the generation of APC-like neutrophils in response to a broad range of microbial pathogens and imply a unique and decisive role for human unconventional T cells in orchestrating local inflammatory events and in shaping the transition of the innate to the adaptive phase of the antimicrobial immune response, with implications for diagnosis, therapy, and vaccination.

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Disclosures

The authors have no financial conflicts of interest.

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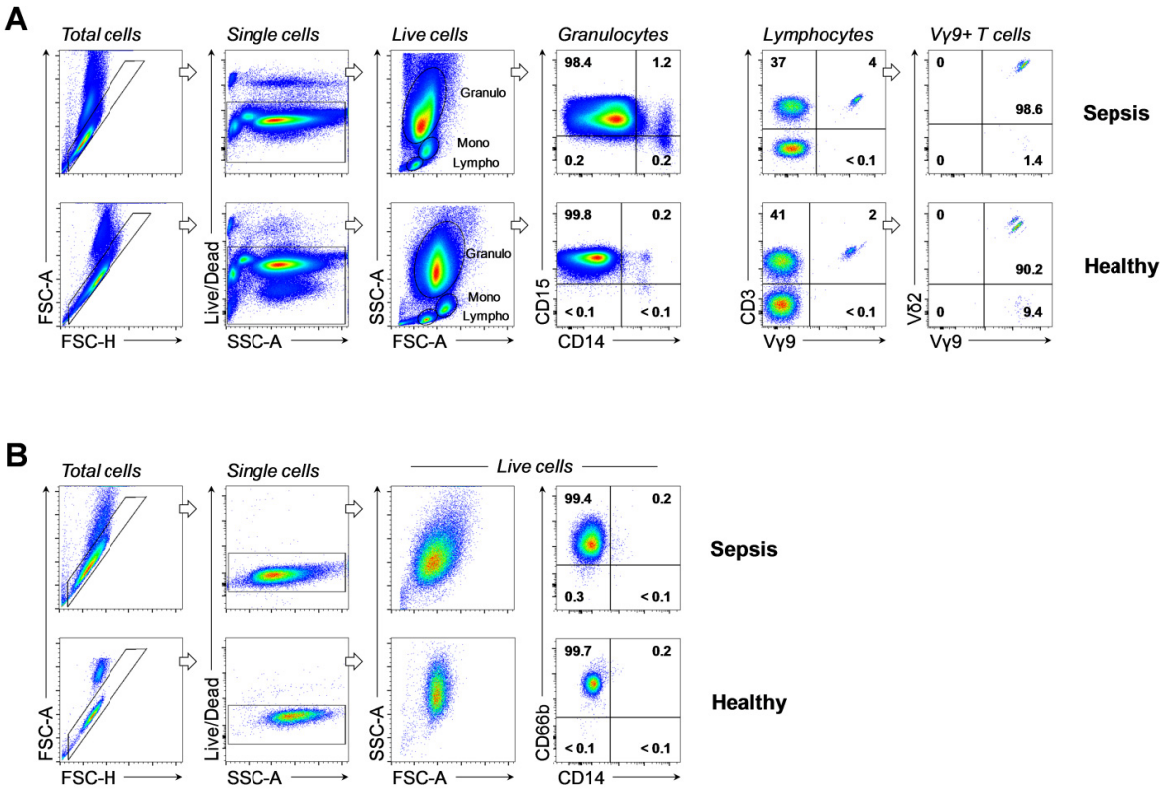
SUPPLEMENTAL INFORMATION



SUPPLEMENTAL FIGURE 1. Flow cytometric gating strategies for human neutrophils and Vγ9/Vδ2 T cells. (***A***) For immunophenotyping of peripheral leukocytes, whole white blood cells were isolated from healthy donors or patients with sepsis. Cells were gated on single live cells and segregated by their appearance in forward and side scatter. Neutrophils were positively identified by their surface expression of CD15 and lack of CD14. Vγ9/Vδ2 T cells were positively identified by their surface expression of CD3, TCR-Vγ9 and TCR-Vδ2. FACS plots are representative of 37 sepsis patients and 10 healthy donors. (***B***) For functional assays, neutrophils were isolated by negative selection from the blood of healthy donors or patients with sepsis, using the EasySep neutrophil enrichment kit that depletes all other human blood cells by specifically targeting CD2, CD3, CD9, CD19, CD36, CD56 and glycophorin A (StemCell Technologies). Purities were confirmed by the percentage of cells expressing CD66b but lacking CD14. FACS plots are representative of 5 sepsis patients and 15 healthy donors.

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Current Opinion in Immunology

Early innate responses to pathogens: pattern recognition by unconventional human T-cells

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Although typically viewed as a feature of innate immune responses, microbial pattern recognition is increasingly acknowledged as a function of particular cells nominally categorized within the adaptive immune system. Groundbreaking research over the past three years has shown how unconventional human T-cells carrying invariant or semi-invariant TCRs that are not restricted by classical MHC molecules sense microbial compounds via entirely novel antigen presenting pathways. This review will focus on the innate-like recognition of non-self metabolites by Vγ9/Vδ2 T-cells, mucosal-associated invariant T (MAIT) cells and germline-encoded mycolyl-reactive (GEM) T-cells, with an emphasis on early immune responses in acute infection.

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Introduction

The human body is constantly exposed to a vast array of microorganisms through contact with environmental species and interactions with commensals, opportunists and pathogens. This microbial bombardment exerts a perpetual evolutionary pressure on the immune system to identify and eliminate potentially dangerous agents. Microbes express a plethora of pathogen-associated molecular patterns that engage with various components of the human immune system, triggering rapid and distinct responses as a first-line defense against specific groups of organisms. The innate recognition of such patterns ultimately induces unique clusters of immune and tissue-related biomarkers that coalesce as pathogen-specific 'immune fingerprints' [1•,2], with widespread implications for point-of-care diagnosis of acute infection.

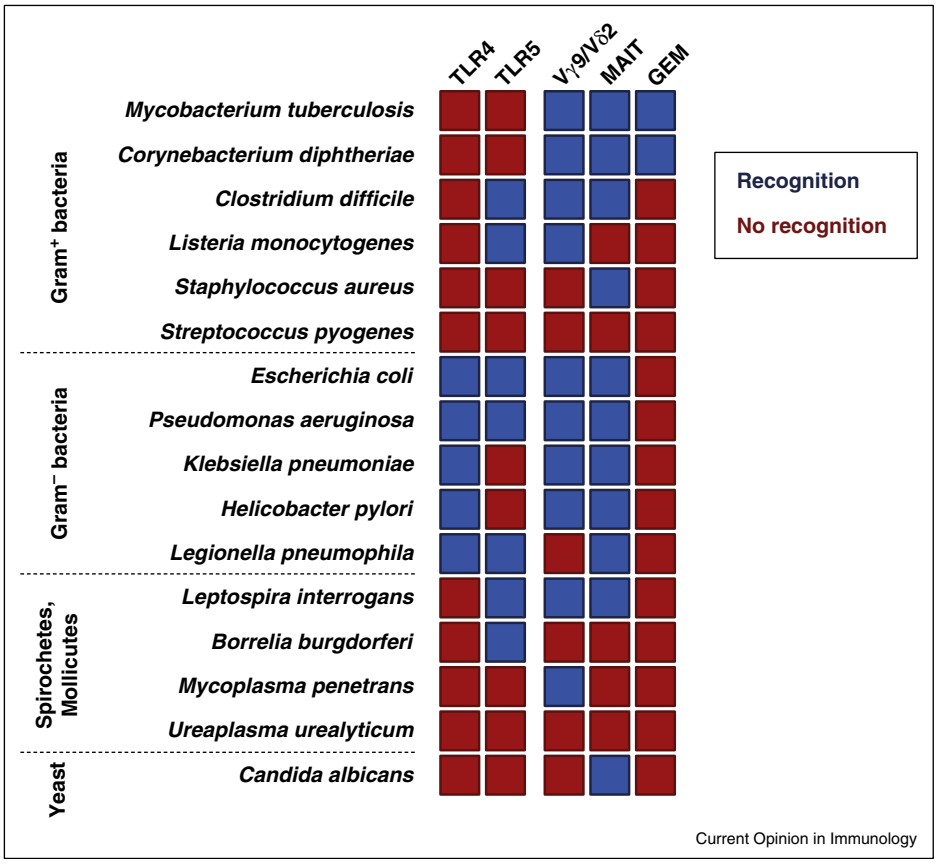
In the adaptive immune system, somatic recombination of V(D)J gene segments and junctional modifications generate a diverse repertoire of clonotypically expressed TCRs, enabling antigenic peptide-specific T-cell responses restricted by MHC class I and class II molecules. Although such genomic rearrangements occur in all T-cells, 'unconventional' populations characterized by semi-invariant, invariant or even germline-encoded TCRs are universally present and serve to recognize alternative antigens that are not restricted by classical MHC molecules. Research over the past three years has exposed how unconventional T-cells detect pathogens by sensing microbial, non-peptidic compounds via entirely novel antigen presenting pathways. High throughput sequencing approaches have also hinted at the existence of further unconventional T-cell subsets [3]. This review will focus primarily on the innate-like recognition of non-self metabolites by human Vγ9/Vδ2 T-cells, mucosal-associated invariant T (MAIT) cells and germline-encoded mycolyl-reactive (GEM) T-cells. The roles of other unconventional T-cells and iNKT cells in tissue homeostasis, stress surveillance and autoimmunity are well described elsewhere [4–6].

Unconventional T-cells: Not based on or conforming to what is generally done or believed (Oxford Dictionary)

Given the energetic costs of somatic recombination and thymic selection (largely unproven for unconventional T-cells), innate-like recognition by certain αβ and γδ T-cells must confer a crucial evolutionary advantage. In this respect, Vγ9/Vδ2 T-cells, MAIT cells and other unconventional T-cells effectively bridge the innate and adaptive immune systems by orchestrating acute inflammatory responses and driving the generation of antigen-presenting cells [7•,8,9]. Akin to the discrimination between 'self' and 'non-self' via TLR4-mediated recognition of lipopolysaccharide (LPS), a cell wall constituent of Gram-negative bacteria, and TLR5-mediated recognition of flagellin, a component of bacterial flagella, the metabolic pathways targeted by Vγ9/Vδ2 T-cells, MAIT cells and

[*] These authors contributed equally.

Figure 1

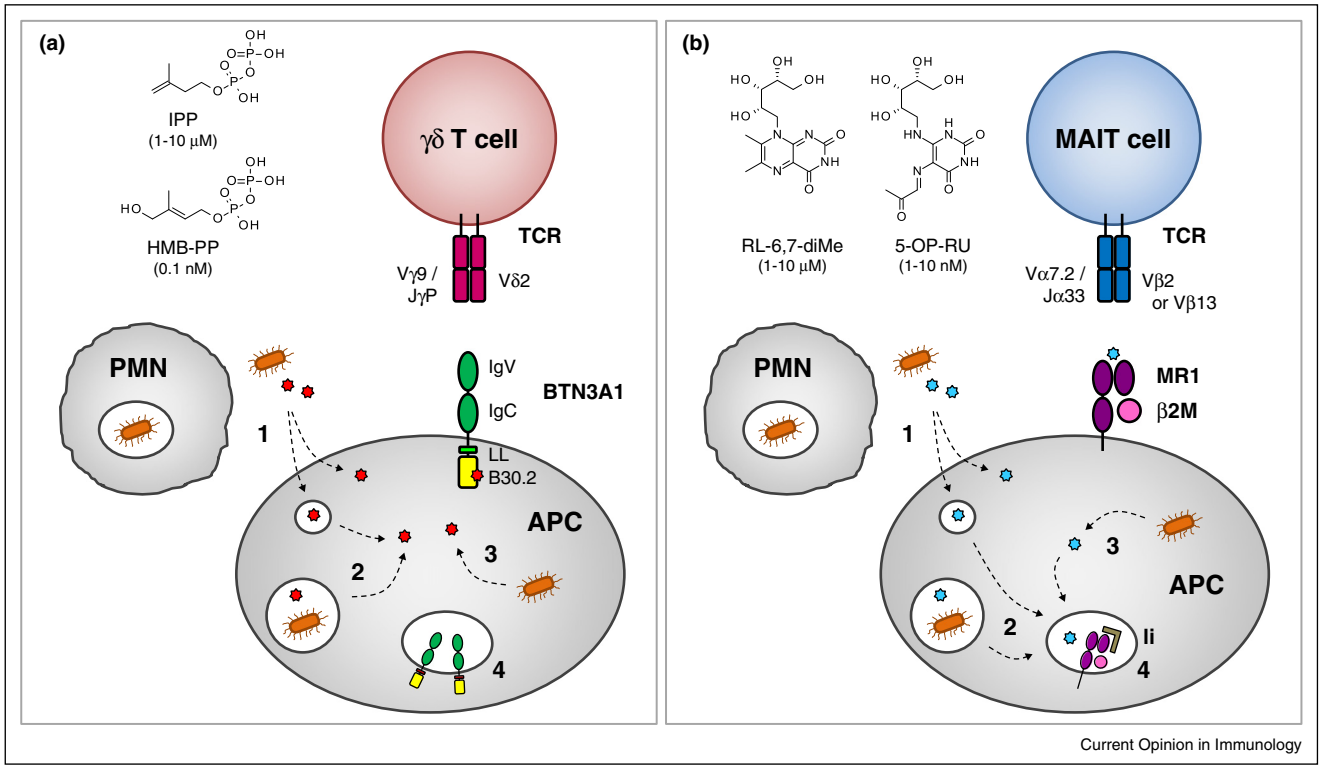


Innate sensing of microbial pathogens by Toll-like receptors and unconventional T-cell receptors. Pattern recognition of clinically relevant microbial pathogens via TLR4 and TLR5, and TCRs expressed by Vγ9/Vδ2 T-cells, MAIT cells and GEM T-cells. Blue symbols, recognition; red symbols, no recognition.

GEM T-cells incorporate key structures that allow the body to sense a wide range of potentially harmful microorganisms and trigger an inflammatory response aimed at effective pathogen control (Figure 1). These biochemical determinants are absent from human cells and include ligands derived from the non-mevalonate pathway, which generates the building blocks of all higher isoprenoids in most Gram-negative bacteria and many Gram-positive species (as well as the protozoa *Plasmodium falciparum* and *Toxoplasma gondii*) [10], components of the riboflavin pathway, which yields vitamin B2 in the vast majority of bacteria as well as yeasts and fungi [11], and certain long-chain fatty acids (mycolic acids) found exclusively in the cell wall of mycobacteria and coryneform bacteria [12••]. Similar principles govern the recognition of microbial α-linked glycolipids by iNKT cells [5,6]. Many unconventional T-cells also respond to cytokines such as IL-1β, IL-12, IL-18 and IL-23 in a TCR-independent manner, and may therefore act similarly to NK cells and other innate lymphoid cells [13–15].

To facilitate innate sensing of microbial pathogens, unconventional human T-cells are thought to undergo extrathymic and presumably antigen-driven expansion in the periphery, consistent with a predominant central or effector memory phenotype and the capacity to mount rapid responses. Unconventional T-cells also localize frequently to specific tissues and may therefore play a role in local immune surveillance. Intriguingly, human Vγ9/Vδ2 T-cell and MAIT cell numbers increase in peripheral blood after birth and subsequently decline in older individuals; they are also more prevalent in women [16,17]. These observations could reflect age-related and gender-dependent exposure to environmental, commensal and/or pathogenic species. However, recent studies show that both Vγ9/Vδ2 T-cells and MAIT cells acquire their anti-microbial responsiveness during fetal development, prior to contact with environmental microbes and commensal microflora [18•,19•]. Of note, an age-related decline associated with changes in peripheral subset composition has also been reported for iNKT cells [20].

Figure 2



Recognition of microbial metabolites by unconventional T-cells. **(a)** 'Presentation' of HMB-PP to the Vγ9/Vδ2 TCR in a BTN3-dependent manner: *1*, Uptake of soluble HMB-PP released by extracellular bacteria, phagocytes or infected cells, via endocytosis and/or active/passive transport across the cell membrane (e.g. *E. coli*); *2*, Transport of HMB-PP from endocytic vesicles across the membrane after phagocytosis or infection (e.g. *Mycobacterium tuberculosis*); *3*, Release of HMB-PP into the cytosol by intracellular pathogens (e.g. *Salmonella* spp.); *4*, Putative intracellular loading compartment for BTN3. High affinity ligand: HMB-PP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; low affinity ligand: IPP, isopentenyl pyrophosphate. Note that HMB-PP is a microbial metabolite, whereas IPP is present in all prokaryotic and eukaryotic cells. **(b)** Presentation of vitamin B2 metabolites to the MAIT TCR by MR1: *1*, Uptake of soluble vitamin B2 metabolites released by extracellular bacteria, phagocytes or infected cells; *2*, Shuttling of vitamin B2 metabolites to late endosomes; *3*, Release of vitamin B2 metabolites into the cytosol; *4*, MR1 loading compartment. High affinity ligand: 5-OP-RU, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil; low affinity ligand: RL-6,7-diMe, 6,7-dimethyl-8-D-ribityllumazine. APC, antigen-presenting cell; PMN, polymorphonuclear cell; IgV, immunoglobulin-like V ectodomain; IgC, immunoglobulin-like C ectodomain; LL, di-leucine motif; β2M, β_2-microglobulin; Ii, MHC class II-associated invariant chain (CD74).

Vγ9/Vδ2 T-cells: Antigen presentation without presentation of an antigen?

Peripheral blood Vγ9/Vδ2 T-cells carrying a *TRGV9*/*TRGJP*-encoded TCRγ chain normally constitute 1–5% of the circulating T-cell population in humans but can increase in frequency to >50% during microbial infections. Despite their prevalence in blood, these cells mobilize rapidly to mucosal surfaces, where they may confer protection against tissue-localized infections [21]. Vγ9/Vδ2 T-cells display a striking responsiveness to bacterial species capable of producing the isoprenoid precursor (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) *in vitro* and *in vivo* [9,22] (Figure 2). In patients infected with a range of pathogens, HMB-PP-producing organisms are associated with higher Vγ9/Vδ2 T-cell frequencies than HMB-PP-deficient species. This appears to be true both for local responses at the site of infection, as demonstrated in patients with acute bacterial peritonitis [1•,10], and for systemic responses during acute sepsis [7•]. These clinical observations are backed up by studies in macaques, where an HMB-PP-deficient vaccine strain of *Listeria monocytogenes* elicited significantly reduced pulmonary and systemic Vγ9/Vδ2 T-cell responses compared with the HMB-PP-producing parental strain [23•]. Similarly, an HMB-PP-overproducing vaccine strain of *Salmonella enterica* serovar Typhimurium stimulated prolonged Vγ9/Vδ2 T-cell expansions in rhesus monkeys, while the avirulent parental strain was less efficient in this respect [24].

The unique responsiveness of Vγ9/Vδ2 T-cells to HMB-PP remains enigmatic as it appears to embody the only case where an antigen is not actually presented to the TCR but instead binds intracellularly to an innate sensor (butyrophilin 3; BTN3/CD277) (Figure 2). Although Vγ9/Vδ2 T-cells are generally portrayed as a population

unique to primates and absent in rodents, immunogenetic studies point to a co-evolution of the Vγ9 and Vδ2 genes together with *BTN3* in other placental mammals such as alpacas [25]. However, functional proof for the presence of HMB-PP-specific and BTN3-dependent Vγ9/Vδ2 T-cells in these species is still lacking. Following the pioneering discovery of BTN3 as a restriction element for human Vγ9/Vδ2 T-cell responses [26,27••] and the observation that anti-BTN3 agonist antibodies and soluble phosphoantigens induce identical signaling pathways [28], at least four independent studies have provided evidence for direct binding of HMB-PP to a positively charged pocket in the cytosolic B30.2 (PRYSPRY) domain of BTN3A1 [29••,30,31•,32•]. These findings contradict an alternative model proposing that HMB-PP binds to the extracellular IgV domain of BTN3A1 and is therefore a truly presented antigen [27••]. Despite this convergence of experimental data, it remains entirely unclear how the Vγ9/Vδ2 TCR actually recognizes the BTN3A1/HMB-PP complex. Inside the cell, HMB-PP sensing might be accompanied by interaction partners such as periplakin, which binds a dileucine motif located proximal to the cytoplasmic B30.2 domain of BTN3A1 [32•]. These intracellular events may subsequently propagate across the cell membrane and induce conformational changes on the cell surface [33], possibly in combination with co-factors or clustering effects that enable recognition by Vγ9/Vδ2 TCRs. Nevertheless, it is challenging to reconcile how the detection of a seemingly ubiquitous and non-polymorphic molecule such as BTN3A1 is facilitated through a rearranged TCR.

MAIT cells: Unconventional pathogen-sensing through conventional TCR diversity?

MAIT cells are innate-like T-cells that populate mucosal tissues such as the intestine and lung, comprising in addition up to 10% of the circulating $CD8^+$ αβ T-cell compartment and as many as half of all T-cells present in the liver [34]. They share phenotypic similarities with iNKT cells, express IL-12Rβ2 and IL-18Rα alongside high levels of CD161 [14,35], and localize to sites of infection via chemokine receptors such as CCR2, CCR6 and CXCR6 [35,36]. Unlike conventional αβ T-cells, MAIT cells possess thymic effector functionality despite a naïve phenotype [34] and are selected by hematopoietic cells [37]. They subsequently expand in the periphery as antigen-experienced, effector memory T-cells upon microbial exposure [36,37]. Of note, the presence of an intact commensal flora and expression of the non-polymorphic MHC-related protein 1 (MR1) by B-cells are both essential for this peripheral expansion, whereas macrophages and dendritic cells are dispensable [37]. Recent data have also revealed an essential role for STAT3 signaling downstream of IL-21R and IL-23R in controlling human MAIT cell numbers [38•].

Pathogen recognition by human MAIT cells is driven by a semi-invariant MR1-restricted TCR that typically incorporates a TRAV1-2/TRAJ33 (Vα7.2-Jα33) TCRα chain paired predominantly with a TRBV20-1 (Vβ2) or TRBV6 (Vβ13) TCRβ chain. Infrequent usage of other *TRAJ* and *TRBV* gene segments has also been described [3,39]. Unlike MHC class I-restricted epitopes, MAIT cell ligand presentation by MR1 is independent of proteasomal degradation and the transporter associated with antigen processing (TAP), but requires the MHC class II chaperones invariant chain (Ii) and HLA-DM [40]. After initial observations that both human and murine MAIT cells respond to species such as enterobacteria, staphylococci and mycobacteria, but not to streptococci [7•,36], key mechanistic advances have shown that MAIT TCRs recognize ligands derived from microbial vitamin B2 metabolism [11,41,42••] (Figure 2). Recent analyses have also revealed that the MAIT cell repertoire is more diverse than initially thought [43,44•], which may allow these cells to discriminate between different microbial pathogens via TCR-dependent 'sensing' of distinct MR1-bound ligands [39,44•]. These findings suggest the existence of other, as yet undiscovered, microbial antigens within the MAIT cell recognition spectrum, a possibility consistent with structural interpretations of MR1 ligand promiscuity [41,45–47]. However, a recent study in mice has challenged this idea of ligand discrimination via the TCRβ chain [48], which may point to species-specific differences between human and murine MAIT cells.

Patients with severe sepsis display an early decrease in circulating MAIT cells compared with healthy controls and uninfected critically ill patients [49•]. In particular, non-streptococcal bacterial infection was identified as an independent determinant of peripheral MAIT cell depletion, suggesting recruitment to the site of infection in response to pathogens with an intact riboflavin pathway [36,50,51]. In HIV-1 infection, circulating $Vα7.2^+$ $CD161^+$ T-cells are depleted and fail to recover with antiretroviral therapy [52,53]. This may indicate a progressive translocation of MAIT cells to peripheral tissues, down-regulation of CD161, functional exhaustion and/or activation-induced apoptosis. In a number of autoimmune and metabolic disorders, MAIT cells typically display similarly decreased levels in peripheral blood [54–56], possibly as a result of low-grade inflammation and alterations of the microbiota.

Other pathogen-specific unconventional T-cells: GEM T-cells and beyond

The MHC class I-related molecule CD1b was found almost 20 years ago to present bacterial glycolipids such as glucose monomycolate (GMM), yet the identity and specificity of CD1b-restricted T-cells has remained elusive until recently [57]. Mycolic acids (MAs) are the predominant cell wall lipids in *Mycobacterium tuberculosis* and represent a major virulence factor for this pathogen. Rare MA-specific T-cells are detectable in tuberculosis

patients at diagnosis but virtually absent in non-infected BCG-vaccinated individuals [58]. These T-cells are CD1b-restricted, exhibit both central and effector memory phenotypes, produce IFN-γ and IL-2 upon stimulation, and appear to localize preferentially at the site of infection. The availability of CD1b tetramers allowed direct visualization of MA-specific T-cells, which were estimated to comprise approximately 0.01% of all circulating T-cells [59]. These advances eventually led to the description of CD1b-restricted T-cells as Vα7.2$^+$ CD4$^+$ germline-encoded mycolyl-reactive (GEM) T-cells, which carry an invariant TRAV1-2/TRAJ9 TCRα chain [12••]. MA-specific T-cells were also shown to decline after successful treatment and therefore appear to correlate with pathogen burden [58], emphasizing the potential importance of these unconventional T-cells as novel diagnostic and prognostic biomarkers of tuberculosis.

As mycolic acids are a hallmark of all Corynebacteriales, it is tempting to speculate that MA-specific T-cells may also sense infections caused by bacteria such as *Corynebacterium* spp. and *Nocardia* spp. (Figure 1). Of note, a second population of GMM-specific T-cells has been identified recently. These cells exhibit lower avidities for CD1b tetramers and, in contrast to GEM T-cells, express TCRs with a marked preference for the *TRAV17* and *TRBV4-1* genes [60]. High throughput sequencing of TRAV1-2$^+$ TCRα chains further suggests that we are only seeing the tip of the iceberg with regard to our knowledge of unconventional T-cell populations [3]. It therefore seems likely that many exciting discoveries will ensue in this hybrid field.

Conclusions and future directions

The last three years have witnessed major advances in our understanding of unconventional T-cell subsets, in part due to the skillful application of cutting-edge experimental techniques to well-defined patient cohorts. Future research can now build on this foundation to define the true extent of these T-cell populations and define the mechanisms that underlie microbial pattern recognition within the adaptive immune system. Many questions remain in this regard. Precisely how do unconventional TCRs interact with non-polymorphic presenting molecules? Are specific gene segments within the TCR locus conserved for this purpose? Does the process of somatic recombination serve to diversify bound ligand recognition? Do unconventional T-cells undergo positive selection in the thymus and does this process involve the engagement of endogenous ligands? What are the molecular processes involved in antigen uptake and intracellular trafficking that allow the presentation of microbial metabolites?

Key pieces of the puzzle are also missing at the functional level. How do unconventional T-cells migrate to and from sites of infection? Do they persist as tissue-resident memory-like cells after pathogen clearance? What is the role of the commensal microbiota? Why do most unconventional T-cells possess a memory phenotype from early life? What mechanisms underlie the pronounced age and gender bias? Are there implications for homeostasis and susceptibility to infections, autoimmunity and malignancy? How do accessory molecules such as CD4, CD8, CD161 and NKG2D contribute in this setting?

It is becoming increasingly clear that unconventional T-cells play a pivotal role in the orchestration of early inflammatory responses. In parallel, emerging mechanistic insights have started to unlock the secrets of innate-like recognition encoded by specific portions of the TCR repertoire. The highly constrained genetic and microbial elements inherent within each of these various systems potentially offer unique molecular targets for the development of novel and universally applicable diagnostics, vaccines and immunotherapeutics. The overarching question is therefore, as always, a humanitarian one. How can we best harness the unique attributes of unconventional T-cells to combat the infectious and malignant plagues of our times?

Conflict of interest statement

The authors declare no competing financial interests.

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Unconventional human T-cells accumulate at the site of infection in response to microbial ligands and induce local tissue remodeling

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Running title: Local γδ and MAIT cell responses in microbial infection

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ABSTRACT

The anti-microbial responsiveness and function of unconventional human T-cells are poorly understood, with only limited access to relevant specimens from sites of infection. Peritonitis is a common and serious complication in individuals with end-stage kidney disease receiving peritoneal dialysis (PD). By analyzing local and systemic immune responses in PD patients presenting with acute bacterial peritonitis and monitoring individuals before and during defined infectious episodes, our data show that Vγ9/Vδ2^{+} γδ T-cells and mucosal-associated invariant T (MAIT) cells accumulate at the site of infection with organisms producing (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) and vitamin B2, respectively. Such unconventional human T-cells are major producers of IFN-γ and TNF-α in response to these ligands that are shared by many microbial pathogens, and affect the cells lining the peritoneal cavity by triggering local inflammation and inducing tissue remodeling with consequences for peritoneal membrane integrity. Our data uncover a crucial role for Vγ9/Vδ2 T-cells and MAIT cells in bacterial infection, and suggest that they represent a useful predictive marker for important clinical outcomes, which may inform future stratification and patient management. These findings are likely to be applicable to other acute infections where local activation of unconventional T-cells contributes to the anti-microbial inflammatory response.

INTRODUCTION

The classical view of the immune response to infection is based on the assumption that 'innate' immune cells sense pathogens via pattern recognition receptors such as the Toll-like receptors (TLRs), while antigen-specific 'adaptive' T-cell responses are restricted by molecules of the major histocompatibility complex (MHC). This simplistic model is being eroded by the growing realization that substantial numbers of T-cells in the body are non-MHC restricted and integrate innate and adaptive features [1,2]. Such innate-like T-cells include γδ T-cells, mucosal-associated invariant T (MAIT) cells, natural killer T (NKT) cells, germline-encoded mycolyl-reactive (GEM) T-cells and other CD1-restricted T-cells [3-8] . Together, these 'unconventional' T-cell populations comprise a sizeable proportion of all T-cells in blood, epithelia, organs such as the liver, and inflamed tissues. Sensing non-peptide antigens by unconventional T-cells endows the body with the capacity to respond to a plethora of foreign and self molecules produced by invading pathogens or released by stressed, infected or metabolically active tissues. In humans, microbial organisms sensed by one or more of these T-cell subsets include the causative agents of tuberculosis, malaria and most hospital-acquired bacterial infections [9].

There is an increasing appreciation of the role played by unconventional T-cells in orchestrating early cellular events in response to invading pathogens, which is likely to contribute to microbial clearance and the development of immunological memory, but which may also result in inflammation-associated tissue damage [1,9-11]. Progress in the understanding of unconventional T-cell biology has been hampered by their responsiveness to relatively poorly defined structures, and the paucity of appropriate experimental research tools. These cells also display striking species-specific differences with respect to their TCR repertoires, activities and anatomical locations, with the restricting elements butyrophilin-3 (BTN3), CD1a, CD1b and CD1c as well as the corresponding T-cell subpopulations being

absent in mice [1,8,9,12]. As a consequence, no small animal model replicates the complex interactions between unconventional T-cells and other immune and non-immune cells in the human body.

The characterization of unconventional T-cell responses *in vivo* and their relevance for homeostasis, immune surveillance and inflammation remains challenging [3-9]. In particular, the microbial and environmental signals that lead to the migration, differentiation, expansion and maintenance of unconventional T-cells under physiological conditions are poorly defined. Studies into human responses during acute infections are notoriously difficult to undertake, with only limited access to relevant specimens, in particular from the site of infection, and to matched samples collected before disease onset. We here addressed this knowledge gap by studying a well-defined cohort of individuals receiving peritoneal dialysis (PD) and presenting with acute peritonitis. PD is a live-saving treatment for people with end-stage kidney disease that permits immunological investigations with direct clinical relevance, where a permanently inserted catheter affords continuous and non-invasive access to localized responses to a range of bacterial species [13-15]. We recently reported elevated numbers of Vγ9/Vδ2 T-cells in a cross-sectional cohort of PD patients with acute peritonitis, particularly in those with infections caused by Gram-negative bacteria and (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) producing bacteria [14-15]. However, it is unclear whether those elevated numbers are due to preferential recruitment of Vγ9/Vδ2 T-cells to the peritoneal cavity in certain infections, and/or a result of ligand-specific local activation and expansion in response to the respective pathogens.

Our present data show that both Vγ9/Vδ2 T-cells and MAIT cells specifically accumulate at the site of infection in response to organisms producing HMB-PP and vitamin B2, respectively, and have the capacity to activate local tissues with consequences for acute inflammation, peritoneal membrane integrity and clinical outcomes. In a wider context, the present study

demonstrates the power of using PD as an experimental and clinical model for monitoring individuals before, during and after defined microbial infections.

MATERIALS AND METHODS

Study approval. Recruitment of PD patients and healthy volunteers for this study was approved by the South East Wales Local Ethics Committee under reference numbers 04WSE04/27 and 08/WSE04/17, respectively, and conducted according to the principles expressed in the Declaration of Helsinki. All individuals provided written informed consent. The PD study was registered on the UK Clinical Research Network Study Portfolio under reference numbers #11838 "Patient immune responses to infection in Peritoneal Dialysis" (PERIT-PD) and #11839 "Leukocyte phenotype and function in Peritoneal Dialysis" (LEUK-PD). Fresh omentum samples from consented patients were obtained from the Wales Kidney Research Tissue Bank.

Patient samples. The local study cohort comprised 101 adults PD patients admitted to the University Hospital of Wales, Cardiff, on day 1 of acute peritonitis between September 2008 and April 2016. 41 stable individuals receiving PD for at least 3 months and with no previous infection served as non-infected controls. Subjects known to be positive for HIV or hepatitis C virus were excluded. Clinical diagnosis of acute peritonitis was based on the presence of abdominal pain and cloudy peritoneal effluent with >100 white blood cells/mm^3. According to the microbiological analysis of the effluent by the routine Microbiology Laboratory, Public Health Wales, episodes of peritonitis were defined as culture-negative (with unclear etiology) or as confirmed bacterial infections caused by specific subgroups of Gram-positive and Gram-negative organisms. The distribution of the non-mevalonate (HMB-PP) and vitamin B2 pathways across microbial species was determined based on the absence or presence of the enzymes HMB-PP synthase (EC 1.17.7.1) and 6,7-dimethyl-8-D-ribityllumazine (DMRL) synthase (EC 2.5.1.78), respectively, in the corresponding genomes, according to the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg). Cases of fungal infection and mixed or unclear culture results were excluded from this analysis.

Outcome analysis. Microbiological and clinical outcome data were obtained from 5,071 adult patients of the Australia and New Zealand Dialysis Transplant (ANZDATA) Registry who developed first-time peritonitis between April 2003 and December 2012, excluding cases of fungal infection, polymicrobial infection or unrecorded culture results. Endpoints of outcome analyses were 30 day mortality and measurements of overall technique failure including catheter removal, interim transfer to hemodialysis and permanent transfer to hemodialysis over 90 days.

Media, reagent and antibodies. Peritoneal leukocytes were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 1% sodium pyruvate, 50 µg/ml penicillin/streptomycin and 10% fetal calf serum (Life Technologies). Mesothelial cells were cultured in Earle's buffered Medium 199 (Life Technologies) containing 10% fetal calf serum, peritoneal fibroblasts were cultured in a 1:1 (*v*/*v*) mixture of DMEM and Ham's F-12 nutrients (Life Technologies) with 20% fetal calf serum; both media were supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Life Technologies) as well as 5 µg/ml transferrin, 5 µg/ml insulin and 0.4 µg/ml hydrocortisone (all from Sigma-Aldrich). Synthetic (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) was provided by Dr Hassan Jomaa, University of Giessen, Germany; synthetic 6,7-dimethyl-8-D-ribityllumazine (DMRL) by Dr Boris Illarionov, Hamburg School of Food Science, Germany. Biotinylated human MR1:rRL-6-CH2OH (active ligand) and MR1:6-FP (negative control) monomers were provided by Dr Lars Kjer-Nielsen, University of Melbourne, Australia, and reconstituted as described before [16]. Recombinant IFN-γ, TNF-α and IL-1β were purchased from Miltenyi. Human T-Activator CD3/CD28 Dynabeads were purchased from Life Technologies. Blocking reagents used included anti-BTN3 (103.2; Dr Daniel Olive, Université de la Méditerranée, Marseille, France); anti-MR1 (26.5; Dr Ted Hansen, Washington University School of

Medicine, St. Louis, Missouri, USA); anti-IFN-γ (B27) and anti-IL-1β (H1b-27) (Biolegend); and sTNFR p75-IgG1 fusion protein (etanercept/Enbrel; Amgen).

Bacteria. Clinical isolates of *Escherichia coli* (Gram$^-$ HMB-PP$^+$ vit.B2$^+$), *Klebsiella pneumoniae* (Gram$^-$ HMB-PP$^+$ vit.B2$^+$), *Pseudomonas aeruginosa* (Gram$^-$ HMB-PP$^+$ vit.B2$^+$), *Corynebacterium striatum* (Gram$^+$ HMB-PP$^+$ vit.B2$^+$), *Listeria monocytogenes* (Gram$^+$ HMB-PP$^+$ vit.B2$^-$), *Staphylococcus aureus* (Gram$^+$ HMB-PP$^-$ vit.B2$^+$), *Streptococcus pneumoniae* (Gram$^+$ HMB-PP$^-$ vit.B2$^-$) and *Enterococcus faecalis* (Gram$^+$ HMB-PP$^-$ vit.B2$^-$) were grown in LB broth, harvested at an OD_{600} of 0.5-0.8, and sonicated in 1/10 (*v*/*v*) PBS, pH 8.0. Insoluble debris was removed by centrifugation, the supernatants were passed through 0.1 μm sterile filter units (Millipore), and the protein concentrations were determined using the BCA protein assay kit (Pierce). Low molecular weight fractions were obtained using cellulose filters with a molecular mass cut-off of 3 kDa (Millipore). Bacterial extracts were used in cell culture at dilutions corresponding to protein concentrations of the original samples (before 3 kDa filtration) of 60-100 μg/ml.

T cells. PBMC were isolated from peripheral blood of healthy volunteers using Lymphoprep (Axis-Shield). Vγ9$^+$ T-cells (>98%) were isolated from PBMC using monoclonal antibodies (mAbs) against Vγ9-PECy5 (Beckman Coulter) and anti-PE magnetic microbeads (Miltenyi); Vα7.2$^+$ T-cells (>98%) were isolated using anti-Vα7.2-APC (Biolegend) and anti-APC microbeads (Miltenyi). To generate unconventional T-cell conditioned medium, purified blood Vγ9/Vδ2 T-cells and MAIT cells were incubated for 24 hours in the presence of 10 nM HMB-PP and anti-CD3/CD28 dynabeads at 0.5 beads/cell, respectively. Human peritoneal leukocytes were harvested from overnight dwell effluents of stable PD patients [13], and cultured in the absence or presence of 1-100 nM HMB-PP, 100 μM DMRL or bacterial extracts at dilutions corresponding to protein concentrations of 60-100 μg/ml. For blocking

experiments, anti-BTN3 and anti-MR1 were used at 10 μg/ml and added 30 min before stimulating the cells.

Mesothelial cells and peritoneal fibroblasts. Human peritoneal mesothelial cells were obtained from fresh omental samples after two cycles of tissue digestion in the presence of trypsin (15 min each); peritoneal fibroblasts were obtained after a third digestion cycle lasting 1 hour [17-19]. All data presented are from experiments performed with confluent mesothelial cells and fibroblasts between the first and third passage. Mesothelial cells were growth arrested for 48 hours in serum-free medium prior to treatment, fibroblasts in medium containing 0.2% FCS. After starvation, cells were exposed for 24 hours to T-cell conditioned medium at the indicated dilutions; recombinant TNF-α and IFN-γ were used as controls. Cell-free peritoneal effluent from stable and infected patients (n=3-4) was added to cell cultures at a dilution of 1:4. In blocking experiments, T-cell conditioned medium or peritoneal effluent were pre-treated for 30 min with anti-IFN-γ, anti-IL-1β and sTNFR, either alone or in combination at 10 μg/ml. Supernatants were harvested and assessed by ELISA; cells were analyzed by qPCR.

Flow cytometry. Cells were acquired on an eight-color FACSCanto II (BD Biosciences) and analyzed with FlowJo 10.1 (TreeStar), using monoclonal antibodies against CD3 (SK7), CD69 (FN50), CCR4 (1G1), CCR5 (2D7) and CCR6 (11A9) from BD Biosciences; anti-TCR-Vγ9 (Immu360) from Beckman Coulter; anti-CD161 (HP-3G10), CCR2 (K036C2) and anti-TCR-Vα7.2 (3C10) from Biolegend; together with appropriate isotype controls. Anti-mouse antibodies reactive beads were used to set compensation (Life Technologies). Intracellular cytokines were detected using anti-IFN-γ (B27; Biolegend) and anti-TNF-α (188; Beckman Coulter). For detection of intracellular cytokines, 10 μg/ml brefeldin A (Sigma) was added to cultures 5 hours prior to harvesting. Leukocyte populations were gated based on their appearance in side scatter and forward scatter area/height and exclusion of live/dead staining

(fixable Aqua; Invitrogen). Unless stated otherwise, peritoneal γδ T-cells were defined as $V\gamma9^+$ $CD3^+$ lymphocytes. Peritoneal MAIT cells were defined as $V\alpha7.2^+$ $CD161^+$ $CD3^+$ lymphocytes; control stainings using MR1 tetramers as reference confirmed the validity of this approach (data not shown).

ELISA. Cell-free peritoneal effluents were analyzed on a SECTOR Imager 6000 (Meso Scale Discovery) for IFN-γ, TNF-α, IL-1β, CCL3, CCL4 and CXCL8. Conventional ELISA kits and a Dynex MRX II reader were used for CCL2 (eBioscience) and CCL20 (R&D Systems). Cell culture supernatants were analyzed using conventional ELISA kits for IFN-γ (Biolegend), TNF-α and CCL2 (eBioscience) as well as for CXCL8, CXCL10 and IL-6 (R&D Systems).

Real-time PCR. Total RNA was isolated from mesothelial cells cultured under the indicated conditions using Trizol (Invitrogen). cDNA was generated from 0.5 µg of RNA using the high capacity cDNA reverse transcription kit (Thermo Fisher), 100 mM dNTPs, 40 U/µl RNase inhibitor (New England Biolabs), 50 U/µl MultiScribe reverse transcriptase and 1 × random primers, according to the manufacturer's recommendations. Quantitative PCRs were run on a ViiA7 real-time PCR system (Thermo Fisher), using the power SYBR green PCR master mix (Thermo Fisher) and 300 nM forward and reverse primers: TCCCAATACATCTCCCTTCACA and ACCCACCTCTAAGGCCATCTTT for E-cadherin; TAAATCCACGCCGGTTCCTGAAGT and AGGTGTCTCAAAGTTACCACCGCT for occludin; CCGAGGTTTTAACTGCGAGA and TCACCCACTCGGTAAGTGTTC for fibronectin; CGAGCCCACCGGGAACGAAA and GGACCGAAGGCGCTTGTGGAG for IL-6; CCTCTGACTTCAACAGCGACAC and TGTCATACCAGGAAATGAGCTTGA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH); TTTACCTTCCAGCAGCCCTA and GGACAGAGTCCCAGATGAGC for Snail; AACTGGGACGACATGGAAA and AGGGTGGGATGCTCTTCAG for α-smooth muscle actin (α-SMA); GGAGAGGTGTTCCGTGTTGT and GGCTAGCTGCTCAGCTCTGT for zona occludens-1

(ZO-1); CGGGTTGCTTGCAATGTGC and CCGGCGACAACATCGTGAC for claudin-1. 10 ng of total RNA were used for cellular microRNAs using the Taqman Universal Master Mix II and specific primers for miR-21 and miR-191 (Applied Biosystems). mRNA and microRNA expression levels were normalized to the endogenous controls GAPDH and miR-191, respectively.

Statistics. Statistical analyses were performed using GraphPad Prism 6.0 software. Data distributions were analyzed using D'Agostino-Pearson omnibus normality tests. Data were analyzed using two-tailed Student's *t*-tests for normally distributed data and two-tailed Mann-Whitney tests for non-parametric data. Differences between groups were analyzed using one way ANOVA with Holm-Sidak's post tests for multiple comparisons of parametric data, or Kruskal-Wallis tests combined with Dunn's post tests for non-parametric data. Matched data were analyzed using paired *t*-tests or Wilcoxon matched pairs tests for two groups, or Friedman tests combined with Dunn's multiple comparisons tests for more than two groups. Differences were considered statistically significant as indicated in the figures and tables: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

RESULTS

Blood unconventional T-cells migrate early into the inflamed peritoneal cavity, at the time of peak neutrophil influx. Acute disease is characterized by a considerable influx of leukocytes to the site of infection, together with locally elevated levels of inflammatory mediators. Here, the effluent of PD patients presenting with acute peritonitis, before commencing antibiotic treatment, contained increased levels of total cells and of chemokines like the neutrophil-attracting molecule CXCL8 (IL-8), compared to effluent from stable non-infected PD patients (**Figure 1A**). Absolute numbers of both Vγ9/Vδ2 T-cells and MAIT cells were increased during acute peritonitis compared to stable patients (**Figure 1B**), indicating rapid co-recruitment of unconventional T-cells from blood to the inflamed peritoneal cavity, alongside neutrophils. These observations were in agreement with the migratory profiles of these cells. Circulating Vγ9/Vδ2 T-cells and MAIT cells in stable PD patients preferentially expressed the chemokine receptors CCR2, CCR5 and CCR6, compared to their non-Vγ9 and non-MAIT counterparts (**Figs. 1C+D**). We detected markedly increased levels of the corresponding chemokines CCL2 (CCR2 ligand), CCL3 and CCL4 (CCR5 ligands) and CCL20 (CCR6 ligand) in early peritonitis (**Figure 1E**), demonstrating a substantial production of inflammatory chemokines with the potential to attract unconventional T-cells to the site of infection, thereby complementing the local pool of tissue-resident Vγ9/Vδ2 T-cells and MAIT cells. In accordance, local frequencies of Vγ9/Vδ2 T-cells and MAIT cells during peritonitis were generally higher than in blood (**Figure 2A**). No systemic increase in blood unconventional T-cell levels was seen in patients with peritonitis, in support of the locally confined nature of the infection.

Unconventional T-cells are locally enriched during acute infections caused by bacterial pathogens producing the corresponding ligands. To avoid confounding resulting from the considerable biological variations between people and the underlying pathologies, we collected

matched samples from the same individuals to examine systemic responses in blood and local responses in the peritoneal cavity, before and during episodes of peritonitis. Such investigations in acutely infected people have not been attempted before, and are a unique advantage of studying individuals receiving PD. These matched analyses in fact identified further ligand-specific effects on local frequencies, as Vγ9/Vδ2 T-cells and MAIT cells were particularly elevated in peritonitis caused by bacteria producing HMB-PP and vitamin B2, respectively, even in individuals with multiple infection episodes (**Figure 2B**).

Across the cohort, proportions of Vγ9/Vδ2 T-cells among all $CD3^+$ T-cells in blood and peritoneal cavity were comparable in the absence of infection, indicating that under homeostatic conditions Vγ9/Vδ2 T-cells are not enriched locally (**Figure 3A**). In contrast, on the day of presentation with acute peritonitis, local levels of Vγ9/Vδ2 T-cells were elevated compared to blood, suggesting preferential recruitment and/or accumulation at the site of infection (**Figure 3B**). This preferential increase in local Vγ9/Vδ2 T-cell levels was apparent in patients infected with HMB-PP^+ bacteria but not in patients with HMB-PP^- infections (**Figure 3B**), despite highly elevated peritoneal chemokine levels in both HMB-PP^+ and HMB-PP^- infections (data not shown). Finally, we performed a longitudinal study and observed a significant increase in local Vγ9/Vδ2 T-cell levels in patients who developed acute peritonitis caused by HMB-PP^+ organisms, over pre-infection baseline levels when the same individuals were stable (**Figure 3C**). As there was no such difference between pre-infection and post-infection levels in individuals presenting with HMB-PP^- infections, these findings show that Vγ9/Vδ2 T-cells accumulate locally at the site of infection in response to HMB-PP^+ but not HMB-PP^- organisms.

Parallel studies on the distribution of MAIT cells revealed analogous patterns. While in stable PD patients local MAIT cell frequencies in the peritoneal cavity were slightly higher than those in blood (**Figure 3D**), such differences between anatomical sites were much more pronounced

in acutely infected individuals (average 3.99% of all T-cells in the peritoneal cavity versus 0.83% in blood), particularly during infections caused by vitamin B2 producing (vit.$B2^+$) bacteria (**Figure 3E**). As observed for peritoneal Vγ9/Vδ2 T-cell responses to HMB-PP^+ bacteria, a comparison with matched pre-infection values revealed a significant local increase in MAIT cell levels in individuals infected with vit.$B2^+$ organisms (**Figure 3F**), confirming the responsiveness of MAIT cells to vitamin B2 derivatives *in vivo*. These findings on Vγ9/Vδ2 T-cells and MAIT cells support a ligand-induced local expansion of unconventional T-cells at the site of infection.

Peritoneal unconventional T-cells respond to bacterial pathogens producing the corresponding ligands. To address the pathogen specificity of local unconventional T-cell responses, we cultured peritoneal leukocytes from stable PD patients in the presence of different microbial stimuli. In agreement with our earlier data on blood cells [10], peritoneal Vγ9/Vδ2 T-cells were highly specific for HMB-PP whereas MAIT cells recognized the vitamin B2 precursor, 6,7-dimethyl-8-D-ribityllumazine (DMRL), as judged by upregulation of CD69 and production of TNF-α by responding cells (**Figure 4A**). When testing extracts from defined clinical isolates covering the majority of pathogens associated with PD-related peritonitis, peritoneal Vγ9/Vδ2 T-cells responded readily to HMB-PP producing Gram-negative (*E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Corynebacterium striatum*, *Listeria monocytogenes*), but not to HMB-PP deficient *Enterococcus faecalis* and *Streptococcus pneumoniae* (**Figure 4B**). Blocking experiments using neutralizing antibodies confirmed that these HMB-PP dependent responses were mediated via BTN3 (**Figure 4C**). Strikingly, peritoneal Vγ9/Vδ2 T-cells also responded to *Staphylococcus aureus* despite this organism's lack of HMB-PP, possibly via superantigens [20]. Peritoneal MAIT cells were activated by the vit.$B2^+$ bacteria *E. coli*, *K. pneumoniae*, *C. striatum* and *S. aureus* but not by the vit.$B2^-$ species *L. monocytogenes*, *E. faecalis* and *S.*

pneumoniae; responses to the vit.B2$^+$ organism *P. aeruginosa* did not reach statistical significance (**Figure 4B**).

Although Vγ9/Vδ2 T-cells and MAIT cells constitute only minor proportions of the peritoneal T-cell pool, these cells represented major producers of pro-inflammatory cytokines in response to bacterial extracts (**Figure 5A**). Using *E. coli* as example of an organism producing both HMB-PP and vitamin B2, responding peritoneal Vγ9/Vδ2 T-cells and MAIT cells together made up a large fraction of TNF-α$^+$ T-cells (median 31.7%) and IFN-γ$^+$ T-cells (median 39.2%) despite considerable variability across PD patients (**Figure 5B**). In contrast, both cell types constituted far lower proportions amongst TNF-α$^-$ and IFN-γ$^-$ T-cells in *E. coli*-stimulated peritoneal leukocytes. Similar results were obtained using HMB-PP$^+$ vit.B2$^+$ *C. striatum* extracts (data not shown). Analyses of supernatants from peritoneal leukocytes exposed to different bacteria demonstrated that only organisms producing HMB-PP and/or vitamin B2 (*S. aureus*, *C. striatum*) but not HMB-PP$^-$ vit.B2$^-$ *E. faecalis* induced secretion of IFN-γ and the IFN-γ inducible chemokine CXCL10 (**Figure 5C**). As control, levels of CXCL8 were comparable in response to all three bacterial species, demonstrating an equal potential to stimulate peritoneal leukocytes other than Vγ9/Vδ2 T-cells and MAIT cells (data not shown). Secretion of TNF-α was not assessed in these experiments as any unconventional T-cell derived TNF-α would have been masked by peritoneal macrophages and neutrophils sensing diverse pathogen-associated molecular patterns. The contribution of Vγ9/Vδ2 T-cells and MAIT cells to the overall secretion of IFN-γ by peritoneal leukocytes in response to HMB-PP$^+$ vit.B2$^+$ organisms was confirmed by anti-BTN3 and anti-MR1 blocking antibodies (**Figure 5D**). Taken together, these findings show that peritoneal unconventional T-cells are major producers of IFN-γ and TNF-α in response to a wide range of microbial pathogens, and that abrogation of ligand recognition through BTN3 and MR1 abrogates this cytokine production.

Crosstalk with local tissue amplifies the pro-inflammatory response to bacterial pathogens. Local activation of unconventional T-cells in acute infection is likely to occur in close proximity to the peritoneal membrane, thus exposing the mesothelial cell layer that lines the peritoneal cavity to T-cell derived mediators. Using supernatants from activated unconventional T-cells, our experiments demonstrate that Vγ9/Vδ2 T-cells and MAIT cells induced secretion of CCL2, CXCL8, CXCL10 and IL-6 by omentum-derived primary mesothelial cells (**Figure 6A**) and peritoneal fibroblasts (**Figure 6B**). This activation of peritoneal tissue cells was dose-dependent (data not shown). Neutralization of TNF-α and/or IFN-γ in the conditioned media prior to addition to peritoneal tissue cells attenuated these responses considerably, with the CXCL8 and IL-6 secretion being particularly sensitive to inhibition of TNF-α while the CXCL10 secretion was mainly driven by IFN-γ (**Figure 6A,B**). These findings were in accordance with control experiments showing that recombinant TNF-α was a potent inducer of CCL2, CXCL8 and IL-6 expression by mesothelial cells and fibroblasts (data not shown). Recombinant IFN-γ on its own was mainly effective at inducing CCL2 and, to a lesser extent, IL-6 expression by mesothelial cells and fibroblasts while having no effect on CXCL8. These findings identify unconventional T-cell derived TNF-α and IFN-γ as major stimulators of peritoneal tissue cells, which is likely to enhance local inflammation and contribute to further recruitment of monocytes, neutrophils and lymphocytes to the site of infection.

Activation of peritoneal tissue cells by effluent from PD patients with acute peritonitis. We next sought to address the physiological relevance of the findings above in more detail. Our previous work already demonstrated that peritoneal effluent of patients presenting with acute peritonitis can contain considerable levels of TNF-α and IFN-γ [13-15]. We therefore tested whether cytokines released into the dialysis fluid during acute infection had similar bioactivity to unconventional T-cell conditioned media and the recombinant proteins

themselves. Here, the effluent from three patients with peritonitis induced CCL2 and CXCL8 secretion by mesothelial cells while effluent from stable patients showed only background activity (**Figure 7A**). This chemokine production was in part blocked by combined pre-treatment of the infected effluent with sTNFR and anti-IFN-γ (**Figure 7B**). These experiments demonstrate that TNF-α and IFN-γ are produced locally in response to bacterial pathogens at concentrations that are sufficiently high to affect the cells lining the peritoneal cavity, and that targeting cytokine production by unconventional T cells may diminish local inflammation.

Clinical outcome from peritonitis depends on the capacity of the causative pathogen to produce unconventional T-cell ligands. Acute inflammatory events make a major contribution to tissue fibrosis. In particular, peritonitis has a direct effect on peritoneal membrane morphology and function [21,22] and is hence a major reason for technique failure in PD patients. Yet, little is known about the role of unconventional T-cells in these processes. Given the contribution of Vγ9/Vδ2 T-cells and MAIT cells to the local immune response to HMB-PP$^+$ and vit.B2$^+$ bacteria, respectively, we investigated the short and mid-term impact of infections by such organisms on the clinical outcome in 5,071 PD patients presenting with first-time peritonitis recorded in the Australia and New Zealand Dialysis and Transplant (ANZDATA) Registry (**Table 1**). In accordance with our earlier findings in a much smaller subgroup of the same cohort [14], infections by HMB-PP$^+$ bacteria were associated with poorer outcomes as determined by higher rates of catheter removal, permanent transfer to hemodialysis and overall technique failure, compared to HMB-PP$^-$ infections (**Figure 8, Table 2**). This was true for episodes of peritonitis caused by both Gram$^+$ and Gram$^-$ species, thereby identifying the utilization of the non-mevalonate pathway of isoprenoid biosynthesis by the causative organism as useful predictive marker, and implying that Vγ9/Vδ2 T-cell driven responses may contribute to overall clinical outcome. Within the HMB-PP$^+$ group, Gram$^-$

organisms caused even more severe complications than Gram$^+$ species, including significant mortality within the first month after the onset of acute peritonitis.

Since all HMB-PP$^+$ bacteria in the ANZDATA cohort were also positive for vitamin B2 (and did not include *L. monocytogenes* as the only relevant HMB-PP$^+$ vit.B2$^-$ pathogen in PD patients), outcome predictions based on the presence of the vitamin B2 pathway followed closely those seen for HMB-PP$^+$ organisms (**Table 2**). However, differential analysis of HMB-PP$^-$ infections allowed us to determine the clinical impact of vit.B2$^+$ species in that patient subgroup. Here, vit.B2$^+$ infections showed a trend toward higher rates of catheter removal, compared to vit.B2$^-$ infections, which was also reflected in total technique failure rates (**Figure 8**). However, no such differences between vit.B2$^+$ and vit.B2$^-$ infections were seen with regard to transfer to hemodialysis or mortality. This relatively benign course of HMB-PP$^-$ vit.B2$^+$ peritonitis may have been due to the high prevalence of infections caused by the comparatively avirulent skin commensal *Staphylococcus epidermidis* and related coagulase-negative species (67.6% of all infections in this group). As expected [23,24], *S. aureus* infections were associated with a considerably greater risk of technique failure and catheter removal than coagulase-negative staphylococcal infections (data not shown). These findings indicate that while the presence of the vitamin B2 pathway alone may not be sufficiently predictive of clinical outcome in that patient group, MAIT cells are nevertheless likely to make a contribution to the overall inflammatory response during acute peritonitis caused by vit.B2$^+$ organisms.

Unconventional T-cell driven epithelial-mesenchymal transition of peritoneal mesothelial cells. Finally, we investigated the functional impact of activated unconventional T-cells on the surrounding tissue. Inflammatory mediators including TNF-α have previously been associated with the induction of an epithelial-mesenchymal transition (EMT)-like process in mesothelial cells [25], and IFN-γ has been identified as major driver of tissue fibrosis in the peritoneal

cavity [22]. Here, primary omentum-derived mesothelial cells exposed to supernatants from activated Vγ9/Vδ2 T-cells (data not shown) and MAIT cells (**Figure 9A**) underwent striking changes from an epithelial-like appearance to a spindled fibroblastic shape within 24 hours. Such pronounced morphological changes were greatly diminished when neutralizing TNF-α and/or IFN-γ in the unconventional T-cell conditioned media prior to addition to mesothelial cells (**Figure 9A**). As controls, similar effects were observed when mesothelial cells were cultured in the presence of TNF-α or IFN-γ, particularly when both cytokines were combined (**Figure 9B**). In agreement with these morphological changes, the expression of epithelial cell-associated markers such as E-cadherin and occludin by mesothelial cells was downregulated upon exposure to MAIT cell conditioned media, whereas the mesenchymal marker fibronectin was upregulated, as determined by RT-qPCR (**Figure 9C**). A similar upregulation of fibronectin expression was seen with Vγ9/Vδ2 T-cell conditioned medium, while effects on E-cadherin and occludin were less pronounced, most likely due to the generally lower levels of TNF-α and IFN-γ in the preparations used for those experiments, compared with MAIT cell conditioned medium (data not shown). As viability control, expression of IL-6 was greatly enhanced by unconventional T-cells at the mRNA level (data not shown), in agreement with the protein data. In addition to fibronectin, MAIT cell stimulated mesothelial cells also expressed elevated levels of the microRNA miR-21, which has been linked to TGF-β induced EMT in mesothelial cells, and to membrane fibrosis in patients receiving PD (Melisa López Antón and Donald J. Fraser, unpublished observations). This miR-21 induction by MAIT cells was decreased upon neutralization of TNF-α and IFN-γ (**Figure 9D**). Further epithelial (zona occludens-1, claudin) and mesenchymal markers (Snail, collagen-1, α-smooth muscle actin) were not significantly affected by unconventional T-cell secreted factors (**Figure 9C** and data not shown), indicating incomplete initiation of the EMT process under these conditions. Notably, while mesothelial cells treated with a combination of TNF-α and IFN-γ downregulated the expression of all epithelial markers tested (data not shown), only Vγ9/Vδ2

T-cells and MAIT cells triggered a parallel upregulation of the mesenchymal marker fibronectin. These data imply that unconventional T-cells may produce as yet unidentified mediators, in addition to TNF-α and IFN-γ, that may affect mesothelial cells and reprogram their morphology and phenotype. Taken together, these findings indicate that microbe-responsive unconventional T-cells have the capacity to trigger local tissue remodeling with potential consequences for peritoneal membrane integrity and long-term clinical outcome.

DISCUSSION

By combining cross-sectional and longitudinal sampling in PD patients together with a functional characterization of peritoneal immune and tissue cells *ex vivo* and an epidemiological analysis of organism-dependent outcomes, we show that Vγ9/Vδ2 T-cells and MAIT cells specifically accumulate locally during infections with bacteria producing HMB-PP and vitamin B2 derivatives, respectively, and that utilization of the HMB-PP and vitamin B2 pathways by the causative organism represents an effective predictive marker for technique failure. Our data thus provide the first conclusive evidence in humans for a ligand-specific role of Vγ9/Vδ2 T-cells and MAIT cells at the site of infection. Due to the limited access to relevant patient samples such a physiological role has so far only been hinted at in complex experimental animal models [26-28]. Our data also show that such unconventional human T-cells are major producers of IFN-γ and TNF-α in response to microbial pathogens, thereby affecting the cells lining the peritoneal cavity and amplifying local inflammation. These findings are likely to be applicable to other acute infections where local activation of unconventional T-cells contributes to the anti-microbial inflammatory response.

The present study demonstrates that PD patients offer exceptional opportunities for immunological studies into acute disease. First, peritoneal effluent can be sampled repeatedly and non-invasively, thus providing continuous access to the site of infection on the first day of microbial infection, before antibiotic therapy commences, to study the development and resolution of acute responses. Second, stable PD patients without overt inflammation serve as age and gender-matched non-infected controls in cross-sectional studies. Third, peritonitis remains a relatively frequent complication of PD therapy, with typical incidences in the UK of one episode per 15-30 patient months [29]. Thorough immunological profiling of a stable PD cohort therefore allows to establish pre-infection baseline parameters in individuals prone to develop peritonitis later on. Fourth, PD-related peritonitis can be caused by Gram-positive and

Gram-negative bacteria as well as fungi, thereby allowing to study the local immune response to a wide spectrum of pathogens under closely related conditions. Since acute infection and associated inflammation remain a major cause of treatment failure and even death in PD patients [30], our findings not only improve our insight into the complex local cell interactions in early infection, but also provide relevant clues to the mechanisms that underpin the clinical severity of infectious episodes, and are readily translatable to improve patient management and outcomes.

Many unconventional T-cell populations are rare in humans, with NKT cells typically representing 0.01–0.5% of T-cells in blood and frequencies of GEM T-cells being even lower [31,32]. Given the rare nature of these populations and the low prevalence of organisms that produce the corresponding ligands in PD patients, we focused on Vγ9/Vδ2 T-cells and MAIT cells, the two most abundant unconventional T-cell populations in the peritoneal cavity. Our data provide *in vitro* and *in vivo* evidence for the specificity of peritoneal Vγ9/Vδ2 T-cells and MAIT cells for the corresponding ligands, HMB-PP [33] and vitamin B2 derivatives [16,34]. In particular, our research suggests that Vγ9/Vδ2 T-cells and MAIT cells discriminate locally between different organisms and accumulate rapidly at the site of infection, where they engage local monocytes and neutrophils as well as tissue cells, and orchestrate early inflammatory events [10,13,14]. While others have suggested that circulating MAIT cells decrease in certain clinical scenarios sufficiently to reach statistical significance [35], our data indicate for the first time that local recruitment of MAIT cells takes place in acute infection. Unconventional T-cell driven responses are likely to contribute to the transition from local innate immunity to antigen-specific adaptive immunity [11], and have potential to be exploited for improved patient management through novel diagnostics, therapeutics and vaccines. As such, we show here that local Vγ9/Vδ2 T-cells and MAIT cells represent dominant sources of IFN-γ and TNF-α in response to diverse bacteria. As these cytokines control leukocyte recruitment to the site

of infection but also possess pro-fibrotic functions, unconventional T-cells represent key regulators of acute immune responses as well as collateral tissue damage, ultimately affecting outcomes.

The present study identified unconventional T-cell derived TNF-α and IFN-γ as potent stimulators of primary mesothelial cells and peritoneal fibroblasts, the two major types of tissue cells constituting the peritoneal membrane. Through induction of IL-6 as well as chemokines such as CCL2, CXCL8 and CXCL10, Vγ9/Vδ2 T-cells and MAIT cells are likely to enhance local inflammation and contribute to further recruitment of monocytes, neutrophils and lymphocytes to the site of infection. This interaction with local tissue cells complements our earlier findings on a potent crosstalk of Vγ9/Vδ2 T-cells and MAIT cells with monocytes and neutrophils triggering chemokine production and leading to monocyte and neutrophil survival and acquisition of APC features [10,11,13,14]. Together, these findings indicate that Vγ9/Vδ2 T-cells and MAIT cells play a crucial role in driving local inflammatory events by engaging both immune and non-immune cells at the site of infection by organisms producing the corresponding ligands. The timely detection of Vγ9/Vδ2 T-cell and MAIT cell responses in PD patients presenting with acute peritonitis may therefore allow an 'immune fingerprint'-based point-of-care definition of the causative pathogen, which would improve early patient management by targeting treatments more efficiently than current empirical approaches, reducing unnecessary exposure to broad-spectrum antibiotics, and identifying individuals at increased risk of subsequent complications who may require prolonged hospitalization [15,36].

The orchestration of early anti-microbial responses by unconventional T-cells is likely to contribute to pathogen clearance and wound healing, and thus be beneficial to the host in many different infectious scenarios. However, the situation is different in individuals receiving PD who are highly susceptible to inflammation-related tissue damage with immediate consequences for their treatment [37-39]. In particular, IL-6 has been linked to tissue fibrosis

via induction of Th1 cell responses as a consequence of peritoneal inflammation [22]. Unconventional T-cells are therefore likely to contribute to scarring in the peritoneal cavity, both directly via the local release of IFN-γ and indirectly via induction of IL-6 production by mesothelial cells and fibroblasts. Our present study identified striking morphological and phenotypical changes of mesothelial cells in response to Vγ9/Vδ2 T-cells and MAIT cell secreted cytokines, changes that are linked to infection-driven peritoneal fibrosis and technique failure. Such local tissue remodeling by downregulating epithelial features and inducing expression of mesenchymal markers will ultimately affect the integrity of the peritoneal membrane and affect long-term clinical outcomes. Together, these complex interactions may, at least in part, explain why organisms that are capable of activating local unconventional T cells are frequently associated with higher rates of technique failure in PD patients. Besides predicting important clinical outcomes, unconventional T-cell driven responses to ligands shared by many microbial pathogens therefore also represent potential targets to suppress overshooting inflammation and limit damage on the peritoneal membrane, with the possibility to deliver treatments locally via the peritoneal catheter. Novel interventions will benefit greatly from the recent elucidation of the BTN3 and MR1 presentation pathway, and the availability of reagents blocking the TCR-mediated recognition of microbial ligands [40,41], antibiotics shutting off the non-mevalonate and riboflavin biosynthesis [42-45], and biologics interfering with key effector cytokines.

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FOOTNOTES

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Abbreviations used in this article: ANZDATA, Australia and New Zealand Dialysis Transplant Registry; BTN3, butyrophilin-3 (CD277); DMRL, 6,7-dimethyl-8-D-ribityllumazine EMT, epithelial-mesenchymal transition; GEM T-cells, germline-encoded mycolyl-reactive T-cells; HMB-PP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; MAIT cells, mucosal-associated invariant T-cells; MR1, MHC-related protein 1; PD, peritoneal dialysis; sTNFR, soluble TNF-α receptor

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Table 1. Characteristics of all PD patients analyzed in the present study.

	Cardiff	**ANZDATA**
Number of stable patients	45	n/a
Age (mean ± SD)	69.1 ± 13.5	n/a
Women (%)	18.6	n/a
Days on PD (mean ± SD)	624 ± 546	n/a
Number of patients presenting with acute peritonitis	101	5,071
Age (mean ± SD)	66.0 ± 13.3	60.1 ± 16.9
Women (%)	32.6	43.9
Days on PD (mean ± SD)	936 ± 856	1010 ± 791
No culture samples obtained (%)	0.0	0.4
Culture negative episodes (%)	22.8	16.6
Fungi (%)	1.0	2.3
Polymicrobial infections (%)	4.0	6.8
HMB-PP^{+} vit.B2^{+} species among single bacteria (%)	34.2	35.3
HMB-PP^{-} vit.B2^{+} species among single bacteria (%)	42.5	50.2
HMB-PP^{-} vit.B2^{-} species among single bacteria (%)	23.3	14.5

Table 2. Odds ratios for risk of technique failure in patients presenting with first-time peritonitis, depending on the causative pathogen. OR, odds ratio of the indicated type of technique failure; CI, confidence interval; HD, hemodialysis.

ANZDATA *n*=5,071	90th day overall technique failure		30th day mortality		90th day catheter removal		30th day transfer to interim HD		90th day transfer to permanent HD	
	OR (95% CI)	***p***	**OR** (95% CI)	***p***	**OR** (95% CI)	***p***	**OR** (95% CI)	***p***	**OR** (95% CI)	***p***
Reference: culture-negative	**1.0**		**1.0**		**1.0**		**1.0**		**1.0**	
$Gram^+$ $HMB\text{-}PP^+$ $vitamin\ B2^+$	2.4 (1.389-4.129)	*0.002*	0.262 (0.043-2.360)	*0.262*	3.2 (1.812-5.637)	***	7.3 (1.606-33.143)	*0.010*	2.3 (1.253-4.398)	*0.008*
$Gram^+$ $HMB\text{-}PP^-$ $vitamin\ B2^+$	1.1 (0.866-1.468)	*0.374*	0.737 (0.455-1.192)	*0.213*	1.2 (0.895-1.637)	*0.215*	3.2 (1.125-9.353)	*0.029*	1.0 (0.763-1.437)	*0.775*
$Gram^+$ $HMB\text{-}PP^-$ $vitamin\ B2^-$	0.9 (0.662-1.381)	*0.812*	0.794 (0.409-1.543)	*0.497*	1.0 (0.658-1.523)	*0.998*	2.7 (0.758-9.615)	*0.126*	1.0 (0.655-1.551)	*0.971*
$Gram^-$ $HMB\text{-}PP^+$ $vitamin\ B2^+$	4.3 (3.318-5.500)	***	2.6 (1.713-4.037)	***	4.4 (3.358-5.946)	***	9.0 (3.242-25.319)	***	3.4 (2.506-4.550)	***
Reference: culture-negative	**1.0**		**1.0**		**1.0**		**1.0**		**1.0**	
$HMB\text{-}PP^+$	4.1 (3.194-5.271)	***	2.43 (1.589-3.737)	***	4.3 (3.285-5.792)	***	8.9 (3.201-24.863)	***	3.3 (2.449-4.425)	***
$HMB\text{-}PP^-$	1.0 (0.843-1.405)	*0.515*	0.75 (0.474-1.184)	*0.217*	1.2 (0.868-1.558)	*0.312*	3.1 (1.099-8.873)	*0.033*	1.0 (0.766-1.408)	*0.808*
Reference: culture-negative	**1.0**		**1.0**		**1.0**		**1.0**		**1.0**	
$Vitamin\ B2^+$	2.1 (1.703-2.734)	***	1.4 (0.939-2.129)	*0.097*	2.3 (1.789-3.074)	***	5.5 (2.021-15.235)	*0.001*	2.0 (1.403-3.049)	***
$Vitamin\ B2^-$	0.9 (0.662-1.381)	*0.812*	0.8 (0.409-1.543)	*0.497*	1.0 (0.658-1.523)	*0.998*	2.7 (0.758-9.615)	*0.126*	0.9 (0.493-1.689)	*0.771*
Reference: $HMB\text{-}PP^-$	**1.0**		**1.0**		**1.0**		**1.0**		**1.0**	
$Gram^+$ $HMB\text{-}PP^+$	2.2 (1.312-3.689)	*0.003*	0.424 (0.058-3.098)	*0.398*	2.7 (1.618-4.670)	***	0.9 (0.700-7.796)	*0.167*	2.3 (1.248-4.095)	*0.007*
$Gram^-$ $HMB\text{-}PP^+$	3.9 (3.263-4.721)	***	3.5 (2.488-4.947)	***	3.8 (3.141-4.700)	***	2.9 (1.821-4.623)	***	3.3 (2.614-4.046)	***
Reference: $Vitamin\ B2^-$	**1.0**		**1.0**		**1.0**		**1.0**		**1.0**	
$Gram^+$ $vitamin\ B2^+$	1.2 (0.892-1.721)	*0.201*	0.9 (0.480-1.688)	*0.742*	1.3 (0.896-1.883)	*0.167*	1.3 (0.526-3.105)	*0.588*	1.1 (0.748-1.617)	*0.629*
$Gram^-$ $vitamin\ B2^+$	4.5 (3.235-6.168)	***	3.3 (1.832-5.985)	***	4.4 (3.108-6.415)	***	3.4 (1.420-7.933)	*0.006*	3.3 (2.302-4.874)	***
Reference: $Gram^-$ $HMB\text{-}PP^+$ $vitamin\ B2^+$	**1.0**		**1.0**		**1.0**		**1.0**		**1.0**	
$Gram^+$ $HMB\text{-}PP^+$ $vitamin\ B2^+$	0.6 (0.335-0.939)	*0.028*	0.12 (0.017-0.877)	*0.037*	0.7 (0.423-1.211)	*0.212*	0.805 (0.245-2.648)	*0.721*	0.228 (0.385-1.255)	*0.695*
$Gram^+$ $HMB\text{-}PP^-$ $vitamin\ B2^+$	0.3 (0.217-0.321)	***	0.3 (0.193-0.407)	***	0.3 (0.219-0.336)	***	0.4 (0.218-0.589)	***	0.3 (0.245-0.392)	***
$Gram^+$ $HMB\text{-}PP^-$ $vitamin\ B2^-$	0.2 (0.162-0.309)	***	0.3 (0.167-0.546)	***	0.2 (0.156-0.322)	***	0.3 (0.126-0.704)	*0.006*	0.3 (0.205-0.434)	***

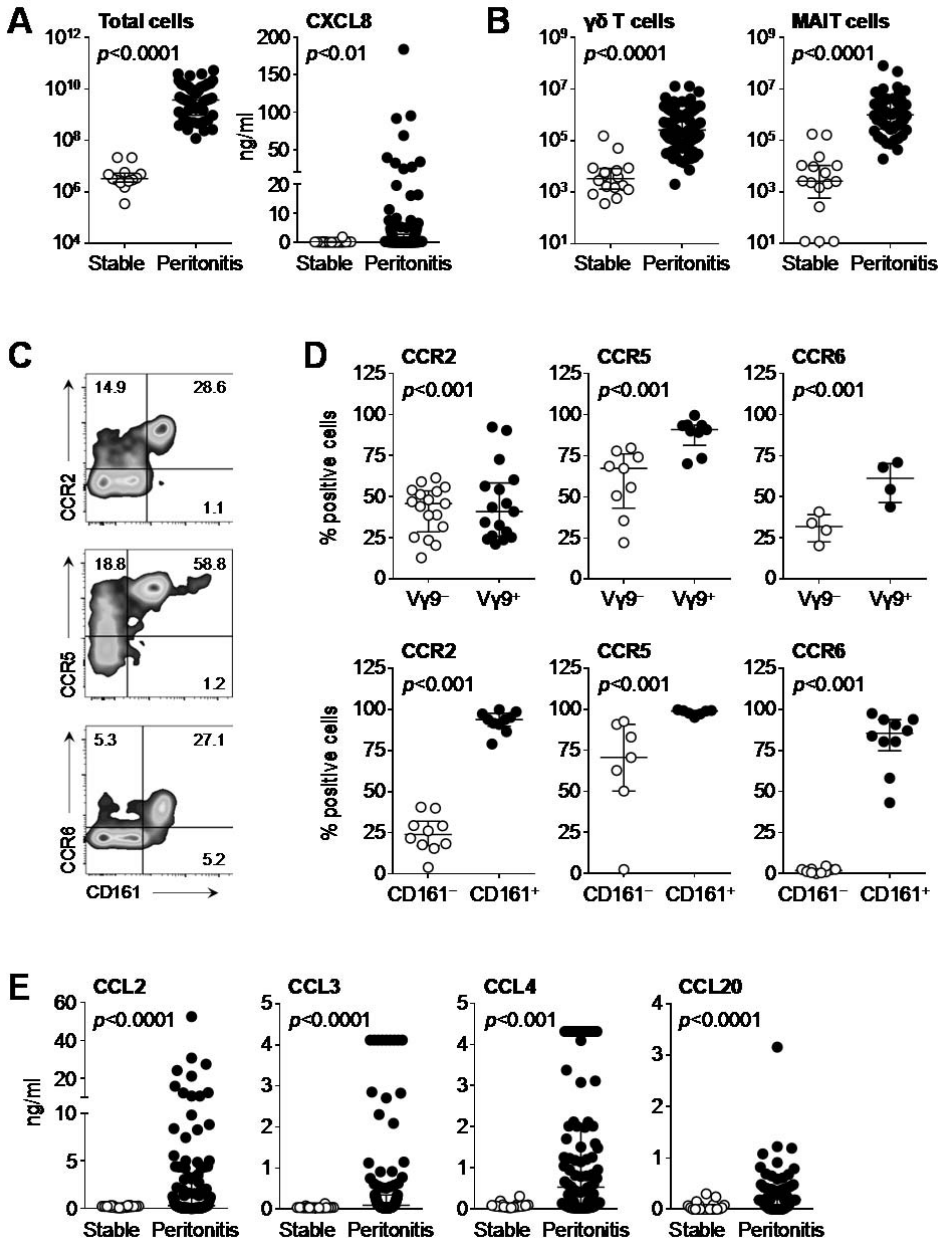

Figure 1. Pro-inflammatory migratory profile of unconventional T-cells. (***A***) Total cell counts and concentration of the neutrophil-attracting chemokine CXCL8 in the peritoneal effluent of stable PD patients and patients presenting with acute peritonitis. (***B***) Total numbers of $V\gamma9^+$ $CD3^+$ T-cells and $V\alpha7.2^+$ $CD3^+$ T-cells within the peritoneal cell population in stable PD patients and during acute peritonitis. (***C***) Representative example for the co-expression of CCR2, CCR5 and CCR6 with CD161 on blood $V\alpha7.2^+$ $CD3^+$ T-cells in a stable PD patient. (***D***) Percentage of $CCR2^+$, $CCR5^+$ and $CCR6^+$ cells amongst $V\gamma9^-$ and $V\gamma9^+$ $CD3^+$ T-cells (upper panels) or amongst $V\alpha7.2^+$ $CD161^-$ and $V\alpha7.2^+$ $CD161^+$ $CD3^+$ T-cells in the blood of stable PD patients (lower panels). (***E***) Concentration of the indicated chemokines in the effluent of patients presenting with acute peritonitis; upper limits of detection for CCL3 and CCL4 were 4.12 ng/ml and for 4.32 ng/ml, respectively. Data were analyzed using Mann Whitney tests (in the case of CCL2 after normalization). Each data point represents an individual patient, error bars depict the median ± interquartile range.

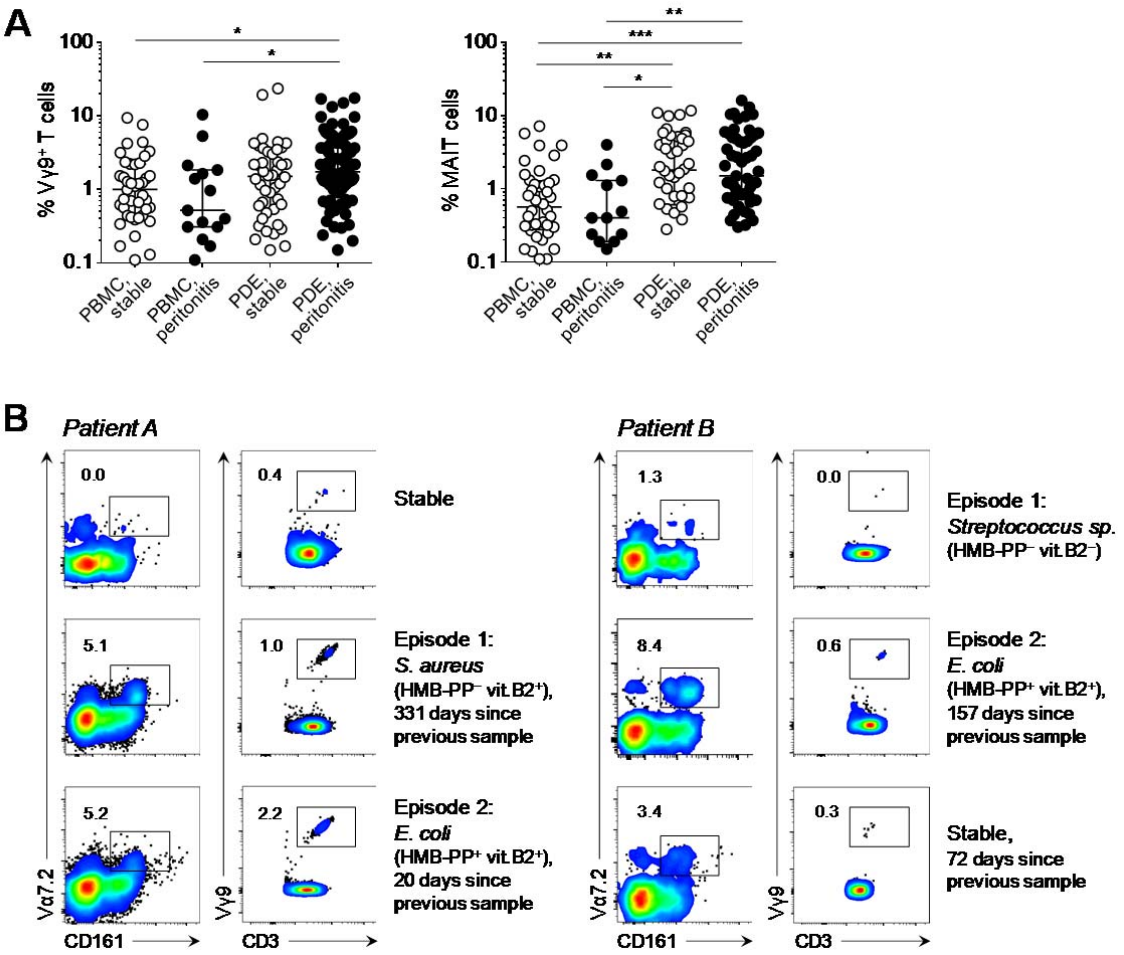

Figure 2. Systemic and local levels of unconventional T-cells in stable PD patients and during acute peritonitis. (***A***) Blood (PBMC) and peritoneal dialysis effluent (PDE) were analyzed by flow cytometry for the proportion of Vγ9/Vδ2 T-cells (identified as $V\gamma9^+$; left) and MAIT cells ($V\alpha7.2^+$ $CD161^+$; right), expressed as percentage of all $CD3^+$ T-cells. Samples were collected from stable PD patients and patients presenting with acute peritonitis (day 1), before commencing antibiotic treatment. Data were analyzed using Kruskal-Wallis tests combined with Dunn's multiple comparisons tests. Each data point represents an individual patient; asterisks indicate significant differences between groups. (***B***) Local levels of unconventional T-cells in the effluent of two PD patients whilst the individuals were stable and when they presented with distinct peritonitis episodes caused by bacteria capable or not of producing HMB-PP or vitamin B2 (day 1).

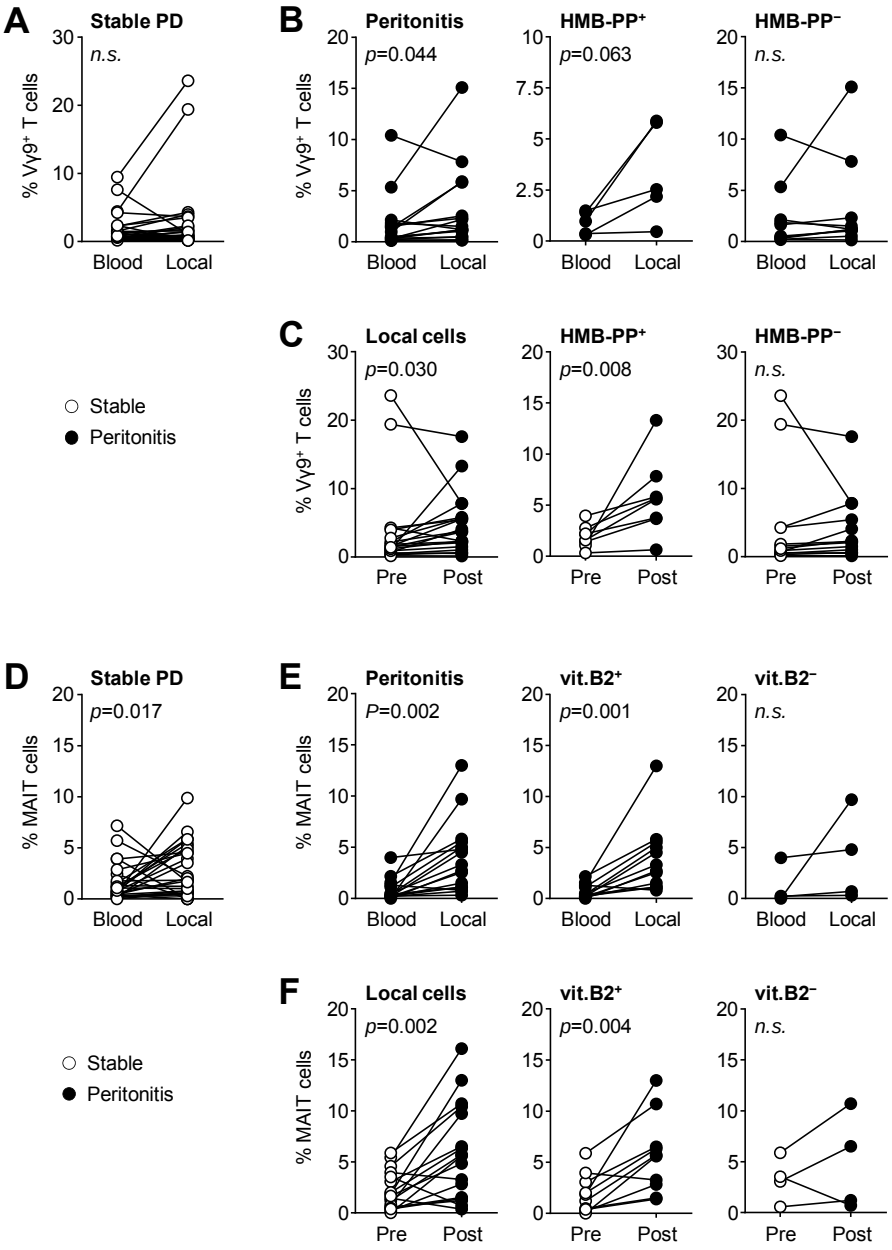

Figure 3. Matched levels of unconventional T-cells in blood and effluent of PD patients before and during acute peritonitis. Blood and peritoneal effluent samples from the same individuals were analyzed by flow cytometry for the proportion of Vγ9/Vδ2 T-cells (identified as $V\gamma9^+$; ***A-C***) and MAIT cells ($V\alpha7.2^+$ $CD161^+$; ***D-F***), expressed as percentage of all $CD3^+$ T-cells. Samples were collected whilst patients were stable and when they presented with acute peritonitis (day 1), before commencing antibiotic treatment. (***A,D***) Unconventional T-cell levels in blood and effluent of stable individuals. (***B,E***) Unconventional T-cell levels in blood and effluent of all patients with acute peritonitis (left), and in subgroups of patients with confirmed infections by bacteria capable or not of producing HMB-PP or vitamin B2 (middle, right). (***C,F***) Local unconventional T-cell levels in the effluent of PD patients before and during acute peritonitis (left), and in subgroups of patients with infections by bacteria producing HMB-PP and/or vitamin B2 (middle, right). Data were analyzed using Wilcoxon matched-pairs signed rank tests. Each data point represents an individual patient.

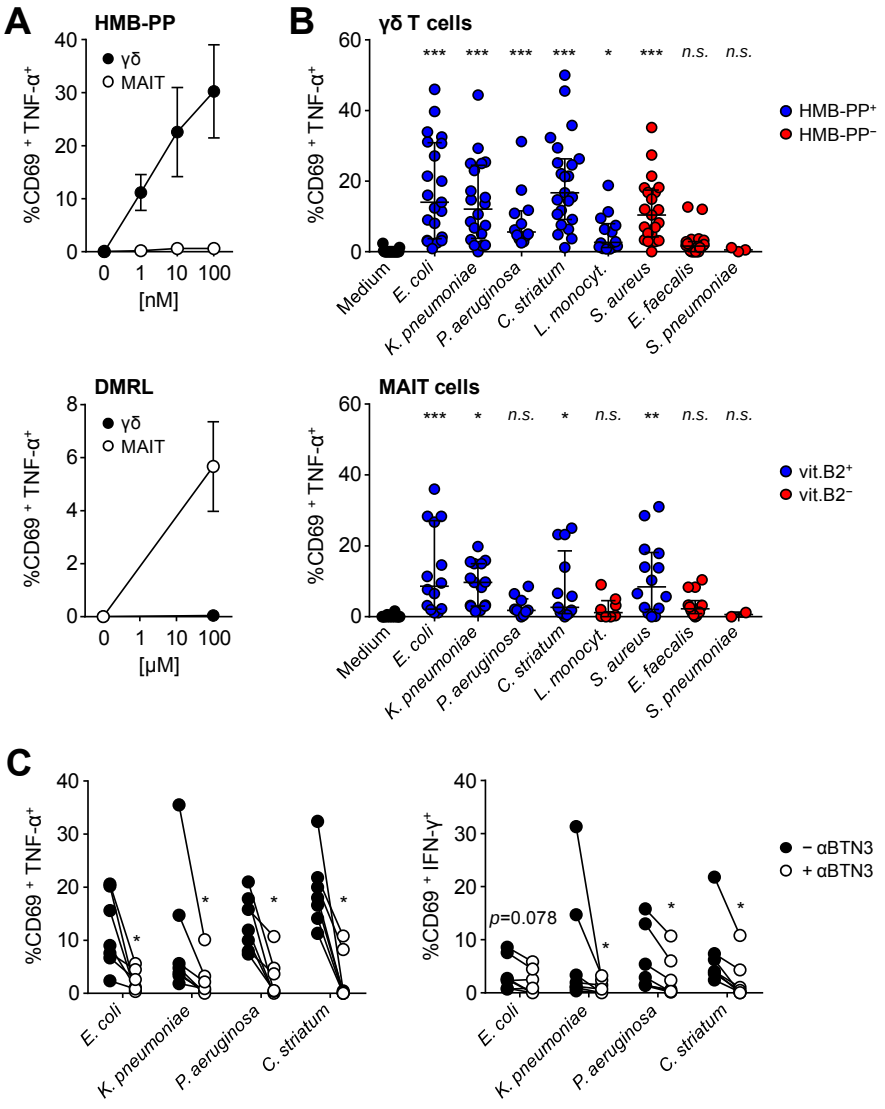

Figure 4. Peritoneal unconventional T-cell responses to microbial metabolites. (***A***) Activation of peritoneal Vγ9$^+$ γδ T-cells and Vα7.2$^+$ CD161$^+$ MAIT cells from stable PD patients upon overnight stimulation with HMB-PP (*n*=4 individual patients) or DMRL (*n*=3), as analyzed by flow cytometry and expressed as proportion of γδ or MAIT cells co-expressing CD69 and TNF-α (means ± SEM). (***B***) Activation of peritoneal Vγ9$^+$ γδ T-cells and Vα7.2$^+$ CD161$^+$ MAIT cells upon overnight stimulation in the presence of extracts from different clinical isolates that produce (in blue) or do not produce (in red) the corresponding ligands (median ± interquartile range): *E. coli* (HMB-PP$^+$ vit.B2$^+$), *Klebsiella pneumoniae* (HMB-PP$^+$ vit.B2$^+$), *Pseudomonas aeruginosa* (HMB-PP$^+$ vit.B2$^+$), *Corynebacterium striatum* (HMB-PP$^+$ vit.B2$^+$), *Listeria monocytogenes* (HMB-PP$^+$ vit.B2$^-$), *Staphylococcus aureus* (HMB-PP$^-$ vit.B2$^+$), *Enterococcus faecalis* (HMB-PP$^-$ vit.B2$^-$), and *Streptococcus pneumoniae* (HMB-PP$^-$ vit.B2$^-$). Each data point represents an individual patient. (***C***) Activation of total peritoneal leukocytes by extracts of the indicated bacteria, in the absence or presence of anti-BTN3 blocking antibodies, shown as co-expression of CD69 and TNF-α (left) or IFN-γ (right) by Vγ9$^+$ T-cells after overnight stimulation. Data were analyzed using Wilcoxon matched-pairs signed rank tests. Each data point represents an individual patient; asterisks depict significant differences of anti-BTN3 treated samples compared to untreated controls.

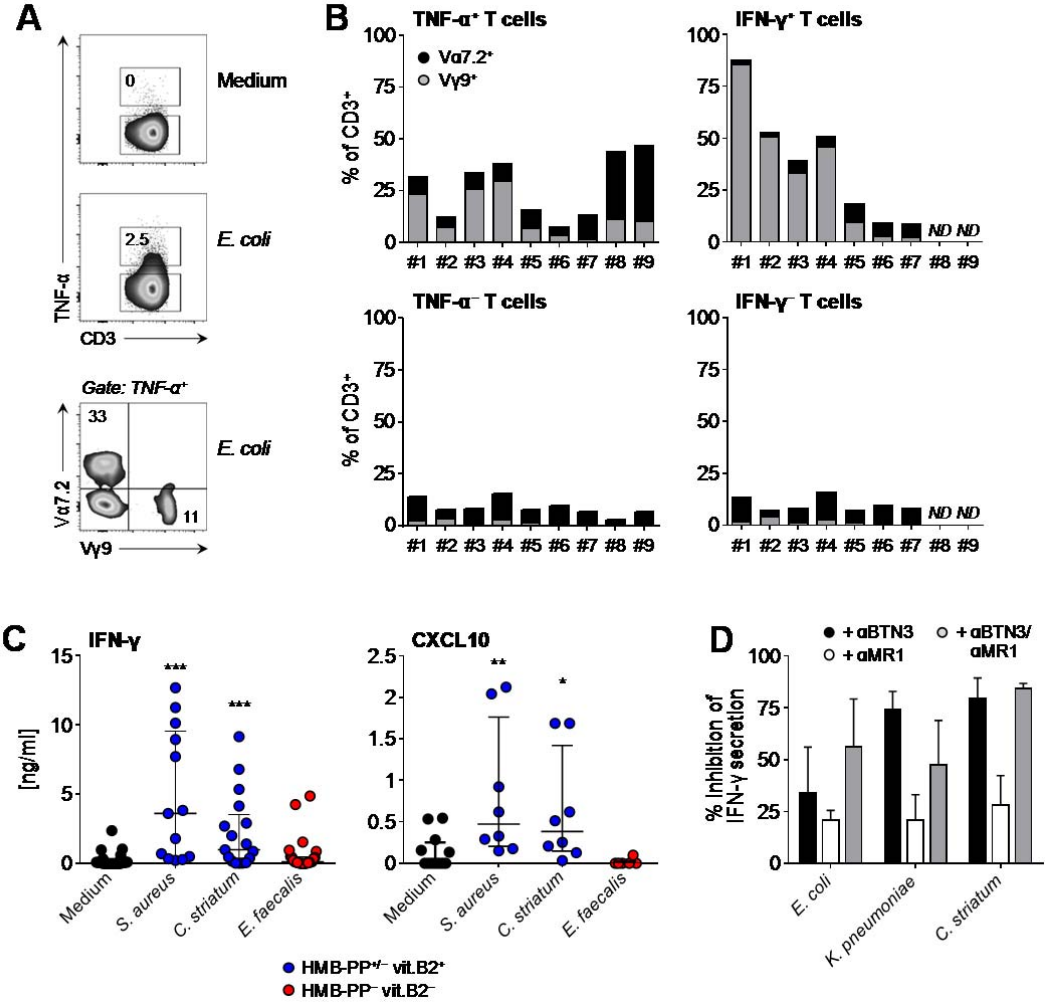

Figure 5. ***Ex vivo*** **responsiveness of peritoneal leukocytes to pathogenic bacteria.** Peritoneal cells were obtained from the effluent of stable patients and exposed overnight to extracts prepared from the indicated bacterial species. (***A***) Representative example of an intracellular staining of TNF-α in peritoneal leukocytes cultured in the absence (medium; top panel) or presence of *E. coli* extract (middle panel), as analyzed by flow cytometry within the $CD3^+$ gate. Bottom panel, distribution of $V\alpha7.2^+$ and $V\gamma9^+$ cells within all $CD3^+$ TNF-α^+ peritoneal cells after stimulation with *E. coli* extract. (***B***) Proportion of $V\alpha7.2^+$ (black) and $V\gamma9^+$ cells (shaded) T-cells amongst peritoneal T-cells producing or not TNF-α and IFN-γ in response to *E. coli*, as analyzed by flow cytometry in nine stable individuals. (***C***) Overnight secretion of IFN-γ, CXCL10 and CXCL8 by peritoneal cells in response to bacteria that produce (*S. aureus*, *C. striatum*; in blue) or do not produce (*E. faecalis*; in red) ligands for Vγ9/Vδ2 T-cells and/or MAIT cells, as analyzed by ELISA (median ± interquartile range). Data were analyzed using Kruskal-Wallis tests combined with Dunn's multiple comparisons tests. Each data point represents an individual patient; asterisks indicate significant differences compared to medium controls. *ND*, not done. (***D***) Specific inhibition of IFN-γ secretion by peritoneal leukocytes in response to bacterial extracts, in the absence or presence of anti-BTN3 and anti-MR1 blocking antibodies, alone or in combination. Data shown are means ± SEM from independent experiments with 3 omental donors.

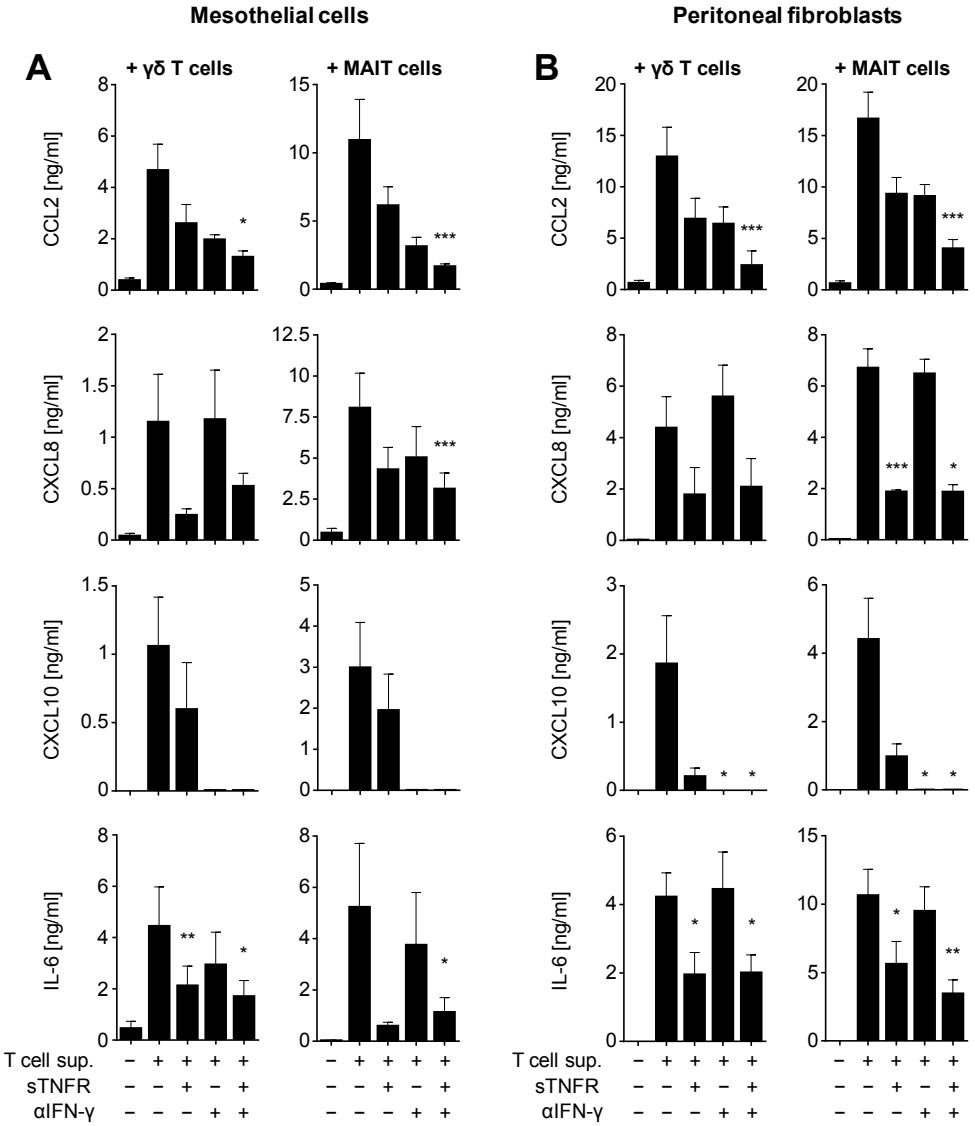

Figure 6. Activation of peritoneal tissue cells by γδ T-cell and MAIT cell derived cytokines. Growth-arrested peritoneal mesothelial cells (***A***) or peritoneal fibroblasts (***B***) from human omentum were exposed to supernatants derived from activated Vγ9/Vδ2 T-cells and MAIT cells at a dilution of 1:4, in the absence or presence of 10 μg/ml sTNFR and 10 μg/ml anti-IFN-γ, alone or together. Data shown are levels of CCL2, CXCL8, CXCL10 and IL-6 secreted into the culture medium over 24 hours by ELISA (means ± SEM from independent experiments with 4-7 omental donors). Data were analyzed using Friedman tests combined with Dunn's multiple comparisons tests. Asterisks indicate significant differences compared to medium controls.

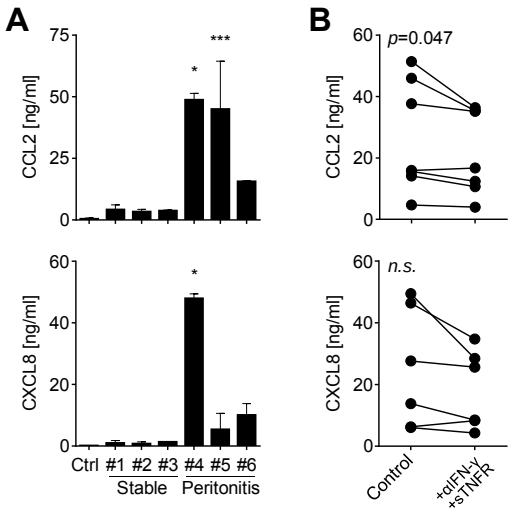

Figure 7. Activation of peritoneal tissue cells by effluent from PD patients with acute peritonitis. (***A***) Growth-arrested peritoneal mesothelial cells from human omentum (*n*=2-4) were exposed to peritoneal effluent obtained from three stable PD patients in the absence of any inflammation (#1-3) and from three patients presenting with acute peritonitis (#4: *Enterobacter sp.*, #5: *E. coli* and #6: *Acinetobacter sp.*). Data shown are levels of CCL2 and CXCL8 secreted into the culture medium over 24 hours by ELISA (median ± interquartile range). Data were analyzed using Kruskal-Wallis tests combined with Dunn's multiple comparisons tests. Asterisks indicate significant differences compared to medium controls (Ctrl). (***B***) Mesothelial cells were exposed to peritoneal effluent from patients presenting with peritonitis, in the absence or presence of 10 μg/ml sTNFR and 10 μg/ml anti-IFN-γ. Data shown are expressed as percent inhibition of CCL2 and CXCL8 secretion over 24 hours, compared to untreated controls (median ± interquartile range). Data were analyzed using Wilcoxon matched-pairs signed rank tests. Each data point represents an independent experiment.

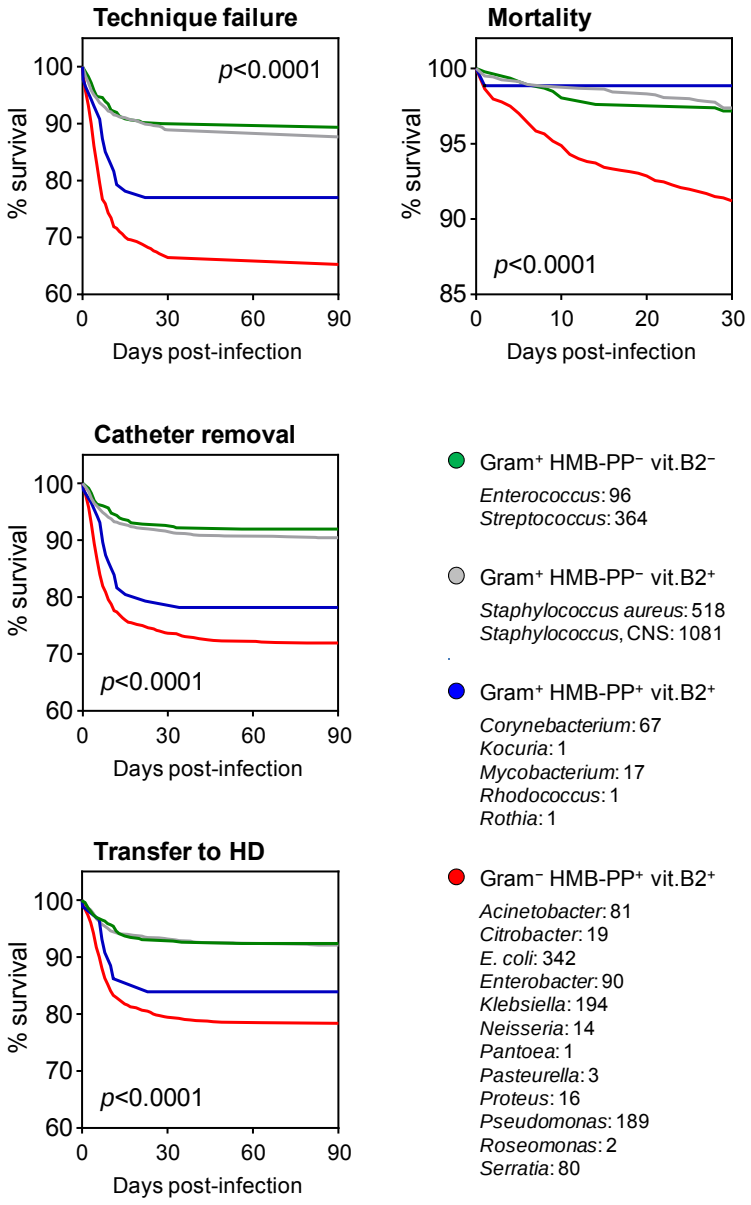

Figure 8. Association of first-time peritonitis caused by HMB-PP$^+$ and vit.B2$^+$ bacteria with poor clinical outcome. Cumulative rates of technique failure (top left), mortality (top right), catheter removal (bottom left) and transfer to permanent hemodialysis (HD; bottom right) of patients from the ANZDATA registry with first-time peritonitis, grouped into infections with Gram$^+$ HMB-PP$^-$ vit.B2$^-$ (green), Gram$^+$ HMB-PP$^-$ vit.B2$^+$ (grey), Gram$^+$ HMB-PP$^+$ vit.B2$^+$ (blue) or Gram$^-$ HMB-PP$^+$ vit.B2$^+$ bacteria (red); episodes caused by Gram$^-$ HMB-PP$^-$ (*e.g. Legionella spp.*) or Gram$^+$ HMB-PP$^+$ vit.B2$^-$ species (*e.g. Listeria monocytogenes*) were not recorded and/or were too rare for this comparison. Numbers indicate the number of cases of acute peritonitis caused by the listed organisms. Comparisons were made using log-rank tests.

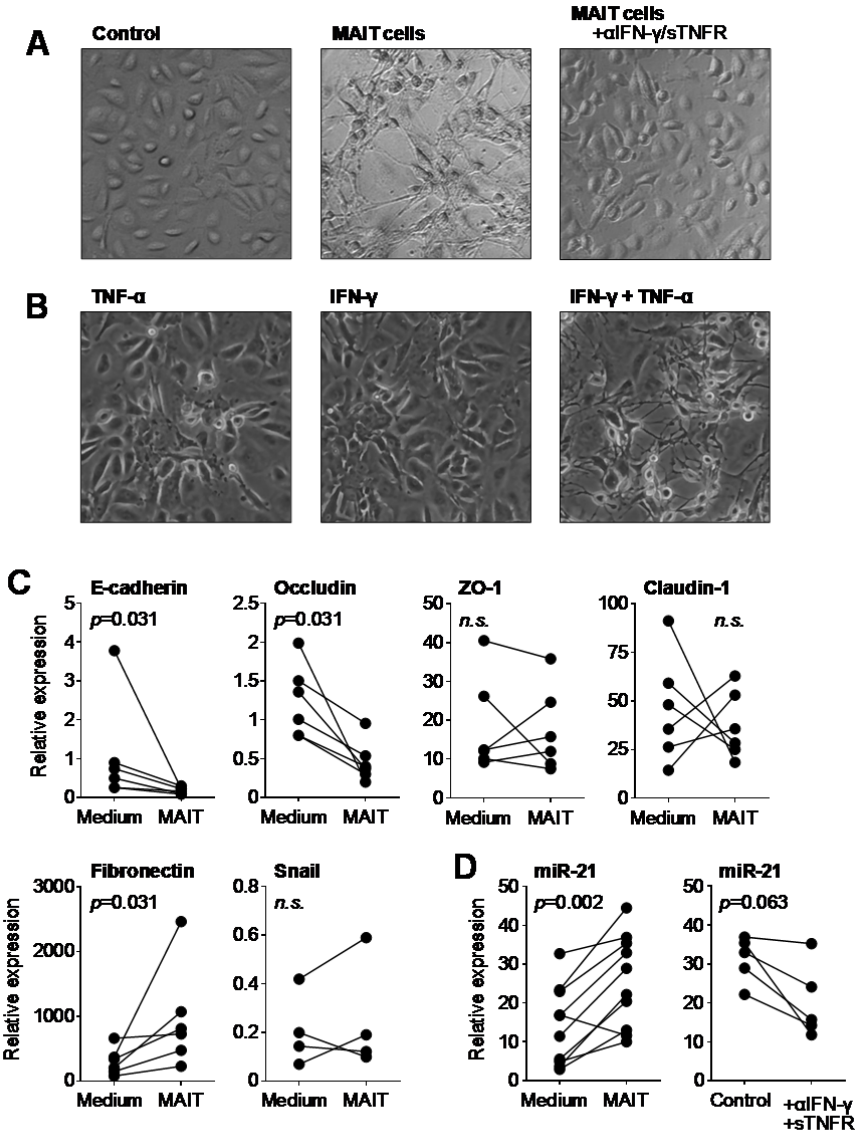

Figure 9. Unconventional T-cell induced reprogramming of peritoneal mesothelial cells. Growth-arrested peritoneal mesothelial cells from human omentum were cultured in medium alone or exposed to supernatants derived from activated MAIT cells, in the absence or presence of 10 μg/ml sTNFR and 10 μg/ml anti-IFN-γ ***(A)***, or stimulated with 5 ng/ml of TNF-α and IFN-γ, alone or in combination ***(B)***. Images were captured after 24 hours in culture with a light microscope at a 20× magnification, and are representative of 3-4 individual donors. ***(B)*** Expression of epithelial (E-cadherin, occludin, zona occludens-1 [ZO-1], claudin-1) and mesenchymal markers (fibronectin, Snail) by mesothelial cells after 24 hour exposure to MAIT cell supernatants, as determined by quantitative PCR as relative expression compared to 1,000 copies of GAPDH as housekeeping gene. ***(C)*** Expression of miR-21 by mesothelial cells after 24 hour exposure to MAIT cell supernatants in the absence or presence of 10 μg/ml sTNFR and 10 μg/ml anti-IFN-γ, as determined by quantitative PCR as relative expression compared to miR-191 as reference microRNA. Data were analyzed using Wilcoxon matched-pairs signed rank tests or paired *t*-tests. Each data point represents an individual patient.